

**The Science and Technology
of Gelatin**

FOOD SCIENCE AND TECHNOLOGY

A SERIES OF MONOGRAPHS

Editorial Board

G. F. STEWART
C. O. CHICHESTER
JOHN HAWTHORN
A. I. MORGAN

E. M. MRAK
J. K. SCOTT
E. VON SYDOW

-
- Maynard A. Amerine, Rose Marie Pangborn, and Edward B. Roessler, PRINCIPLES OF SENSORY EVALUATION OF FOOD. 1965.
- C. R. Stumbo, THERMOBACTERIOLOGY IN FOOD PROCESSING, second edition. 1973.
- Gerald Reed (ed.), ENZYMES IN FOOD PROCESSING, second edition. 1975.
- S. M. Herschdoerfer, QUALITY CONTROL IN THE FOOD INDUSTRY. Volume I - 1967. Volume II - 1968. Volume III - 1972.
- Hans Riemann, FOOD-BORNE INFECTIONS AND INTOXICATIONS. 1969.
- Irvin E. Liener, TOXIC CONSTITUENTS OF PLANT FOODSTUFFS. 1969.
- Martin Glicksman, GUM TECHNOLOGY IN THE FOOD INDUSTRY. 1970.
- L. A. Goldblatt, AFLATOXIN. 1970.
- Maynard A. Joslyn, METHODS IN FOOD ANALYSIS, second edition. 1970.
- A. C. Hulme (ed.), THE BIOCHEMISTRY OF FRUITS AND THEIR PRODUCTS. Volume 1 - 1970. Volume 2 - 1971.
- G. Ohloff and A. F. Thomas, GUSTATION AND OLFACTION. 1971.
- George F. Stewart and Maynard A. Amerine, INTRODUCTION TO FOOD SCIENCE AND TECHNOLOGY. 1973.
- Irvin E. Liener (ed.), TOXIC CONSTITUENTS OF ANIMAL FOODSTUFFS. 1974.
- Aaron M. Altschul (ed.), NEW PROTEIN FOODS: Volume 1, TECHNOLOGY PART A - 1974. Volume 2, TECHNOLOGY PART B - in preparation.
- S. A. Goldblith, L. Rey, and W. W. Rothmayr, FREEZE DRYING AND ADVANCED FOOD TECHNOLOGY. 1975.
- R. B. Duckworth (ed.), WATER RELATIONS OF FOOD. 1975.
- A. G. Ward and A. Courts (eds.), THE SCIENCE AND TECHNOLOGY OF GELATIN. 1977.

The Science and Technology of Gelatin

Edited by

A. G. WARD

Proctor Department of Food and Leather Science,
The University of Leeds, Leeds, England

A. COURTS

Imperial College of Science and Technology,
London, England

1977

ACADEMIC PRESS

London New York San Francisco

A Subsidiary of Harcourt Brace Jovanovich, Publishers



ACADEMIC PRESS INC. (LONDON) LTD
24-28 Oval Road,
London NW1

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue,
New York, New York 10003

Copyright © 1977 By ACADEMIC PRESS INC. (LONDON) LTD

All Rights Reserved

No part of this book may be reproduced in any form by photostat, microfilm, or any other means, without written permission from the publishers

Library of Congress Catalog Card Number: 74 5672
ISBN: 0 12 735050 0

Printed in Great Britain by
Adlard & Son Ltd
Bartholomew Press, Dorking, Surrey

Contributors

- G. BALIAN, University of Washington, Seattle, Washington, U.S.A.
J. H. BOWES, Retired.
R. C. CLARK, 321 Main Street, Waterkloof, Pretoria, South Africa.
A. COURTS, Imperial College of Science and Technology, London SW7, England.
J. E. EASTOE, Department of Dental Science, Royal College of Surgeons of England, Lincoln's Inn Fields, London W.C.2, England.
C. A. FINCH, Croda Polymers Limited, 153 New Bedford Road, Luton, Bedfordshire, England.
R. HINTERWALDNER, 8 Munchen 90, Postfach 90 0425, Germany.
A. JOBLING, Croda Polymers Limited, 153 New Bedford Road, Luton, Bedfordshire, England.
P. JOHNS, Department of Experimental Pathology, University of Birmingham, England.
N. R. JONES, British Food Manufacturing Industries Research Association, Randalls Road, Leatherhead, Surrey, England.
A. M. KRAGH, Ilford Limited, Ilford, Essex, England.
A. A. LEACH, "Melbrae", Millers Lane, Outwood, Redhill, Surrey, England.
G. STAINSBY, Procter Department of Food and Leather Science, The University of Leeds, Leeds, Yorkshire, England.
F. W. WAINWRIGHT, Canada Packers Limited Research and Development Division, 2211 St. Clair Avenue West, Toronto, Ontario, Canada.
P. D. WOOD, Gelatine and Glue Research Association, Warwick Street, Birmingham 12, England.

Foreword

The present volume was originally conceived when many of the authors of the chapters were, or had recently been, members of the staff of the British Gelatine and Glue Research Association (BGGRA). This institute was founded in 1948 to serve the collective research needs of the U.K. gelatin and animal glue industries and of firms using these products. The term "British" was later dropped from its title when a number of overseas firms were included in its membership, and which quickly became a force in the councils of the organization.

After 22 years of independent existence, the GGRA was incorporated into the British Food Manufacturing Industries Research Association. During its life as the (B)GGRA, it had three Directors of Research, two of whom are the editors of this volume.

Those authors having a period of service with the (B)GGRA are Dr. Clark, Dr. Eastoe, Dr. Johns, Mr. Kragh, Dr. Leach, Dr. Stainsby, Mr. Wainwright and Mrs. Woods. Authors who have been on the staff of sister research associations are Dr. Balian, Dr. Bowes and Mr. Jones, while Drs. Finch and Jobling have worked in the U.K. gelatin and glue industry and Dr. Hinterwaldner is a consultant who contributed to the 1957 Cambridge International Conference of the BGGRA.

Few people with knowledge of this highly specialist area would wish to argue other than that the Research Association has made a significant contribution to advances in the study of gelatin and collagen fundamental structure and properties over the last 25 years. It has also played a leading role in the development of testing standards for gelatin and glue which have proved meaningful for manufacturer and user alike. This volume is intended, in part, to consolidate these contributions, but it gives equal place to the many advances made in this subject by academic and industrial scientists.

The inevitable problems of preparing a collective volume with several authors have delayed publication longer than was first hoped. This is especially true for collagen research where recent progress has been much more rapid than with gelatin. An example of this progress is the $\alpha 1$ chain sequence analysis overpage, published by Hulme *et al.* and referred to in Chapter 4.

A. G. Ward
A. Courts

pGlu-Met-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys-Ser-Ala-Gly-Val-Ser-Val-Pro-

1 Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
28 Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Met-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Lys-Asn-Gly-Asp-Asp-
55 Gly-Glu-Ala-Gly-Lys-Pro-Gly-Arg-Hyp-Gly-Gln-Arg-Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Leu-Hyp-Gly-Thr-Ala-
82 Gly-Leu-Hyp-Gly-Met-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Asp-Gly-Ala-Lys-Gly-Asn-Thr-Gly-Pro-Ala-Gly-Pro-Lys-
109 Gly-Glu-Hyp-Gly-Ser-Hyp-Gly-Glx-Asx-Gly-Ala-Hyp-Gly-Gln-Met-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Glu-Arg-Gly-Arg-Hyp-
136 Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ala-Arg-Gly-Asp-Asp-Gly-Ala-Val-Gly-Ala-Ala-Gly-Pro-Hyp-Gly-Pro-Thr-Gly-Pro-Thr-
163 Gly-Pro-Hyp-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Ala-Lys-Gly-Glu-Ala-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Ser-Glu-Gly-Pro-Gln-
190 Gly-Val-Arg-Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Ala-Ala-Gly-Pro-Ala-Gly-Asn-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-
217 Gly-Ala-Lys-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Ile-Ala-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Ala-Arg-Gly-Pro-Ser-Gly-Pro-Gln-
244 Gly-Pro-Ser-Gly-Ala-Hyp-Gly-Pro-Lys-Gly-Asn-Ser-Gly-Glu-Hyp-Gly-Ala-Hyp-Gly-Asn-Lys-Gly-Asp-Thr-Gly-Ala-Lys-
271 Gly-Glu-Hyp-Gly-Pro-Ala-Gly-Val-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Glu-Gly-Lys-Arg-Gly-Ala-Arg-Gly-Glu-Hyp-
298 Gly-Pro-Ser-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Glu-Arg-Gly-Gly-Hyp-Gly-Ser-Arg-Gly-Phe-Hyp-Gly-Ala-Asp-Gly-Val-Ala-
325 Gly-Pro-Lys-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Ser-Hyp-Gly-Pro-Ala-Gly-Pro-Lys-Gly-Ser-Hyp-Gly-Glu-Ala-Gly-Arg-Hyp-
352 Gly-Glu-Ala-Gly-Leu-Hyp-Gly-Ala-Lys-Gly-Leu-Thr-Gly-Ser-Hyp-Gly-Ser-Hyp-Gly-Pro-Asp-Gly-Lys-Thr-Gly-Pro-Hyp-
379 Gly-Pro-Ala-Gly-Gln-Asp-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Ala-Arg-Gly-Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp-
406 Gly-Pro-Lys-Gly-Ala-Ala-Gly-Glu-Hyp-Gly-Lys-Ala-Gly-Glu-Arg-Gly-Val-Hyp-Gly-Pro-Hyp-Gly-Ala-Val-Gly-Pro-Ala-
433 Gly-Lys-Asp-Gly-Glu-Ala-Gly-Ala-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Glu-Gln-Gly-Pro-Ala-
460 Gly-Ser-Hyp-Gly-Phe-Gln-Gly-Leu-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Glu-Ala-Gly-Lys-Hyp-Gly-Glu-Gln-Gly-Val-Hyp-
487 Gly-Asp-Leu-Gly-Ala-Hyp-Gly-Pro-Ser-Gly-Ala-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-Pro-Hyp-
514 Gly-Pro-Ala-Gly-Pro-Arg-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Asn-Asp-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Ala-Hyp-Gly-Ala-Hyp-
541 Gly-Ser-Gln-Gly-Als-Hyp-Gly-Leu-Gln-Gly-Met-Hyp-Gly-Glu-Arg-Gly-Ala-Ala-Gly-Leu-Hyp-Gly-Pro-Lys-Gly-Asp-Arg-
568 Gly-Asp-Ala-Gly-Pro-Lys-Gly-Aln-Asp-Gly-Ala-Pro-Gly-Lys-Asp-Gly-Val-Arg-Gly-Leu-Thr-Gly-Pro-Ile-Gly-Pro-Hyp-
595 Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Asp-Lys-Gly-Glu-Ala-Gly-Pro-Ser-Gly-Pro-Ala-Gly-Thr-Arg-Gly-Ala-Hyp-Gly-Asp-Arg-
622 Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Phe-Ala-Gly-Pro-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-Gly-Ala-Lys-Gly-Glu-Hyp-
649 Gly-Asp-Ala-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Pro-Ile-Gly-Asn-Val-
676 Gly-Ala-Hyp-Gly-Pro-Hyl-Gly-Ala-Arg-Gly-Ser-Ala-Gly-Pro-Hyp-Gly-Ala-Thr-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Arg-Val-
703 Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Asn-Ala-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Lys-Glu-Gly-Ser-Lys-Gly-Pro-Arg-
730 Gly-Glu-Thr-Gly-Pro-Ala-Gly-Arg-Hyp-Gly-Glu-Val-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Lys-Gly-Ala-Hyp-
757 Gly-Als-Asp-Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Thr-Pro-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp-
784 Gly-Gln-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Pro-Ser-Gly-Glu-Hyp-Gly-Lys-Gln-Gly-Pro-Ser-Gly-Ala-Ser-
811 Gly-Glu-Arg-Gly-Pro-Hyp-Gly-Pro-Met-Gly-Pro-Hyp-Gly-Leu-Ala-Gly-Pro-Hyp-Gly-Glu-Ser-Gly-Arg-Glu-Gly-Ala-Hyp-
838 Gly-Ala-Glu-Gly-Ser-Hyp-Gly-Arg-Asp-Gly-Ser-Hyp-Gly-Ala-Lys-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-Gly-Ala-Hyp-
865 Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Ala-Hy-Gly-Pro-Val-Gly-Pro-Ala-Gly-Lys-Ser-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-
892 Gly-Pro-Ile-Gly-Pro-Val-Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly-Pro-Gln-Gly-Pro-Arg-Gly-Asx-Hyl-Gly-Glx-Thr-
919 Gly-Glx-Glx-Gly-Asx-Arg-Gly-Ile-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Gln-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Ser-Hyp-
946 Gly-Glu-Gln-Gly-Pro-Ser-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ser-Hyp-Gly-Lys-Asp-
973 Gly-Leu-Asn-Gly-Leu-Hyp-Gly-Pro-Ile-Gly-Hyp-Hyp-Gly-Pro-Arg-Gly-Arg-Thr-Gly-Asp-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-
1000 Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Pro-

calf skin

Ser-Gly-Gly-Tyr-Asp-Leu-Ser-Phe-Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Lys-Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr

The amino acid sequence of the $\alpha 1$ chain from rat (residues 1–402) and calf (residues 403–1011) skin collagen. The N-terminal (from rat) and the C-terminal (from calf) non-helical regions are separated from the triplet region and are not numbered. From Hulme, D. S. S. *et al.* (1973) *J. Molec. Biol.* 79.

Introduction

A. G. Ward and A. Courts

The conversion of the collagenous component of connective tissue (skin, bone, sinew) into a soluble, gel forming material, either as a food component or as a glue, dates back to prehistory. The preparation of clear jellies, using egg white for clarification, was long a cherished art of the housewife until industrial production of gelatin rendered it unnecessary. The range of textures provided by gelatins of different manufacture, the ease of solution and the thermal reversibility of the changes on gelation, tempted the decorative and culinary skills of chefs. Their simpler inventions in due course became the factory products of the food industry. So intriguing a material also early caught the imagination of scientists, and gelatin played a major role in the development of colloid chemistry.

Gelatin is used as a commodity because of its gel forming, film forming and surface active properties. It was perhaps in the development of photographic emulsions that the widest range of these properties, was employed. Formation of an even film coating, the control of silver halide crystallisation, power of swelling after drying, hardening properties and the presence of traces of compounds directly affecting the sensitivity of halide grains made possible the form of photography with which we are familiar. Although today knowledge of photographic chemistry has reduced the dependence of the photographic industry on the sensitizing characteristics of gelatin, as yet it has not proved possible to match synthetically the complete range of properties needed for photographic use.

Not surprisingly, much of the early research on gelatin properties was carried out or stimulated by scientists in the photographic industry. In the interwar years, S. E. Sheppard and co-workers (1921, 1932) of Eastman Kodak determined many of the rheological properties, their dependence on pH, temperature, concentration and salts present and the changes in these properties resulting from thermal degradation.

The rapid development of technique and theory for the study of macromolecules in solution from 1920 to 1950 enabled gelatin to be characterized as a macromolecular substance, with a polypeptide chain virtually identical in composition with the collagen from which it was made. It was expected that the solution and gel properties would depend, at least in large measure, on molecular weight. For solution viscosity this was shown to be so, especially

in the work of J. Pouradier and A. M. Venet (1952). J. D. Ferry (1948) established for low molecular weight gelatins (M_n 45,000 and less) that gel "strength" (i.e. elasticity modulus of the gel under constant conditions) depended on molecular weight. Meanwhile the advance of protein chemistry, especially the refined techniques for amino acid analysis of A. C. Chibnall, allowed a reasonably accurate picture of the amino acid composition to be established.

It was clear to gelatin chemists concerned with the manufacture of gelatin that the process of gelatin making and the properties of the gels of gelatins of differing manufacture and source material were too complex to be explained by molecular weight changes alone.

The use of two distinct manufacturing processes provides a first point of difference. One involves extraction of the gelatin at a neutral or slightly acid pH, after cold alkaline pretreatment of the raw material. The second process, applied especially to pigskin, uses a brief soak in acid followed by extraction at a moderate temperature at about pH 4. The work of W. M. Ames (1953) in particular showed that the pretreatment with cold alkali hydrolysed ammonia from glutamine and asparagine residues leaving collagen with an isoionic point, pI, of 4.8 to 5.0. Acid pigskin gelatins retained glutamine and asparagine so giving a high pI, later shown to be as high as 9.0 to 9.3.

It had long been known that fish glue from fish skin and bone or even a fish gelatin made in the laboratory lacked much of the gelling ability of mammalian gelatins. In contrast, fish collagens of cartilaginous fish gave gelatins of better gelling power. These anomalies were shown by the amino acid determinations of J. E. Eastoe, A. A. Leach (1958) and others to relate to the contents of the amino acids, proline and hydroxyproline. A low content indicated poor gelling power.

The combination of the fractionation techniques and molecular weight determinations of G. Stainsby (1954) with gel rigidity studies by P. R. Saunders and A. G. Ward (1955, 1958) of these fractions and of materials degraded by heat or enzymes showed conclusively that gel rigidity for gelatins of molecular weight more than c. 50,000 hardly depended at all on molecular weight, but was determined by some unknown structural feature, which was reduced in its gelling effect by neutral or alkaline thermal degradation. A. Courts (1959) also showed that, if the gelatins had multichain molecules, the gelling power did not depend on the average chain length. This problem of the identity of the structural feature controlling gel strength has still, despite extensive further structural information (see A. Veis, 1964), not been fully resolved. An indication that it may arise from the scission of the polypeptide chains at the specific points of the sequence most involved in the initiation of gel formation has been put forward by G. Stainsby and R. J. A. Grand (1973). So there are still practical and theoretical problems

to be resolved in explaining the behaviour of the varied types of gelatin. The combination of the sequencing studies of K. A. Piez and others, with the physical studies on gelatins derived from homogeneous collagen molecules may greatly aid this work.

Side by side with the advances in knowledge of the structure and properties of gelatin has been some progress in understanding and improving the conversion of collagen to gelatin without loss of useful properties. Although mature mammalian collagens contain a small soluble collagen component, convertible to gelatin by heating briefly at 40–45°C, the bulk of the collagen in skin, bone and sinew is prevented from dissolving by covalent crosslinks whose frequency, in general, increases with age. Treatment at ambient temperatures with lime suspensions removes many of these crosslinks but the process is slow and the accompanying rupture of peptide bonds results in progressive losses of collagen into the lime pretreatment liquors. Young pigskin has fewer crosslinks and so responds to a mild acid extraction process with little pretreatment.

In making gelatin from collagen, the process must (i) separate impurities in the raw material from the gelatin produced, (ii) free collagen fragments of suitable size by breaking crosslinks and/or peptide bonds, (iii) leave untouched the gelling power of the gelatin obtained, (iv) break stabilizing hydrogen bonds (e.g. by raising the temperature) to allow fragments to pass into solution. New short duration processes have made possible the conversion of a high proportion of collagen into gelatin of high gelling power and of sufficiently high molecular weight. An attempt to achieve this was made by the Deutsche Gelatin Fabriken Co. (1952), using a high concentration of sodium sulphate to limit the action of hot sulphuric acid on collagen in the form of skin or demineralized bone. A temperature of 80°C was used for some 15–30 minutes. The hot acid treated skin or demineralized bone swelled extensively on washing out the acid and salt, showing the reduction in crosslinking and bonding which had occurred. Gelatin was then readily obtained by warming the treated collagen with water. This process, although reproducible in the laboratory, was difficult to apply on a works scale.

A. G. Ward (1953) developed a pretreatment process with saturated sodium sulphate and 1.5 to 7 per cent caustic soda for 2 to 16 days at 12–27°C. The more extensive pretreatments made washing difficult as the collagen swelled extensively to give a very fragile material. By choosing optimal pretreatment conditions, high yields of high grade gelatin were obtained. This process and variants derived from it have since been applied to industrial production. A. Courts and G. Stainsby (1959) have used it to pretreat collagen to enable high yields of solubilized collagen (eu collagen) to be obtained, resembling in many respects neutral and acid soluble collagens. In these soluble collagens, the final stage of conversion to gelatin, rupture of

hydrogen bonds, has not occurred but gentle warming allows it to take place. Other attempts to solve the same problems have involved the use of enzymes and of combined acid/alkali pretreatments.

Before 1950 most studies on collagen and gelatin originated from the leather, gelatin and gelatin using industries or from scientists using gelatin as a model colloidal system. Today the importance of collagen, which constitutes about one third of the protein of the human body and which is the major factor in the so-called collagen diseases, justifies major biochemical research efforts in many laboratories. The information from these researches, although referring in the first instance to collagen, is also relevant to gelatin research. It will also exert a powerful influence on the manufacture of the commercial product, gelatin, by suggesting new methods or a more efficient use of old methods, to effect the collagen gelatin conversion. Similarly, a more efficient utilization of gelatin has become possible, whilst a recognition of its chemical reactivity could lead to uses which had not previously been considered.

REFERENCES

Note. Only a selection of key references are given here.

- Ames, W. M. (1953). *J. Sci. Fd. Agric.* **3**, 454.
Courts, A. and Stainsby, G. (1959) in Ward, A. G. (1959). *Nature, Lond.* **183**, 440.
Courts, A. (1959). *Biochem. J.* **73**, 596.
Eastoe, J. E. and Leach, A. A. (1958) In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 193, Pergamon Press, London.
Ferry, J. D. (1948). *Adv. Protein Chem.* **4**, 21.
Pouradier, J. and Venet, A. M. (1952). *J. Chim. phys.* **49**, 238.
Saunders, P. R. and Ward, A. G. (1955). *Nature, Lond.* **176**, 26.
Saunders, P. R. and Ward, A. G. (1958) In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 197, Pergamon Press, London.
Sheppard, S. E. and Houck, S. S. (1932). *J. phys. Chem.* **36**, 2319.
Sheppard, S. E. and Sweet, S. S. (1921). *J. Am. chem. Soc.* **43**, 539.
Stainsby, G. (1954). *Disc. Far. Soc.* **18**, 288.
Stainsby, G. and Grand, R. J. A. (1973). Private communication, see also Grand, R. J. A. (1972). Thesis "The N-terminal imino acids of gelatin", University of Leeds.
Veis, A. (1964). "The Macromolecular Chemistry of Gelatin", Academic Press, New York.

Contents

Contributors	v
Foreword	vii
Introduction	ix

1. The Structure and Properties of Collagen

G. BALIAN AND J. H. BOWES

I Introduction	1
II Composition	2
III Molecular Structure and Fibril Formation	4
IV Properties and Subunit Structure of Soluble Collagens	10
V Reactivity and Stability	20
VI Summary	26
References	27

2. The Structure and Composition of Collagen Containing Tissues

P. JOHNS

I Introduction	32
II Composition of Mammalian Tissue	33
III Skin and Hide	35
IV Bone	48
V Tendon	61
VI Cartilage	63
VII Other Mammalian Collagen Tissues	64
VIII Non mammalian Collagens	65
References	66

3. Chemical Constitution of Gelatin

J. E. EASTOE AND A. A. LEACH

I Introduction	73
II Influence of Collagen Source on Gelatin Composition	77
III Relationship of Gelatin Composition to that of Parent Collagen	83
IV The Main Protein Component—Gelatin	91
V Contaminants	100
References	105

4. The Physical Chemistry of Gelatin in Solution

G. STAINSBY

I Introduction	109
II Solubility	111
III Dilute Solutions	113
IV Concentrated Solutions	128
References	135

5. Relationship Between Collagen and Gelatin

P. JOHNS AND A. COURTS

I Introduction	138
II Preparation of Raw Material	138
III Breakage of Non-covalent Bonds	140
IV Formation of Non-covalent Bonds	146
V Mechanical Breakage of Collagen Tissue	151
VI Breakage of Intermolecular Crosslinks	151
VII Breakage of Crosslinks and Non-covalent Bonds	156
VIII Formation of Covalent Crosslinks	166
IX Breakage of Peptide Bonds	167
References	173

6. The Gelatin Gel and the Sol-Gel Transformation

G. STAINSBY

I Introduction and Scope	179
II Factors Influencing Gelation	181
III The Events in Gelation	186
IV A Theory of Gelation	203
V Isoelectric Gels	205
References	206

7. The Chemical Reactivity of Gelatin

R. C. CLARK AND A. COURTS

I Introduction	209
II The Constitution and Occurrence of the Side-chain Groups in Gelatin	210
III Gelatin Modification Reactions	212
References	241

8. The Physical Properties of Gelatin

C. A. FINCH AND A. JOBLING

I Introduction	250
II Swelling and Solubility	251
III Mechanical Properties	260
IV Diffusion through Gelatin Films and Gels	273
V Optical Properties	275
VI Electrical Properties	279
VII Thermal Properties	283
VIII Surface Active Properties	286
References	288

9. Raw Materials

R. HINTERWALDNER

I General Aspects	295
II Preparation of Bones	297
III Preparation of Hide Stock	306
IV Preparation for Extraction	307
References	313

10. Technology of Gelatin Manufacture

R. HINTERWALDNER

I Special Methods of Gelatin Manufacture	315
II General Operating Procedures of Commercial Manufacture of Gelatin	320
References	361

11. Uses of Gelatin in Edible Products

N. R. JONES

I Quality Standards	366
II Properties Important in Choice of Gelatins	367
III Behaviour of Gelatin in Relation to Food Use	372
IV Functions of Gelatin in Food	373
V Handling of Gelatin	374
VI Use of Gelatin as a Jellying Agent	376
VII Use of Gelatin as a Whipping Agent	384
VIII Use of Gelatin as a Stabilizer	388
IX Use of Gelatin as an Emulsifier	390
X Use of Gelatin to Increase Viscosity	390
XI Use of Gelatin as an Adhesive	391
XII Use of Gelatin as a Binder	391
XIII Use of Gelatin as a Fining Agent	391
References	392

12. Uses of Collagen in Edible Products

A. COURTS

I Sausage Skins	396
II Clarifying Reagent for Beer	405
References	412

13. Technical and Pharmaceutical Uses of Gelatine

P. D. WOOD

I Introduction	414
II Pharmaceutical and Medical Uses	415
III Microencapsulation	419
IV Sizing of Paper	422
V Emulsification	424
VI Flocculation	425

VII Printing Applications	426
VIII Coated Abrasives	429
IX Adhesive Uses	430
X Miscellaneous Uses	433
References	436

14. Swelling, Adsorption and the Photographic Uses of Gelatin

A. M. KRAGH

I Introduction	439
II Properties of Gelatin—Importance in Photography	440
III General Constitution of Photographic Films	460
IV Use of Gelatin in Emulsion Technology	461
V Conclusions	470
References	471

15. The Chemical Examination of Gelatins

A. A. LEACH AND J. E. EASTOE

I Introduction	476
II Measurement of the Gelatin Content of Solids and Solutions	476
III Amino Acid Analysis of Gelatins	481
IV Examination of the End Groups of Protein Chains	486
V Examination of Side Chain Groups	488
VI Examination of Other Constituents of the Gelatin Molecule	491
VII Examination of Chemically Modified Gelatins	493
VIII Gelatin Contaminants	496
References	502

16. Physical Tests for Gelatin and Gelatin Products

F. W. WAINWRIGHT

I Introduction	508
II Methods for Determining Gelatin Gel Strength	508
III Dessert Gel Strength Tests	519
IV Viscosity Determinations	521
V Gelatin Viscosity Degradation Test	525
VI Methods for Determining Gelatine pH	527
VII Methods for Determining the Gelatin Isoionic Point	528
VIII The Determination of Gel Melting Point	529
IX Gelation Time Tests	531
References	533
Author Index	535
Subject Index	557

Chapter 1

The Structure and Properties of Collagen

G. BALIAN

Department of Animal Husbandry, University of Bristol

AND

J. H. BOWES

*British Leather Manufacturers' Research Association,
Milton Park, Egham, Surrey*

I	Introduction	1
II	Composition	2
	A. Amino Acid Composition	2
	B. Other Constituents	4
III	Molecular Structure and Fibril Formation	4
	A. X-ray Diffraction Studies	5
	B. Electron Microscope Studies	7
IV	Properties and Subunit Structure of Soluble Collagen	10
	A. Physicochemical Studies	10
	B. Subunit Structure	11
	C. Nature and Location of Crosslinks	17
V	Reactivity and Stability	20
	A. Functional Groups	20
	B. Stability to Chemicals and Enzymes	21
	C. Denaturation	23
	D. Swelling	25
VI	Summary	26
	References	27

I. INTRODUCTION

Collagen is an important constituent of the supporting structures of both vertebrates and invertebrates. In the mammal it is the most abundant body protein forming the major protein constituent of skin, tendon, cartilage, bone and connective tissue generally. In birds and fish it plays a similar role and in the invertebrates it is an important constituent of the body wall.

In addition to being of great physiological importance collagen is also the basic raw material of the leather and the gelatin and glue industries. It is not surprising, therefore, that it has attracted the interest of many workers.

Collagen, *in vivo*, generally occurs as white, opaque, nonbranching fibrils

embedded in a matrix of mucopolysaccharide and other proteins, the amounts of these depending on the type of tissue and the age of the animal. The fibres can be readily recognized by histological staining techniques, their tendency to swell and the sharp contraction which they undergo when heated to about 60°C.

Regardless of the species and tissue from which they come, collagen fibres have many features in common. Firstly, an amino acid composition in which glycine constitutes approximately one-third of the total residues and the imino acids proline and hydroxyproline a further 15–30%. Secondly, a wide angle X-ray pattern with a characteristic set of spacings, in particular a meridional reflection at 2.9Å and an approximately 11Å hydration sensitive reflection on the equator and thirdly, a characteristic banded structure when viewed in the electron microscope.

Several reviews dealing with various aspects of collagen structure have appeared in recent years, e.g. Harrington and von Hippel (1961), Harding (1965), Bailey (1968a) and Piez (1968); also the first two volumes of an extensive work, "Treatise on Collagen" edited by Ramachandran containing chapters by many of the prominent workers in the collagen field. The present authors have drawn freely on these works and are much indebted to the contributors for lightening their task and enabling them to concentrate on the more recent advances.

II. COMPOSITION

A. Amino Acid Composition

Numerous complete amino acid analysis of collagen from a variety of tissues have been made in recent years and results are now available for nearly every class of vertebrates and for a number of invertebrates also (see Tables I–IV, pp. 78–81). In view of the difficulties inherent in the purification of an insoluble protein, analyses made on preparations of the soluble collagens and on gelatin are probably the most reliable. However, even with these, too much significance should not be placed on small variations from one preparation to another.

The chief features of the amino acid composition of collagen are an unusually high content of glycine and of the imino acids, proline and hydroxyproline, and very small amounts of the aromatic and sulphur containing amino acids. Hydroxyproline is of very limited occurrence in proteins, the only other mammalian protein in which it occurs being elastin (2%). Collagen is also the only protein reported to contain more than about 0.1% hydroxylysine.

At one time the small amounts of tyrosine present were thought to arise

from impurities but the isolation by Grassmann *et al.* (1960) of a large peptide containing both tyrosine and hydroxyproline showed that it formed part of the basic molecule and more recent studies indicate that some collagens contain about 3 residues of tyrosine per 1000 residues. Traces of cystine found in some collagen preparations probably arise from contamination with other proteins, notably keratin in the case of skin.

However, basement membrane collagen is unusual in that it contains cystine as an integral part of the molecule (Kefalides, 1968).

The amino acid composition of the mammalian collagens is remarkably constant. Glycine, the simplest amino acid accounts for about one-third of the total residues, proline and hydroxyproline for about one-fifth and alanine for about one-ninth. In all, these four account for approximately two out of every three amino acid residues in collagen.

Limited analyses of avian collagens indicate that their composition is similar to that of mammalian collagen. Fish collagens show a wider variation in composition, their hydroxyproline and, to a lesser extent, proline contents are lower than that of mammalian collagens and this is compensated for by higher concentrations of the other hydroxyamino acids, serine and threonine. The composition of amphibian and reptile collagens is also rather variable but in the main it is intermediate between that of mammals and fish. Invertebrate collagens show still wider variations but the general pattern of amino acid composition is still clearly recognizable as that of the collagen group. For a full account of the amino acid composition of collagen and gelatin from various sources (see Eastoe, 1967).

Partial hydrolysis studies using acid or enzymes have demonstrated the widespread distribution of glycine, and the frequent occurrence in the polypeptide chain of the sequence Gly-Pro-X, and in particular of the tripeptide Gly-Pro-Hypro. (for further details of sequence studies see Hannig and Nordwig, 1967).

The general picture which emerges is of a chain built up of sequences of peptides containing predominantly neutral amino acids, alternating with more polar sequences containing acidic and basic amino acids. Through much of the chain glycine constitutes every third residue and Gly-Pro-X is the most frequently occurring sequence in the neutral areas.

The alternating areas of polar and nonpolar amino acids can also be distinguished by electron microscopy using suitable differential staining techniques. It is encouraging to find that in the segment long spacing form of precipitated collagen the width of the light non-staining bands corresponding to the nonpolar areas, is of the same order as the length of the neutral sequences as estimated from molecular weight determinations on the isolated neutral peptides, namely 17–85Å (Grassmann *et al.*, 1963) and 35Å (Funakoshi and Noda, 1964).

B. Other Constituents

The presence of hexosamines and hexoses in collagen preparations has frequently been reported. As collagen generally occurs in association with mucopolysaccharides and mucoproteins from which separation is a problem, it is difficult to decide whether they form an integral part of the molecule or not.

Extensive purification of soluble collagens by reprecipitation reduces the hexosamine content to negligible proportions and it is now generally considered that hexosamines are not linked to collagen. With hexoses, however, the situation is different. It has proved impossible to reduce the hexose content below about 0.5% and the presence of about twelve residues of hexose per collagen macromolecule, molecular weight about 300,000, appears to be established.

Butler and Cunningham (1966) have isolated a glycopeptide from the acid-soluble collagen of guinea-pig skin having the composition, Gly-Met-Hyl-(Gal. Glc)-Gly.His.Arg thus establishing, without doubt, that hexoses form an integral part of the molecule. Cunningham and Ford (1968) followed this with the isolation of a further heterogeneous peptide fraction from insoluble collagen containing glucose, galactose, mannose and glucosamine. In view of the difficulties of obtaining a pure preparation of insoluble collagen, it is perhaps open to question whether this fraction really originates from the collagen. More recently however, Morgan *et al.* (1970) have isolated homologous peptides from carp swim bladder and human collagens.

Small amounts of covalently bound phosphate have been found in the hard tissue collagens and the possibility of the involvement of this phosphate in the crosslinking of hard tissue collagen and in nucleation of mineralization has been the subject of speculation (see Veis, 1967).

III. MOLECULAR STRUCTURE AND FIBRIL FORMATION

Present knowledge of the structure of collagen is mainly derived from detailed X-ray diffraction and electron microscope studies. Such work has been greatly facilitated by the recognition of the existence of soluble forms of collagen and the discovery that these could be reprecipitated in various forms according to the conditions used.

Any proposed structure for collagen at the molecular level must firstly provide a reasonable explanation of the characteristic X-ray diffraction pattern and secondly, it must be able to accommodate the high proportion of proline and hydroxyproline known to be present and which, by reason of their stereochemical configuration, impose certain restrictions on the conformation of the polypeptide chain.

A. X-ray Diffraction Studies

The principal features of the wide-angle X-ray diffraction pattern are the 2.86\AA meridional arc and the 11\AA equatorial reflection. Further arcs can also be seen at 9.5\AA and 4.0\AA . The 11\AA equatorial spacing is sensitive to hydration and is related to the lateral separation of the molecules.

There is also a small angle X-ray spacing of about 640\AA along the fibre axis but as will be seen later this is associated with the mode of packing of the collagen macromolecules rather than their structure (see page 8).

In the years preceding 1950 various structures were proposed for collagen but the studies of Pauling and Corey (1951) on bond angles and lengths and on the coplanar nature of the peptide grouping in proteins together with data on the distances of closer approach showed these to be untenable. Following the publication of a greatly improved X-ray diffraction pattern obtained by stretching the collagen (Cowan *et al.*, 1953), a number of similar structures were proposed almost simultaneously by three groups of workers (Ramachandran and Kartha, 1954, 1955; Rich and Crick, 1955; and Cowan *et al.*, 1955) and upon these the present generally accepted model for collagen is based.

Essentially the model is made up of three polypeptide chains each coiled in a three-fold left-handed helix with three residues per turn. These three helices are arranged parallel to each other so that when viewed from above their axes are set at the corners of an equilateral triangle of side 5\AA and then

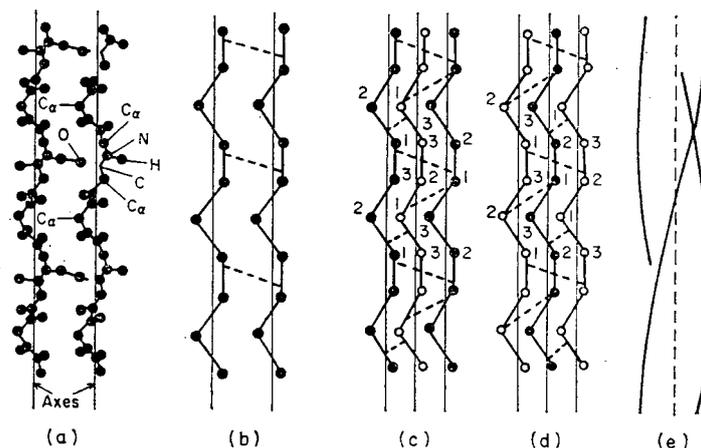


FIG. 1. Diagram illustrating the formation of the collagen model from the polyglycine II lattice. (a) Two polypeptide chains each helically wound with a left-handed three-fold screw axis. (b) simplified version of (a) showing only α carbon atoms. (c) third chain added behind as in collagen I structure. (d) third chain added in front as in collagen II. (e) showing twisting of the minor axis into the collagen super helix. Intrachain H-bonds are shown dotted (from Rich and Crick, 1961).

further twisted about a common axis to give a slight right-handed super helix with an overall repeat distance of about 86Å and a translation of 2.86Å from a residue on one chain to the corresponding residue on another, thus accounting for the strong meridional X-ray reflection (see Fig. 1).

Two structures are possible, one in which the third chain is placed behind the other two (Fig. 1c) and the other with the third chain in front (Fig. 1d). Structure I will accommodate only glycine in positions 1 and 3 and any residue in position 2, including proline and hydroxyproline. With slight deformation, however, any residue except hydroxyproline or proline can be accommodated at 1 and all residues at 3. Structure II also, will only accommodate glycine in position 1 but any residue in positions 2 and 3. In structure I the hydroxyl groups of hydroxyproline project inwards and are thus available for intramolecular hydrogen bonding and in structure II projects outwards from the central axis. It has not been possible to distinguish between the two forms and both may exist in collagen. On the whole structure II is preferred on the grounds that it is stereochemically more favourable and will accommodate the Gly-Pro-Hydro sequence without deformation.

The maintenance of the rather steep three-fold left-handed helix characteristic of the individual peptide chains is considered to involve two factors. First, the steric hindrance to rotation imposed by the high content of pyrrolidine residues as exemplified by the structure of poly-l-proline II and secondly the occurrence of glycine at every third residue which allows the chains to come close enough for hydrogen bonding to occur as in poly-l-glycine. It may be noted here that, in contrast to proteins assuming the α -helical configuration, hydrogen bonds in collagen are at right angles to the main axis and hence are interchain (Ambrose and Elliott, 1951). This interchain hydrogen bonding of the glycine residues allows the poly-l-proline configuration to be maintained across regions of α -amino acids. Whether a second hydrogen bond is involved in triplets containing only one pyrrolidine residue is still open to question. In the original Rich and Crick model only one such bond was considered possible because of stereochemical restrictions but Ramachandran and co-workers have always maintained that there are two such bonds. Recent physicochemical studies on thermal denaturation and rates of deuterium and tritium exchange have now produced evidence in favour of the two bonded structure. (For full discussion of the molecular structure see Ramachandran, 1967.)

The wide-angle diffraction pattern is largely destroyed by dehydration but is partially restored by rewetting. This has led to suggestions that water may be involved in stabilizing the collagen structure. Rougvie and Bear (1953) and Esipova *et al.* (1958) both find that orientation increases with hydration and the latter workers suggest that the crystalline portions of the structure may be stabilized by doubly hydrogen-bonded water bridges between C=O

groups giving rise to continuous chains of structurally incorporated water along the fibre axis. From nuclear magnetic resonance and dielectric studies Chapman and McLaughlan (1969) also conclude that water exists in the form of continuous chains which may be fully extended or in a helical or other periodic form and Ramachandran and Chandrasekharan (1968) suggest water as an intermediary in interchain hydrogen bonding. Infrared studies (Bradbury *et al.*, 1958; Fraser and MacRae, 1959) also indicate that water is an integral part of the collagen structure.

B. Electron Microscope Studies

Native collagen fibrils, when suitably shadowed or stained show a characteristic banded structure when viewed in the electron microscope. Alternating light and dark bands can be seen with a marked axial period at about 640Å corresponding to the low angle X-ray meridional spacing. As early as 1945 Schmitt *et al.*, using electron dense stains such as phosphotungstic acid were able to distinguish five bands within each period and to show that the intraperiod banding was asymmetric about the midpoint. With improved techniques and higher resolution up to thirteen intraperiod bands have now been distinguished.

Improved resolution of the main banding has also been obtained by negative staining techniques demonstrating clearly the alternation of dense and less dense areas (see Fig. 3a).

Although collagen is essentially an insoluble protein small amounts can be extracted from some tissues, by neutral salt solutions and by dilute acids and the discovery that this soluble collagen could be precipitated in various forms by varying the conditions was the prelude to rapid advancement in electron microscope studies.

Precipitation from dilute acid solution with 1% sodium chloride gives fibrils with the characteristic banding pattern of native fibrils, addition of negatively charged compounds, such as glycoproteins or chondroitin sulphate, give fibrils with a symmetrical intraband pattern with a repeating unit of 2600–2800Å (fibrous long spacing or FLS) and under yet other conditions, notably, the addition of adenosine triphosphate, shorter, polarized aggregates of 2600Å (segment long spacing or SLS) can be obtained. Other forms with minor variations in their banding structure have also been obtained by varying salt concentration and pH. (For a summary of this work see Schmitt *et al.*, 1955.) Schmitt and his collaborators interpreted these findings in terms of a fundamental unit or macromolecule about 2600Å long and 15–20Å wide which they called tropocollagen. Their hypothesis was soon given support by the physicochemical studies of collagen in solution by Boedtker and Doty (1956) who reported the existence of rod-like particles of these dimensions

with a molecular weight of about 360,000 (see p. 17 for more recent estimates). From a study of the band patterns of the native, FLS and SLS type fibrils it was deduced that SLS represented the tropocollagen molecule in parallel array, while the FLS pattern arose from an anti-parallel arrangement in which the molecules were placed alternately head to head and tail to tail. The native pattern could then be obtained by postulating that each molecule overlaps its neighbour by a quarter of its length. Further support for this hypothesis came from more detailed studies of the staining of SLS and FLS with phosphotungstic acid, uranyl acetate and other metal salts. The presence of regions containing predominantly basic or acidic amino acids was clearly indicated and by multiple photographic exposure of the SLS pattern with appropriate displacement by one quarter length between exposures, Hodge and Schmitt (1960) obtained a simulated band pattern in close agreement with that of the native type of fibril. Kühn and Zimmer (1961) obtained the same results by similar addition of microdensitometer tracings of stained SLS. Further confirmation of this quarter stagger arrangement came from precipitation of SLS segments on native fibrils. The segments aligned themselves all in one direction along the fibril covering four repeat periods with their intra-band pattern in register with those of the native fibril (Hodge and Schmitt, 1960), (Fig. 2).

More careful measurements of the length of the SLS segments using improved negative staining techniques (Hodge and Petruska, 1962) showed that the length of the tropocollagen molecule was more than four times that of the native banding period indicating an end to end overlap of about 300\AA , i.e.

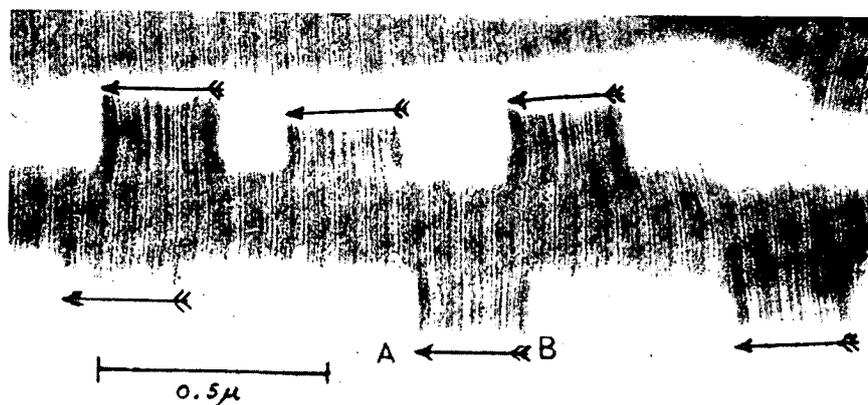


FIG. 2. Electron micrograph showing SLS type aggregates formed as outgrowths from the native fibril (stained with phosphotungstic acid). Each SLS aggregate covers approximately four repeat periods of the native fibril (from Hodge and Schmitt, 1960).

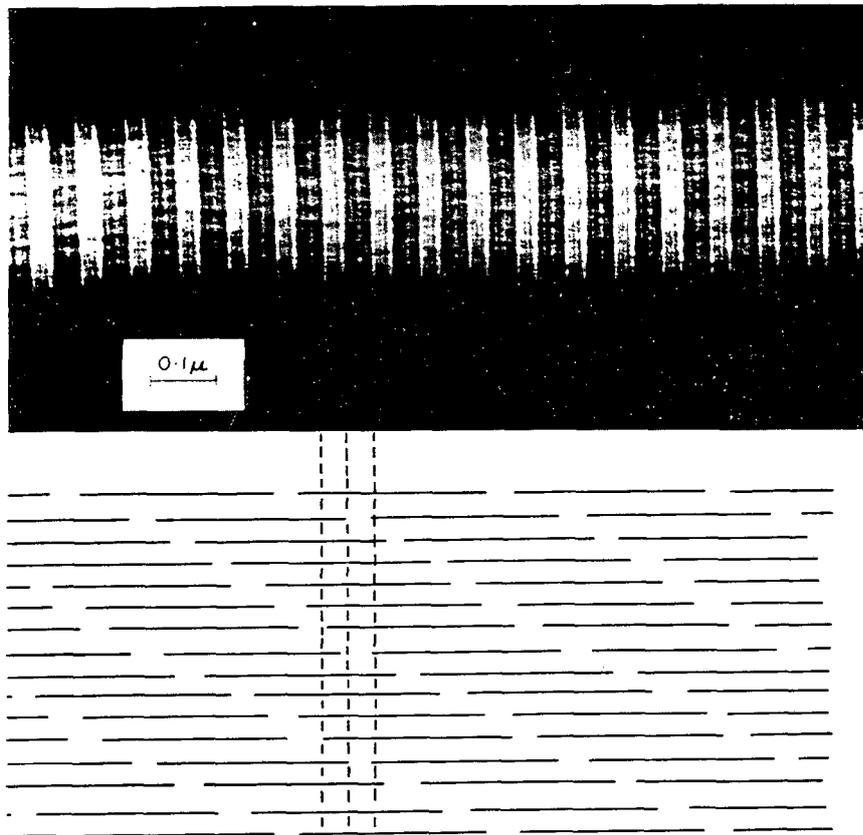


FIG. 3. (a) Native type fibril negatively stained with sodium phosphotungstate. (b) Two dimensional schematic representation of the packing arrangement of the tropocollagen molecules in the native fibril (Hodge *et al.*, 1965). Dashed lines indicate hole and overlap zones and their correspondence with dark and light staining zones.

about 0.4 of the native period. Olsen (1963) working independently came to similar conclusions. Later Petruska and Hodge (1964) pointed out that as a consequence of the non-integral relation between molecular length and the periodic structure found in native-type fibrils the only feasible structure for the native-type fibril was one in which there were "hole" and "overlap" regions within each period (see Fig. 3b). Indications of such areas can be seen in negatively stained electron micrographs (Fig. 3a).

Recently there has been some criticisms of the quarter stagger theory (McGavin, 1964) and Grant *et al.* (1965) have suggested an alternative model

based on the assumption that there are five bonding and four non-bonding zones along the molecule but so far no confirmatory evidence for this has been produced.

Smith (1965) has pointed out the limitations of the quarter stagger theory when applied to a three dimensional arrangement. In a fibre of average size only 66–68% of the contents can be of this type. However, this does not necessarily rule out the hypothesis particularly if the remaining 32–34% of the contacts are multiples of one quarter.

One factor governing precipitation of collagen in various forms is considered to be the “charge profile” of the molecule, i.e. the arrangement of the acidic and basic residues in the macromolecule. The addition of salts and charged compounds by modifying the charged centres allows different directive forces to come into play so affecting the form in which the fibrils are precipitated. A non-helical region at the N-terminus of the molecule (see p. 15) is also thought to play a part since degradation of this region by ultrasonication or by the action of enzymes impairs the ability to form native type fibrils and favours the formation of SLS fibres. Wood (1960) and Cassel *et al.* (1962) suggest fibril formation is a two stage process, first end to end aggregation of the molecules and then side by side arrangement in the quarter stagger array. They also find that there is a “lag phase” during which no precipitation occurs. This presumably represents a stage during which the collagen molecules form nuclei which then grow into fibrils by the addition of more molecules. Wood (1960) suggests that about three molecules are involved in these initial aggregates. The work of Fitton Jackson (1956) on fibril growth also suggests an initial core of about this size.

IV. PROPERTIES AND SUBUNIT STRUCTURE OF SOLUBLE COLLAGENS

A. Physicochemical Studies

1. *Size and shape*

Most of the work on physical properties has been carried out on acid soluble collagen purified from non-collagenous protein and mucopolysaccharide material by repeated precipitation and solution. Extensive studies on the size and shape of the molecule have been made using osmotic pressure, viscosity, light scattering, flow birefringence and sedimentation methods. The most recent of these indicates a rod-like molecule about 2800Å in length and 15Å in diameter with a molecular weight of rather less than 300,000. Each of the three chains making up the helix is the same length with a molecular weight of about 95,000. Vertebrate collagens from a variety of species

and tissues appear to be similar but limited results obtained on invertebrate collagens indicate that these may contain larger and less well defined soluble components. For a full summary and discussion of these physicochemical studies see Harrington and von Hippel (1961) and von Hippel (1967).

2. *Optical rotation*

Collagen has unusual optical rotatory properties, whereas most proteins in solution have a specific optical rotation, α_D , of about -30° to -60° , close to the mean residue rotation of their constituent amino acids, native collagen in solution has a value of -350° to -400° . This large negative rotation is primarily due to the conformation of the polypeptide chains resulting from the Gly-Pro-X sequences in the chain. The slight right-handed twist of the super helix appears to have little effect. It may be noted that the experimental value for the optical rotation of gelatin in solution at temperatures at which the chains have no helical content is close to that calculated from the amino acid composition.

Optical rotatory dispersion data have been shown to fit the single-term Drude equation $[\alpha]_\lambda = (A\lambda^2 - \lambda^2c)$ where α is the specific rotation at wavelength λ and A and c are the constants characteristic of the system.

The constants have been shown to be essentially the same for the helical, partially helical and random coil forms of collagen and gelatin at constant temperature, a finding applicable to few other proteins (von Hippel and Wong, 1963b). Changes in the specific rotation therefore give a direct measure of the helical content of the system and provide a very sensitive method for detecting conformational changes in solutions of collagen and gelatin. (See also Chapter 4 and von Hippel (1967).)

B. Subunit Structure

1. *Fractionation of denatured collagen*

When solutions of collagen are heated at about 40°C or above, denaturation occurs and the helical structure is lost (see p. 23). Examination of the products in the ultracentrifuge shows that two main components are obtained, one β , with a molecular weight twice that of the other, α . Most recent values for the molecular weight of the α component lie between 91,000 and 95,000 (Kang *et al.*, 1966; Piez *et al.*, 1968 and Katz *et al.*, 1969). Another component γ , which sediments faster than β and has a molecular weight similar to that of the tropocollagen molecule can also be separated. For fuller discussion see Piez (1967).

Chromatography on carboxymethyl cellulose leads to the separation of two α and two β components. The two α components, designated α_1 and α_2 differ

in amino acid composition. α_1 contains less histidine and slightly less hydroxylysine than α_2 and is consequently less basic. It, also, contains less tyrosine, fewer large hydrophobic side chains, i.e. valine, leucine and isoleucine residues, than α_2 and more hydroxyproline. From the relative amounts of the two β components and their differing composition, it is concluded that these are dimers of two α_1 chains and designated β_{11} , and of one α_1 and one α_2 chain, designated β_{12} . The tropocollagen molecule is made up of two α_1 and one α_2 chains designated γ_{112} . These findings were first demonstrated by Piez *et al.* (1961) for rat skin collagen, and further studies have shown that the same is true for soluble collagens from a variety of species and tissues (see Piez, 1967 for refs and details). With codfish collagen a third α chain, α_3 , differing very slightly from α_1 was isolated (Piez, 1965). In recent years cartilage collagen has been shown to consist predominantly of three identical α chains designated $\alpha 1$ type II (Miller and Matukas, 1969; Trelstad *et al.*, 1970). Cartilage type II collagen contains more hydroxylysine and exhibits a higher degree of glycosylation when compared with the more common, type I ($\alpha 1$)₂ $\alpha 2$ collagen.

Using more drastic methods of extraction Veis and Anesey (1965) were able to demonstrate the presence of a wide range of components in denatured extracts of steer hide collagen. These included a β_{22} and γ_{111} , γ_{122} and γ_{222} as well as the expected γ_{112} . Higher molecular weight compounds containing two or three of these were also detected, thus demonstrating the presence of intermolecular crosslinks.

Similar separations can also be obtained by electrophoresis on polyacrylamide gel, or by molecular sieve chromatography. The first of these is a particularly useful and convenient method for characterization of the components of collagen extracts.

The proportion of α to β components tends to vary with the tissue, and the method of extraction. In salt-soluble collagen the proportion of β tends to be lower than in the biologically older acid-soluble collagen, and as the severity of the extraction procedure is increased the proportion of β becomes still higher. The proportion of β is also low in lathyrctic collagen, a toxic condition produced in animals by eating the sweet pea. A variety of defects in the connective tissue are observed in this disease and it is now generally agreed that impaired crosslinking of collagen and elastin is involved (see Tanzer, 1965). Lathyrism can be induced artificially by the administration of β amino propionitrile and by a variety of other compounds which have the common property of reacting with aldehydes (see Section IVC3).

2. Subunit structure within the α chains

Various suggestions have been made concerning the occurrence of subunits within the individual α -chains. Such a model was proposed by Gallop (1964)

on the basis of results obtained from breakdown of esterlike bonds in collagen by hydroxylamine.

The presence of esterlike bonds in collagen was suggested by Grassmann in 1954 on the basis of reaction with lithium borohydride and a few years later Gallop *et al.* (1959) and Blumenfeld and Gallop (1962) produced further evidence from a study of the reaction of hydroxylamine and hydrazine. Other workers, notably Hörmann and co-workers have studied the reaction further and it is generally agreed that there are about six esterlike bonds in acid-soluble collagens. Hörmann (1962) reports rather higher values for insoluble collagen and lower values for collagens treated with alkali. (For a full discussion see Harding, 1965.)

It now seems doubtful whether ester-bonds as such are present in collagen. Hydroxylamine sensitive bonds have been located in the α_1 -chain of rat collagen (Butler, 1969; Bornstein, 1970) and evidence is cited for the existence of this bond as a cyclic imide formed by cyclization of an asparaginy (or aspartyl) side chain with the adjacent amide-nitrogen of a glycy (or aspartyl) residue. It is suggested that the susceptibility of collagen to hydroxylamine cleavage is related to its high glycine content and the increased probability of asparaginy-glycy sequences.

Petruska and Hodge (1964) have suggested a subunit model based on mathematical analysis of microdensitometer tracings of the SLS banding pattern. To derive this model they postulate that each α -chain consists of five identical subunits and α_2 of seven identical subunits. Attempts to obtain evidence of such units by reaction of α_1 and α_2 chains with hydroxylamine gave molecular weight fragments of the right size, i.e. 20,000 and 14,000 (Hodge *et al.*, 1965).

Specific cleavage studies, however, have so far yielded no evidence for a repeating structure of this type or for that matter, for the existence of ester bonds within the chain (see below).

3. *Specific cleavage*

The isolation of a collagenase of high specificity from the tissues of the tadpole undergoing rapid metamorphosis has led to some interesting studies on the specific cleavage of the tropocollagen molecule (Gross and Nagai, 1965; Kang *et al.*, 1966; Sakai and Gross, 1967). This enzyme was found to split the molecule into two portions, one equivalent to 25% of the molecule and the other to 75%. Both fragments were helical and could be precipitated as two SLS type aggregates, one a quarter and the other three quarters the length of the normal segment, thus establishing that all three chains were split at one level. Staining indicated that the longer fragment designated TC^A came from the A end of the molecule and the shorter TC^B came from the B end (see Fig. 4). On denaturation TC^B gave a single species of molecular

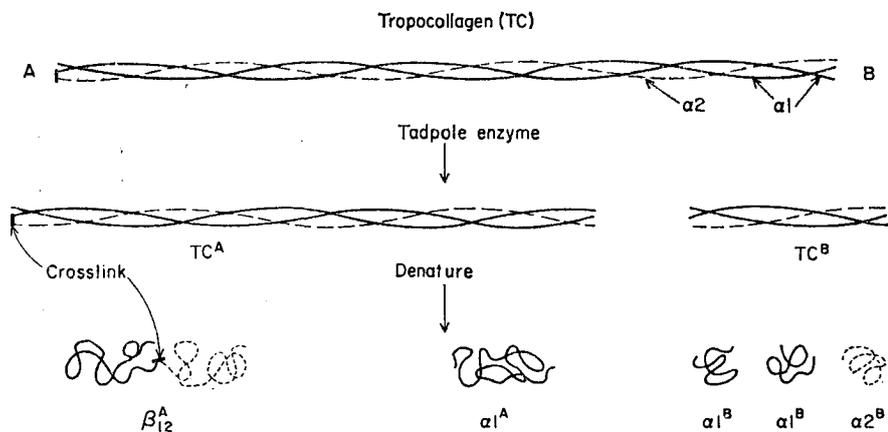


FIG. 4. Diagrammatic representation of the selective cleavage of tropocollagen with tadpole collagenase. Letters A and B distinguish the ends of the molecules as seen in SLS aggregates in the electron microscope (from Kang *et al.*, 1966).

weight 24,000 and TC^A two species of molecular weight 70,000 and 150,000, thus locating a crosslink in the A end of the molecule.

It is of interest to note that Drake and Davison (1968) cite evidence for attack by trypsin at what they describe as a weak point, about one quarter from the B end of the molecule.

All the newly formed N-terminal residues arising from treatment with the tadpole collagenase were on the shorter B fragment locating this as coming from the carboxyl terminus of the molecule and showing that all three chains are orientated in the same direction. Leucine and/or isoleucine was located as a new N-terminal residue on the B fragment and glycine as a C-terminus on the A fragment (Nagai *et al.*, 1965). Analysis of the separated fragments showed that many of the amino acids were unevenly distributed between the A and B portions. For example α_1^B contained a high proportion of leucine and a low proportion of methionine and valine, while α_2^B contained a high proportion of tyrosine and histidine and a low proportion of valine. Such findings do not support the idea of a repeating subunit molecule (Kang *et al.*, 1966).

Further information on the structure within the α -chains comes from specific cleavage studies using cyanogen bromide. This work was summarized by Piez *et al.*, in 1968 and has recently been reviewed (Traub and Piez, 1971). Two main lines of investigation have been followed, first characterization of a single collagen in detail, in this case rat skin collagen and secondly, the collection of comparative data on collagen from different tissues and species. Briefly, eight unique peptides have been isolated from the

α_1 chain of rat skin collagen in approximately equimolecular amounts and their amino acid composition determined. From information derived from a variety of sources it is possible to place these in a specific order. The glycopeptide isolated by Butler and Cunningham (1966) was located on α_1 CB5 near the middle of the chain. Peptides obtained from α_1 -chains of other collagens, e.g. rat tendon, human skin, chick skin and chick bone were found to be homologous with those of rat skin. Splitting of the α_2 chains gave rise to six peptides. The peptides of the four collagens studied were again homologous with respect to one another.

The amino acid sequence of the α_1 chain and part of the α_2 chain has been determined. Hulmes *et al.* (1973) have compiled the sequence of α_1 from various original publications.

Neither of the CB1 peptides are typical of collagen (see Fig. 5) but the presence of the typical sequence Gly-Pro-X at the carboxyl end of both CB1 peptides and the regular occurrence of this triplet in the next α_1 peptide (α_1 CB2) demonstrates that these peptides are adjacent to and contiguous with the main body of the molecule. Rat skin collagen appears to differ from other collagens in that the short terminal sequence found in them containing tyrosine, serine, methionine and glutamic acid (isolated as the pyrrolidine carboxylic acid) is missing. Whereas the main body of the collagen molecules in the rat, chick and human have great structural similarity, the N-terminal sequences, especially that of the α_2 chains show some interspecies differences (Bornstein, 1968 and Bornstein and Kang, 1970). In particular the degree of hydroxylation of lysine residues in α_1 CB1 shows appreciable variation from tissue to tissue, in rat skin collagen 5% of the residues are hydroxylated, in bone collagen 50% and in chicken skin none at all (Bornstein, 1969). It seems probable that this higher degree of hydroxylation is related to the greater crosslinking of bone-collagen (Bailey *et al.*, 1969).

The presence of an uncharacteristic peptide at the N-terminal end of the molecule accords with early speculations concerning the existence of telopeptides external to the collagen helix. The possibility of such appendages was first suggested by Boedtke and Doty (1956) from physicochemical studies and by Hodge and Schmitt (1958) from changes in the aggregation forms of soluble collagen following ultrasonication. The release of a variety of peptides following treatment with enzymes without apparently greatly affecting the bulk of the molecule (see p. 21) has also been taken as indication of the existence of telopeptides.

Drake *et al.* (1966) have made a study of the peptides released by the action of a number of enzymes on acid soluble and insoluble collagen and suggest that telopeptides occur not only at the end but also along the length of the chain leading to the concept of a "hairy" molecule. However, as a result of cleavage studies of native collagen with cyanogen bromide and

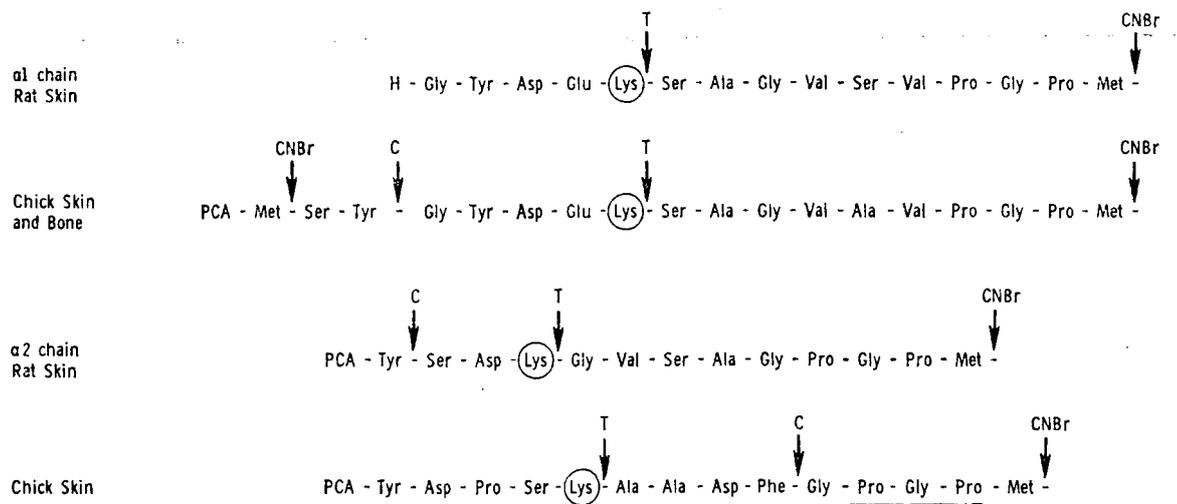


FIG. 5. Amino acid sequences from the N-terminal regions of the α -chains of collagen (from Piez *et al.*, 1968 and Kang and Gross, 1970). Points of cleavage with cyanogen bromide (CNBr), chymotrypsin (C) and trypsin (T) are indicated. The lysyl residue (ringed) in position 5 is the one converted to an aldehyde as a preliminary to crosslinking.

chymotrypsin (Bornstein and Piez, 1966; Bornstein, Kang and Piez, 1966b; Rauterberg *et al.*, 1972). Such non-helical regions (telopeptides) have been shown to exist only at the NH₂- and COOH-ends of the collagen molecule.

C. Nature and Location of Crosslinks

The question of the unusual bonds in collagen and their possible relevance to crosslinking were reviewed by Harding (1965). Among the bonds considered were those involving esters, carbohydrates, aldehydes and ϵ -amino and γ -glutamyl groups. With the exception of aldehydes, no direct evidence for participation of any of these in interchain crosslinking has since been reported.

The existence of γ -glutamyl bonds in collagen was demonstrated by Gallop *et al.* in 1960. Recent enzyme studies by Bensusan (1969) now throws some doubt on their existence. If they do exist, they are almost certainly intrachain, the α -carboxyl group remaining free. The existence of ester bonds as such now appears doubtful and the hydroxylamine sensitive bonds have been located in the α -chain itself (see p. 13).

1. Bonds involving ϵ -amino and tyrosyl groups

Doubt concerning the complete availability for reaction of ϵ -amino groups in collagen have led various workers to speculate on the possibility of crosslinks involving these. Support was lent to this view by the isolation of an ϵ -amino tripeptide by Mechanic and Levy in 1959. No confirmation of this work or of the isolation of similar peptides have been made and consideration of the more recent evidence regarding the availability of ϵ -amino groups indicates that only a small number, say 1 or 2 out of a total of about 34 residues can be involved in such crosslinks (see p. 21).

Changes in physical properties of acid-soluble collagen following treatment with iodine, oxidizing agents, tyrosinase and peroxidase are indicative of crosslinking through tyrosyl residues (LaBella and Paul, 1965; Bensusan, 1966; LaBella *et al.*, 1968; Dabbous, 1966) and has led to suggestions regarding their possible involvement in crosslinking *in vivo*. While it is possible that this may occur in ageing or be induced by exposure to ultraviolet light or γ -irradiation, there is no direct evidence that this occurs in the normal maturation process.

2. Carbohydrate

It has been suggested at various times that hexoses are involved in the crosslinking of collagen but in spite of numerous efforts to demonstrate this no direct evidence is forthcoming. It is however, generally agreed that hexoses are bound to the tropocollagen molecule (see p. 4). Butler and

Cunningham (1966) state that the glycopeptide isolated from acid-soluble collagen is not involved in a crosslink and no evidence was found for the involvement of this or the other dipeptides in the crosslinking of insoluble collagen (Cunningham and Ford, 1968). The function of the bound hexoses, therefore, remains obscure.

3. Aldehydes

Evidence for the implication of aldehydes in the crosslinking of collagen has been accumulating during the past few years and it is now established that the main inter- and intramolecular crosslinks are of this type (see Piez, 1968; Bailey *et al.*, 1974).

The first suggestion of such involvement came from Levene (1962) who pointed out that lathyrin collagen is deficient in both aldehydes and crosslinks and that a common property of all lathrogens is their ability to react with carbonyl compounds. A few years later Gallop (1964) showed that the tropocollagen of several species contained about 2-3 moles of covalently bound aldehyde. After certain chemical treatments, e.g. with hydroxylamine, or following long reaction periods, further aldehyde groups could be detected. Rojkind *et al.* (1964, 1966) isolated an aldehyde containing peptide from a collagenase digest of carp swim bladder collagen. Spectral characteristics indicated a β -hydroxyaldehyde which readily dehydrated to give an α - β unsaturated aldehyde.

More direct evidence for the involvement of aldehydes in intramolecular crosslinks was obtained almost immediately from work by Piez and his collaborators (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966), on the specific cleavage of isolated α and β components of ratskin collagen with cyanogen bromide.

The N-terminal peptides of the α_1 and α_2 chains were shown to occur in a second form in which the lysyl residue was replaced by an aldehyde. Isotopic studies showed this aldehyde to be derived from lysine. A peptide specific to the β_{12} component was also isolated and found to give reactions characteristic of an α - β unsaturated aldehyde. On the basis of this evidence it was postulated that a crosslink was formed between α_1 and α_2 by the aldol condensation of two lysine derived aldehydes. Further studies confirmed the presence of a peptide containing an intramolecular link of this type (Rojkind *et al.*, 1968, 1969; Kang *et al.*, 1969).

The location of the crosslinked region near the N-terminus or A end of the molecule also followed from work on specific cleavage with tadpole collagenase (see p. 13) and from treatment of native collagen with chymotrypsin and cyanogen bromide. Peptides released from the N-terminal non-helical region without affecting the bulk of the molecule were shown to contain the crosslink (Bornstein *et al.*, 1966a, b).

So far the evidence cited has been primarily concerned with the intramolecular bond in collagen. Intermolecular crosslinks are thought to be of a similar type and may be competitive, since lathrogens inhibit both forms of crosslinking. Present views favour the formation of a Schiff's base between an aldehyde group and an ϵ -amino group in another chain as the first step, (Piez, 1968; Bailey, 1969).

The presence of such labile bonds would explain the marked effect of aldehyde reacting reagents such as cysteamine, thiosemicarbazide and penicillamine on solubility and strength and the increased stability resulting from reduction with sodium borohydride which would be expected to stabilize the crosslink (see for example, Tanzer *et al.*, 1966, 1967; Nimni, 1966; Harkness and Harkness, 1968, Bailey, 1968b). The intermolecular nature of the crosslink stabilized by borohydride reduction is indicated by the increase observed in isometric tension developed on heating (Balian *et al.*, 1968; Bailey and Lister, 1968) and by the fact that the effect of borohydride is greatest with intact fibrils and is not evident when collagen is treated in dilute solution (Tanzer, 1968).

Both hydroxylysinoxorleucine (Bailey and Peach, 1969; Tanzer *et al.*, 1970) and lysinoxorleucine (Tanzer and Mechanic, 1970; Kang *et al.*, 1970) have been identified in collagen treated with sodium borohydride and it is deduced that these arise from the stabilization of aldimine bonds formed between the lysine derived aldehyde in one molecule and hydroxylysine and lysine, respectively, in another.

The changes associated with borohydride reduction are similar to those associated with the increased stability of collagen occurring during maturation and ageing (see, for example, Verzar, 1964). The number of reducible crosslinks decreases with age (Bailey, 1969) and although it is tempting to suggest that these become reduced *in vivo*, it has not been possible to isolate the *in vivo* reduced form of these crosslinks from non-reduced collagens (Bailey and Peach, 1971). Furthermore, a recent study has confirmed that *in vivo* reduction of the intermolecular bonds does not occur (Robins *et al.*, 1973).

Kang *et al.* (1970) report the disappearance of the intramolecular aldol crosslink on aging of collagen fibrils *in vitro* suggesting its possible involvement in further intermolecular crosslinking. Bailey *et al.* (1970) have noted the presence of larger complexes containing several lysine residues. Such a component has been isolated from borohydride reduced skin and tendon (Tanzer *et al.*, 1973) and identified as histidino-hydroxymerodesmosine. This is formed by the Michael addition of histidine to the intramolecular aldol followed by condensation with hydroxylysine. Its structure suggests that it can potentially link three or four peptide chains.

However, Robins and Bailey (1973) have recently demonstrated the acid

lability of histidino-hydroxymerodesmosine suggesting that its non-reduced form does not exist as an intermolecular crosslink *in vivo*.

With elastin it has been found that crosslinking occurs as a result of condensation of four lysine derived aldehydes and a lysine residue to form the heterocyclic structures, desmosine and isodesmosine (see Partridge, 1968 for a review of this subject). So far, however, there have been no reports of such compounds in collagen in spite of the fact that the initial steps in the formation of the crosslink seem to be similar.

Recently two crosslinked peptides have been isolated from vertebrate collagens. Kang (1972) isolated a peptide containing a crosslink between α_1 CB1 and α_1 CB6 from borohydride reduced tendon collagen and Miller (1971) isolated α_1 (II) CB4 \times 9 from cartilage type II collagen. More recently, Miller and Robertson (1973) have shown that the crosslinked peptide from cartilage can be isolated from non-reduced collagen and upon subsequent reduction of the isolated peptide yields the crosslink dihydroxy-lysinyonorleucine. This confirms that the reducible component exists *in vivo* as an intermolecular crosslink. Moreover, from the order of the cyanogen bromide peptides in cartilage α_1 (II) (Miller *et al.*, 1973) it is clear that the crosslink occurs between the N-terminal non-helical region of one molecule and the helical region of another such that the two are overlapped as predicted by Hodge and Petrushka (1962).

V. REACTIVITY AND STABILITY

A. Functional Groups

The reactive amino acid side chains all project outwards from the main body of the triple helix and in soluble collagens should, therefore, be accessible to all chemical reagents. In the compact fibrous forms of collagen, however, there is no guarantee that this will be so.

The early acid-base binding studies of Bowes and Kenten (1948) indicated that essentially all the groups were free but more recently Hartman and Bakerman (1966) and Bakerman and Hartman (1966) report that in native salt and acid soluble calf skin collagen not all groups are readily titrated, and even after denaturation approximately 5 carboxyl, 0.5 histidyl and 6 ϵ -amino groups in acid-soluble collagen remain unreacted.

Most interest has been centered on the availability of ϵ -amino groups in view of their possible implication in the crosslinking of the protein. Amino-nitrogen determinations by the Van Slyke procedure return essentially all the amino groups (Bowes and Kenten, 1948), but it has been argued that the conditions of the determination are rather drastic and may cause breakdown. Early work on the interaction of 2,4-fluorodinitrobenzene (FDNB) suggested

that only 60–80% of the ϵ -amino groups in collagen and acid-collagen were free to react. Later work, however, has indicated that all except 1 or 2 per 10^5 g are free (see Hörmann, 1962). Reaction with acetylating and guanidating reagents also indicate nearly 100% reaction (see Veis, 1967). None of these procedures gives a very precise measure of the number of groups reacting, but on balance it would seem that at least 90%, probably more, of the ϵ -amino groups in insoluble collagens are free to react.

Only 60–90% of the guanidyl residues react with hypobromite according to the conditions used but this is probably attributable to electrostatic effects rather than to involvement of these residues in covalent binding with other groups.

B. Stability to Chemicals and Enzymes

Native collagens, even the soluble forms, are very resistant both to the action of enzymes and chemicals, a property almost certainly related to the stable helical conformation of the molecule and the protection this affords to the peptide bonds of the individual chains.

Dilute acids lead to solubilization of varying amounts of collagen and on the basis of current hypotheses this would appear to be due to the action of the acid on labile intermolecular links of the Schiff's base type (see p. 19). Attack on the collagen molecule itself appears to be negligible even at low pH values provided the temperature is below 20°C.

A long treatment in alkali is the traditional prelude to the conversion of collagen to gelatin. The main chemical changes noted have been loss of amide-nitrogen and conversion of a small fraction of the arginine to ornithine. Loss of amide leads to lowering of the isoelectric point, that of alkaline pretreated gelatin generally being between pH 4.8 and 5. Treatment of denatured acid-soluble collagen in dilute alkali at pH 11.0 at 40°C for short periods leads to limited breakdown, two main fractions being obtained corresponding in composition to α_1 and α_2 but with a molecular weight approximately half that of the normal α -chains (Schleyer, 1962). Native acid-soluble collagen, as might be expected, is more resistant, treatment in dilute alkali at 20°C for periods up to 60 days leading only to decrease in β components with concomitant increase in α and some loss of ability to reconstitute as native fibrils. Treatment of insoluble hide and ossein collagen with 5–8% sodium hydroxide saturated with sodium sulphate leads to greatly increased solubility in acid. Physicochemical determinations on this product, eucollagen, indicate that it has many of the attributes of acid-soluble collagen, though ability to reconstitute to native fibrils is largely lost and the hydroxyproline content is increased from 14.0–14.3 to about 14.9% (see Chapter 5).

The general conclusions to be drawn from these results is that provided the swelling and dispersing action of the alkali is reduced by the addition of a

helix stabilizing salt such as sodium sulphate, the action of alkali is restricted to the nonhelical telopeptide area and the removal of the crosslinked region.

Other chemical reagents increase collagen solubility but in the main their effect is primarily on specific non-peptide bonds, e.g. hydroxylamine and hydrazine break ester-like bonds and aldehyde reacting reagents have an effect on labile bonds involving aldehyde groups.

The relative inaccessibility of the peptide bonds in the main helical part of the molecule is also illustrated by the results of experiments on selective cleavage with cyanogen bromide, chymotrypsin and pepsin. With denatured collagen or individual α and β components the expected number of peptides corresponding to cleavage at the methionyl residues can be identified (Butler *et al.*, 1967) but with native collagen the N-terminal peptides only are released, the peptide bonds in the main body of the molecule being apparently protected by the triple helical conformation (Bornstein *et al.*, 1966a, b).

For many years it was believed that proteolytic enzymes were without effect on native collagen. Recent studies, however, have shown that their action, although restricted, may have quite profound effects on some properties. The main body of the tropocollagen molecule apparently remains intact but non-helical peptide areas may be attacked leading to the elimination of both intra- and intermolecular crosslinks. Above the denaturation temperature or in the presence of denaturing reagents the loss of helical structure opens up the molecule to general attack according to the specificity of the enzyme.

Native soluble collagens are resistant to trypsin below about 20°C but above this temperature viscosity decreases and aggregation properties are affected (see, for example Kühn *et al.*, 1966 and Martin *et al.*, 1966). There appears to be no definite end point and it is suggested that attack only becomes possible because of slight unfolding of the helix. Changes in SLS banding pattern suggest that attack is at the B-end of the molecule (Olsen, 1964). Also Drake and Davison (1968) cite evidence for attack of the tropocollagen molecule at what they describe as a weak point, about one quarter length from the B-end.

Chymotrypsin is more specific in its action and less temperature dependent. The viscosity falls to a definite level, the β components are decreased and evidence points to attack in the non-helical N-terminal peptide area (Drake *et al.*, 1966; Martin 1966 and Bornstein *et al.*, 1966b). Pepsin also attacks in this area and is particularly effective in removing intermolecular crosslinks. Pronase acts similarly, solubility is increased and end to end aggregation of soluble collagen is affected. The appearance of SLS aggregates suggests that the B-end of the molecule as well as the A-end is affected (Drake *et al.*, 1966).

How far the non-helical peptides are attacked in native fibrous collagen is less certain. Prolonged action of pronase or pepsin leads to increased solubility but with some tissues solution is far from complete. Changes in

tensile strength occur but it is difficult to differentiate between effects due to attack on collagen from those arising from breakdown of interfibrillary material.

Complete breakdown of native collagen to small peptides can only be achieved by the action of a group of bacterial enzymes, the collagenases, the best documented being that isolated from *Cl. histolyticum* (see Mandl, 1961). These enzymes are specific for the -Gly-Pro-X-Gly-(Pro or Hypo-) sequence, cleavage occurring to give an N-terminal glycine. Even with such enzymes however, complete solubilization and breakdown of many collagenous tissues, e.g. mature ox hide collagen is difficult. Tadpole collagenase (see p. 13) is even more specific in its action.

C. Denaturation

When soluble collagen is heated in dilute solution marked changes in properties occur over a relatively narrow temperature range. The Negative optical rotation decreases sharply, reflecting loss of the poly-l-proline II configuration of the polypeptide chain, decrease in specific viscosity indicates collapse of the rod like structure and changes in the sedimentation pattern and light scattering properties indicate disruption to lower molecular weight components.

Studies of the transition by Engel (1962) using light scattering techniques (which enable simultaneous measurements to be made of number average molecular weight and molecular shape) show that two steps are involved. First there is a collapse of the helical structure as indicated by a rapid fall in optical rotation, specific viscosity and angular dependence of light scattering and then disentanglement of the chains leading to a slower decrease in molecular weight. On cooling, the viscosity and the negative optical rotation increase again indicating partial reversal of the process. (For fuller discussion of these changes see Harrington and von Hippel, 1961; Veis, 1964; von Hippel, 1967.)

The abrupt loss of molecular structure occurring on heating in solution is manifested in collagen fibrils as a sharp contraction to about one third of their original length. This outstanding characteristic of collagen has been known for many years. On shrinking the fibres become elastic, appear swollen and translucent and both wide and small angle X-ray patterns are lost. The process is partially reversible on cooling and stretching, the wide but not the small-angle pattern, being restored.

The denaturation temperature of collagen in solution, T_m (defined as the midpoint of the transition) varies between 37°C and 40°C for mammalian collagens, is lower for fish collagens and varies considerably with the invertebrate collagens. The shrinkage temperature of the native fibres, T_s , varies correspondingly but is about 27°C higher. This higher temperature is

attributed to increasing concentration of collagen within the fibril and electrostatic interactions between the molecules (Gross, 1964). The formation of intermolecular crosslinks may also be a factor (see however below). Weir (1949) considered that the contraction was a rate process but the studies of Flory and Garrett (1956, 1958) have now led to the more generally accepted view that it is in fact a melting phenomenon. From a review of earlier literature and measurement, by a dilatometer method, of the melting temperature of collagen containing various concentrations of diluent (in this case, ethylene glycol), they were able to demonstrate that the change had all the characteristics of a first order phase transition and was typical of that occurring on the fusion or melting of the crystalline regions of polymers. By extrapolating to zero diluent concentration they obtained a "melting temperature" of 145°C for the mammalian collagen used in the investigation. They also detected a slight volume increase at about 40°C characteristic of a polymer-glass transition.

T_m and T_s have been determined for a number of collagens and variations with chemical composition noted. Gustavson (1953) was the first to draw attention to the fact that the shrinkage temperature varied with the hydroxyproline content and at that time it was considered that the additional hydrogen bonds formed by the hydroxyl group were responsible. However, a better correlation between shrinkage temperature and total pyrrolidine residue content can be demonstrated (see Von Hippel and Wong, 1963a), and it is now generally agreed that the constraint exerted by the ring structure is a primary factor governing the stability of the helical collagen configuration and, hence, the shrinkage temperature.

The presence of interchain crosslinks within the molecule has little effect on T_m , the introduction of up to 10 interchain bonds into ichthyocol tropocollagen by treatment with formaldehyde in very dilute solution leading to only 1.4°C increase in T_m (Veis and Drake, 1963). The stability of the helix, therefore, appears to be primarily dependent on the special features of the peptide bonds involving the imino acids, supplemented by interchain hydrogen bonds at the positions occupied by glycine.

The transition temperature and shrinkage temperature are affected by the pH of the solution, by the addition of denaturing agents known to facilitate breaking of hydrogen bonds such as guanidine or urea, and by the salt concentration. Effects at low or high pH values probably involve increased electrostatic repulsion between molecules. The mechanism of salt action is less clear; effects depend both on the nature and concentration of the salt and in the main follow the Hofmeister Series. Bello *et al.* (1956) made an extensive study of the effect of various additives on the melting point of gelatin gels and von Hippel and Wong (1962, 1963a) have followed the effect of salts on the rate of formation and stability of the collagen type helix in collagen and

gelatin solutions. These studies indicate that anions and cations operate independently and effects are not clearly related to sign or magnitude of the ionic charge or to specific ion binding. Sulphate appears to be particularly effective in increasing T_m or T_s and nitrate, iodide, thiocyanate, calcium and lithium ions in decreasing these. (For fuller discussion see von Hippel, 1967.)

Increases in shrinkage temperature are obtained by intermolecular cross-linking and such reactions form the basis of tanning processes. After thermal denaturation collagen fibres become elastic and have been shown to behave essentially as a random elastic network (Weiderhorn and Reardon, 1952; Flory and Spurr, 1961). This approach has been used by a number of workers for the determination of crosslinks introduced by tanning agents. Most recently, Bowes and Cater (1964) have used this technique to assess the cross-linking efficiency of dialdehydes and other bifunctional compounds. With untanned kangaroo tail tendon results indicated the presence of 3-4 crosslinks per tropocollagen molecule, presumably two intramolecular links and one or two intermolecular bonds. After storage for several years the number increased, indicating crosslinking *in vitro*.

D. Swelling

The swelling of collagen fibres in tissues such as tendon or skin is of two types osmotic and lyotropic (see Gustavson, 1956). The first occurs in acid or alkaline solutions and is related to the positive or negative charge on the protein reaching a maximum at pH 2.0 and 12.0 and then decreasing again at more extreme pH values at the rising ion concentration reduces the change effect. The fibres swell laterally, contract in length and become glassy and translucent in appearance. The swelling is reversed by neutralization, by the addition of salts which reduce the effect of charge or by the presence of anions (or cations) having a specific affinity for the charged groups. This type of swelling has been considered in terms of the Donnan equilibrium (Procter and Wilson, 1916) which provides a satisfactory explanation in practical terms. X-ray diffraction studies (Burge *et al.*, 1958) showed that the lateral spacing of about 11Å, attributed to the distance between the molecules was increased to 13.5Å in salt free water in the pH range of minimum swelling but increased to 15Å at pH 2.0. Structural stability, as indicated by fall in shrinkage temperature, is also affected suggesting that water actually penetrates into the tropocollagen molecule but it is difficult to disentangle the effects of swelling, pH and ion concentration.

Swelling in neutral salt solutions has rather different effects, the fibres become opaque and flaccid, length is relatively unaffected and cohesion between fibrils is reduced. The uptake of water varies greatly with the salt,

increasing with its tendency to disrupt hydrogen bonds. Dimensional changes probably first occur in the less ordered polar areas of the molecule leading to more general disruption under favourable conditions, i.e. rise of temperature. (For fuller discussion of the effect of salts on the collagen triple helix see von Hippel, 1967.)

The physical and mechanical properties of collagen fibres has been the subject of two reviews, Harkness (1967) and Elden (1968) and will not be considered here.

VI. SUMMARY

It is now generally accepted that the collagen macromolecule, tropocollagen, is a thin rod-like structure about 2800Å long and 14–15Å in diameter with a molecular weight of about 300,000. It is composed of three polypeptide chains of equal length, each in the form of a poly-L-proline II type left handed helix, and these three chains are coiled about a common axis in a slight right-handed super helix. Glycine constitutes every third residue through the greater part of the structure and the characteristic sequence within the chains is Gly-Pro-X, where X is often hydroxyproline. Peptide sequences of this type are interspersed with sequences containing a high proportion of acidic or basic amino acids. At the N-terminal or A end of the molecule there is an area of uncharacteristic amino acid composition and in this area is located an intramolecular crosslink involving two lysine-derived aldehydes. The exact mechanism by which this crosslink is formed is not known but there are indications that it involves the aldol condensation of two lysine derived aldehydes to give an α, β unsaturated aldehyde. Some evidence has been reported for a subunit structure within the individual chains and for the presence of other non-helical peptides protruding from the body of the molecule. So far no direct support for these hypotheses exists.

Aggregation of the rod-like macromolecules to give fibrils occurs in a highly specific manner with an approximately quarter overlap of molecules in parallel array. This arrangement is thought to be partially governed by the charge profile of the molecule and partly by the telopeptide region of the molecule, modification of the latter generally impairing the ability to form native type fibrils. Further stabilization occurs by intermolecular cross-linking—between a lysine or hydroxylysine residue and a lysine (or hydroxylysine-) derived aldehyde forming a labile aldimine bond which later becomes stable as the collagen ages.

The poly-L-proline II helical configuration of the individual chains is responsible for many of the unusual properties of the molecule, e.g. the high optical rotation and is the main factor governing hydrothermal properties. The coiled-coil conformation also imparts great stability to chemicals and

enzymes and, provided this is not disrupted, attack is limited to the telopeptides.

Chemical and enzymatic attack on these areas, however, may lead to decrease in intramolecular and intermolecular crosslinking and so have a profound effect on subsequent stability and solubility.

REFERENCES

- Ambrose, E. J. and Elliott, A. (1951). *Proc. R. Soc. A* **206**, 206.
- Bailey, A. J. (1968a). In "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, eds) Vol. 26, Part B, p. 297. Elsevier Publishing Co., Amsterdam, London, New York.
- Bailey, A. J. (1968b). *Biochim. biophys. Acta* **160**, 447.
- Bailey, A. J. (1969). *Gerontologia* **15**, 65.
- Bailey, A. J. and Lister, D. (1968). *Nature, Lond.* **220**, 280.
- Bailey, A. J. and Peach, C. M. (1971). *Biochem. J.*, **121**, 257.
- Bailey, A. J., Fowler, L. J. and Peach, C. M. (1969). *Biochem. Biophys. Res. Commun.* **35**, 663.
- Bailey, A. J., Peach, C. M. and Fowler, L. J. (1970). *Biochem. J.* **117**, 819.
- Bailey, A. J., Robins, S. P. and Balian, G. (1974). *Nature*, **251**, 105.
- Bakerman, S. and Hartman, B. K. (1966). *Biochemistry* **5**, 3488.
- Balian, G. A., Bowes, J. H. and Cater, C. W. (1969). *Biochim. biophys. Acta* **181**, 331.
- Bello, J., Riese, H. C. and Vinograd, J. R. (1956). *J. phys. Chem. Ithaca* **60**, 1299.
- Bensusan, H. B. (1966). *Archs. Biochem. Biophys.* **115**, 77.
- Bensusan, H. B. (1969). *Biochemistry* **8**, 4716, 4723.
- Blumenfeld, O. and Gallop, P. M. (1962). *Biochemistry* **1**, 947.
- Boedtker, H. and Doty, P. (1956). *J. Am. Chem. Soc.* **78**, 4267.
- Bornstein, P. (1968). *Science* **161**, 592.
- Bornstein, P. (1969). *Biochemistry* **8**, 63.
- Bornstein, P. (1976). *Biochemistry* **9**, 2408.
- Bornstein, P. and Kang, A. H. (1970). In "Chemistry and Molecular Biology of the Intercellular Matrix" (E. A. Balaz ed.), p. 99. Academic Press, New York.
- Bornstein, P. and Piez, K. A. (1966). *Biochemistry* **5**, 3460.
- Bornstein, P., Kang, A. H. and Piez, K. A. (1966a). *Proc. natn. Acad. Sci. U.S.A.* **55**, 417.
- Bornstein, P., Kang, A. H. and Piez, K. A. (1966b). *Biochemistry* **5**, 3803.
- Bowes, J. H. and Cater, C. W. (1964). *J. appl. Chem., Lond.* **14**, 296.
- Bowes, J. H. and Kenten, R. H. (1948). *Biochem. J.* **43**, 358.
- Bradbury, E. M., Burge, R., Randall, J. T. and Wilkinson, G. R. (1958). *Discuss. Faraday Soc.* **25**, 173.
- Burge, R. E., Cowan, P. M. and McGavin, S. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 25. Pergamon Press, London.
- Butler, W. T. (1969). *Biochemistry* **9**, 44.
- Butler, W. T. (1969). *J. biol. Chem.* **244**, 3415.
- Butler, W. T. and Cunningham, L. W. (1966). *J. biol. Chem.* **241**, 3882.
- Butler, W. T., Piez, K. A. and Bornstein, P. (1967). *Biochemistry* **6**, 3771.
- Cassel, J. M., Mandlkern, L. and Roberts, D. E. (1962). *J. Am. Leath. Chem. Ass.* **57**, 556.

- Chapman, G. E. and McLaughlan, K. A. (1969). *Proc. Roy. Soc.* **B173**, 223.
- Cowan, P. M., McGavin, S. and North, A. C. T. (1955). *Nature, Lond.* **176**, 1062.
- Cowan, P. M., North, A. C. T. and Randall, J. T. (1953). In "The Nature and Structure of Collagen" (J. T. Randall, ed.), p. 241. Butterworths, London.
- Cunningham, L. W. and Ford, J. D. (1968). *J. biol. Chem.* **243**, 2390.
- Dabbous, M. (1966). *J. biol. Chem.* **241**, 5307.
- Drake, M. P., Davison, P. F., Bump, S. and Schmitt, F. O. (1966). *Biochemistry* **5**, 301.
- Drake, M. P. and Davison, P. F. (1968). *J. biol. Chem.* **243**, 2890.
- Eastoe, J. E. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.), Vol. I, p. 1. Academic Press, New York.
- Elden, H. R. (1968). *Int. Rev. Connective Tissue Res.* **4**, 283.
- Engel, J. (1962). *Archs. Biochem. Biophys.* **97**, 150.
- Esipova, N. G., Andreeva, N. S. and Gatovskaia, T. V. (1958). *Biofizika* **3**, 529.
- Fitton Jackson, S. (1956). *Proc. R. Soc.* **B144**, 556.
- Flory, P. J. and Garrett, R. R. (1956). *Nature, Lond.* **177**, 176.
- Flory, P. J. and Garrett, R. R. (1958). *J. Am. chem. Soc.* **80**, 4836.
- Flory, P. J. and Spurr, O. K. (1961). *J. Am. chem. Soc.* **83**, 1309.
- Fraser, R. D. B. and MacRae, T. P. (1959). *Nature, Lond.* **183**, 179.
- Funakoshi, H. and Noda, H. (1964). *Biochim. Biophys. Acta.* **86**, 106.
- Gallop, P. M. (1964). *Biophys. J.* **4**, 79.
- Gallop, P. M., Seifter, S. and Meilman, E. (1959). *Nature, Lond.* **183**, 1659.
- Gallop, P. M., Seifter, S., Lukin, M. and Meilman, E. (1960). *J. biol. Chem.* **235**, 2619.
- Grassmann, W., Endres, H. and Steber, A. (1954). *Z. Naturforsch.* **9B**, 513.
- Grassmann, W., Hannig, K. and Nordwig, A. (1963). *Hoppe-Seyler's Z. physiol. Chem.* **333**, 154.
- Grassmann, W., Hannig, K. and Schleyer, M. (1960). *Hoppe-Seyler's Z. physiol. Chem.* **322**, 71.
- Gross, J. (1964). *Science, N.Y.* **143**, 960.
- Gross, J. and Nagai, Y. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1197.
- Gustavson, G. H. (1953). *Svensk kem. Tidskr.* **65**, 70.
- Gustavson, G. H. (1956). In "Chemistry and Reactivity of Collagen" (G. H. Gustavson, ed.), p. 155. Academic Press, New York.
- Hannig, K. and Nordwig, A. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.) Vol. I, p. 73. Academic Press, New York.
- Harding, J. J. (1965). *Adv. Protein Chem.* **20**, 109.
- Harkness, R. D. (1968). In "Treatise on Collagen" (G. N. Ramachandran, ed.), Vol. 2, Part A, p. 248. Academic Press, New York.
- Harkness, M. L. R. and Harkness, R. D. (1968). *Biochim. biophys. Acta.* **154**, 553.
- Harrington, W. F. and von Hippel, P. H. (1961). *Adv. Protein Chem.* **16**, 1.
- Hartman, B. K. and Bakerman, S. (1966). *Biochemistry* **5**, 2221.
- Hodge, A. J. and Petruska, J. A. (1962). In "Electron Microscopy" (S. S. Breese, ed.), Vol. I, Paper QQ-1. Academic Press, New York.
- Hodge, A. J. and Schmitt, F. O. (1958). *Proc. natn. Acad. Sci. U.S.A.* **44**, 418.
- Hodge, A. J. and Schmitt, F. O. (1960). *Proc. natn. Acad. Sci. U.S.A.* **46**, 186.
- Hodge, A. J., Petruska, J. A. and Bailey, A. J. (1965). In "Structure and Function of Connective and Skeletal Tissue" (S. Fitton Jackson, R. D. Harkness, S. M. Partridge and G. R. Tristram, eds), p. 31. Butterworths, London.
- Hörmann, H. (1962). *Leder* **13**, 79.

- Hulmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A. and Galloway, J. W. (1973). *J. molec. Biol.*, **79**, 137.
- Kang, A. H. (1972). *Biochemistry* **11**, 1828.
- Kang, A. H., Nagai, Y., Piez, K. A. and Gross, J. (1966). *Biochemistry* **5**, 509.
- Kang, A. H., Faris, B., and Franzblau, C. (1969). *Biochem. biophys. Res. Commun.* **36**, 345.
- Kang, A. H., Faris, B. and Franzblau, C. (1970). *Biochem biophys. Res. Commun.* **39**, 175.
- Kefalides, N. A. (1968). *Biochemistry* **7**, 3103.
- Kühn, K. and Zimmer, E. (1961). *Naturforsch* **166**, 648.
- Kühn, K., Fietzek, P. and Kühn, J. (1966). *Biochem. Z.* **344**, 418.
- LaBella, F. S. and Paul, G. (1965). *J. Geront* **20**, 54.
- LaBella, F. S., Waykole, P. and Queen, G. (1968). *Biochem. biophys. Res. Commun.* **30**, 333.
- Levene, C. I. (1962). *J. exp. Med.* **116**, 119.
- Lewis, M. S. and Piez, K. A. (1964). *Biochemistry* **3**, 1126.
- Mandl, I. (1961). *Adv. Enzymol.* **23**, 163.
- Martin, G. R., Mecca, C. E. and Piez, K. A. (1966). In "Environmental Variables in Oral Diseases" (C. J. Kreshover and F. J. McClure, ed.), p. 155. American Ass. for Advmt. Sci., Washington, D.C.
- McGavin, S. (1964). *J. Mol. Biol.* **9**, 60.
- Mechanic, G. L. and Levy, M. (1959). *J. Am. chem. Soc.* **81**, 188.
- Miller, E. J. (1971). *Biochem. biophys. Res. Commun.* **45**, 444.
- Miller, E. J. and Matukas, V. J. (1969). *Proc. natn. Acad. Sci. U.S.* **64**, 1264.
- Miller, E. J. and Robertson, P. B. (1973). *Biochem. biophys. Res. Commun.* **54**, 432.
- Miller, E. J., Woodall, D. L. and Vail, M. S. (1973). *J. biol. Chem.*, **248**, 1666.
- Morgan, P. H., Jacobs, H. G., Segrest, J. P. and Cunningham, L. W. (1970). *J. biol. Chem.*, **245**, 5042.
- Nagai, Y., Piez, K. A. and Cross, J. (1965). Proc. 16th Conf. Structure of Proteins, Fuknoka, Japan, p. 94. Japanese Chemical Society.
- Nimni, M. E. (1966). *Biochem. biophys. Res. Commun.* **25**, 434.
- Olsen, B. R. (1963). *Z. Zellforsch.* **59**, 199.
- Olsen, B. R. (1964). *Z. Zellforsch.* **61**, 913.
- Partridge, S. M. (1968). In "Fibrous Proteins" (W. G. Crewther, ed.), p. 246. Butterworths, London.
- Pauling, L. and Corey, R. B. (1951). *Proc. natn. Acad. Sci. U.S.A.* **37**, 236.
- Petruska, J. A. and Hodge, A. J. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51**, 871.
- Piez, K. A. (1965). *Biochemistry* **4**, 2590.
- Piez, K. A. (1967). In "Treatise on Collagen" (G. M. Ramachandran, ed.), Vol. I, p. 207. Academic Press, New York.
- Piez, K. A. (1968). *A. Rev. Biochem.* **37**, 678.
- Piez, K. A., Lewis, M. S., Martin, G. R. and Gross, J. (1961). *Biochim. Biophys. Acta.* **53**, 596.
- Piez, K. A., Bladen, H. A., Lane, J. M., Miller, E. J., Bornstein, P., Butler, W. T. and Kang, A. H. (1968). *Brookhaven Symp. Biol.* **21**,
- Procter, H. R. and Wilson, J. A. (1916). *J. Chem. Soc.* **109**, 307.
- Ramachandran, G. N. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.), Vol. I, p. 103. Academic Press, New York.
- Ramachandran, G. N. and Chandrasekhanan, R. (1968). *Biopolymers* **6**, 1649.

- Ramachandran, G. N. and Kartha, G. (1954). *Nature, Lond.* **174**, 269.
- Ramachandran, G. N. and Kartha, G. (1955). *Nature, Lond.* **176**, 593.
- Rauterberg, J., Fietzek, P., Rexrodt, F., Becker, U., Stark, M. and Kühn, K. (1972). *F.E.B.S. Letters* **21**, 75.
- Rich, A. and Crick, F. H. C. (1955). *Nature, Lond.* **176**, 915.
- Rich, A. and Crick, F. H. C. (1961). *J. molec. Biol.* **3**, 483.
- Robins, S. P. and Bailey, A. J. (1973). *Biochem. J.* **135**, 657.
- Robins, S. P., Shimokomaki, M. and Bailey, A. J. (1973). *J. Biol. Chem.* **248**, 771.
- Rojkind, M., Blumenfeld, O. O. and Gallop, P. M. (1964). *Biochem. biophys. Res. Commun.* **17**, 320.
- Rojkind, M., Blumenfeld, O. O. and Gallop, P. M. (1966). *J. biol. Chem.* **241**, 1830.
- Rojkind, M., Rhi, M. and Aguire, M. (1968). *J. biol. Chem.* **243**, 2266.
- Rojkind, M., Gutierrez, A. M., Zeichner, M. and Lent, L. W. (1969). *Biochem. biophys. Res. Commun.* **36**, 350.
- Rougyie, M. A. and Bear, R. S. (1953). *J. Am. Leath. Chem. Ass.* **48**, 735.
- Sakai, T. and Gross, J. (1967). *Biochemistry* **6**, 518.
- Schleyer, M. (1962). *Hoppe-Seyler's Z. physiol. Chem.* **329**, 97.
- Schmitt, F. O., Hall, C. E. and Jakus, M. A. (1945). *J. appl. Phys.* **16**, 263.
- Schmitt, F. O., Gross, J. and Highberger, J. H. (1955). *Symp. Soc. exp. Biol.* **9**, 148.
- Smith, J. W. (1965). *Nature, Lond.* **205**, 356.
- Tanzer, M. L. (1965). *Int. Rev. Connect. Tissues Res.* **3**, 91.
- Tanzer, M. L. (1967). *Biochim. biophys. Acta.* **133**, 584.
- Tanzer, M. L. (1968). *J. biol. Chem.* **243**, 4045.
- Tanzer, M. L. and Mechanic, G. (1970). *Biochim. biophys. Res. Commun.* **39**, 183.
- Tanzer, M. L., Monroe, D. and Gross, J. (1966). *Biochemistry* **5**, 1919.
- Tanzer, M. L., Mechanic, G. and Gallop, P. M. (1970). *Biochim. biophys. Acta.* **207**, 548.
- Tanzer, M. L., Housley, T., Berube, L., Fairweather, R., Franzblau, C. and Gallop, P. M. (1973). *J. biol. Chem.* **243**, 393.
- Traub, W. and Piez, K. A. (1971). *Adv. Prot. Chem.* **25**, 243.
- Trelstad, R. L., Kang, A. H., Igarashi, S. and Gross, J. (1970). *Biochemistry* **9**, 4993.
- Veis, A. (1964). In "The Macromolecular Chemistry of Gelatin". Academic Press, New York.
- Veis, A. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.), p. 367. Academic Press, New York.
- Veis, A. and Anesey, J. (1965). *J. biol. Chem.* **240**, 3899.
- Veis, A. and Drake, M. P. (1963). *J. biol. Chem.* **238**, 2003.
- Verzar, F. (1964). *Int. Rev. Connective Tissue Res.* **2**, 243.
- Von Hippel, P. H. (1967). In "Treatise on Collagen" (R. N. Ramachandran, ed.), Vol. I, p. 253. Academic Press, London.
- Von Hippel, P. H. and Wong, K-Y. (1962). *Biochemistry* **1**, 664.
- Von Hippel, P. H. and Wong, K-Y. (1963a). *Biochemistry* **2**, 1387.
- Von Hippel, P. H. and Wong, K-Y. (1963b). *Biochemistry* **2**, 1399.
- Weiderhorn, N. M. and Reardon, G. U. (1952). *J. Polym. Sci.* **9**, 315.
- Weir, C. E. (1949). *J. Am. Leath. Chem. Ass.* **44**, 108.
- Wood, G. C. (1960). *Biochem. J.* **75**, 698.

Chapter 2

The Structure and Composition of Collagen Containing Tissues

P. JOHNS

*Department of Experimental Pathology, University of Birmingham,
Birmingham, England*

I	Introduction	32
II	Composition of Mammalian Tissue	33
	A. Collagen and Elastin	33
	B. Other Components	34
III	Skin and Hide	35
	A. Overall Structure	35
	B. Components of Epidermis	38
	C. Insoluble Collagen	39
	D. Reticulin	40
	E. Elastin	41
	F. Soluble Collagen	41
	G. Serum-Like Proteins	43
	H. Other Non-Collagen Proteins	45
	J. Mucopolysaccharides	45
	K. Nucleic Acids	47
	L. Lipids	47
	M. Sialic Acids	48
IV	Bone	48
	A. Overall Composition	48
	B. Inorganic Phase	50
	C. Collagen	53
	D. Mucopolysaccharides	55
	E. Peptide Components	58
	F. Cellular Components	59
	G. Calcification	59
V	Tendon	61
VI	Cartilage	63
VII	Other Mammalian Collagen Tissues	64
VIII	Non Mammalian Collagens	65
	A. Fish Collagens	65
	B. Invertebrate Collagens	65
	References	66

I. INTRODUCTION

The importance of collagen containing tissue in the manufacture and study of gelatin ties in with the fact that the manufacturer must use these materials rather than pure collagen. Thus the manufacture of gelatin involves both conversion of collagen to gelatin, and removal of as much non-collagenous material as possible. Studies of gelatin properties similarly must take into account the non-collagen components and their degradation products some of which remain associated with the gelatin throughout processing.

Changes in these components during processing are not well characterized, but Pouradier (1967) has discussed changes in the carbohydrate containing components of bovine hide and ossein during liming. Changes in the total carbohydrate content of ossein and the gelatin extracted from it after various periods of liming have been studied by Zimkin *et al.* (1965) and the results are shown in Table I. Similar changes have been noted during liming of bovine hide (Blake and Plaster, 1950) and these results are also included in Table I.

TABLE I. Fall in carbohydrate content of bovine hide and ossein during liming

Hide		Liming time	Ossein	
Liming time	Carbohydrate content ^a %		Ossein %	Extracted gelatin %
0 days	0.95	0 days	0.65	0.66
7 days	0.82	10 days	0.38	0.57
14 days	0.68	30 days	0.34	
		50 days	0.25	0.36
		70 days	0.23	0.27
		90 days	0.23	0.22

^a Blake and Plaster (1950).

^b Zimkin *et al.* (1965).

Non-gelatin components have been studied, but their exact relationship to non-collagenous tissue components is not clearly defined. The mucoprotein present in gelatin (Courts, 1959; Leach, 1961, Williams, 1961) is probably derived from tissue muco-polysaccharides and the uronic acid (Venet *et al.*, 1957) and aldehyde (Armes, 1966) also present in gelatin is probably derived from the same source. All these components must suffer degradation during processing, so that the non-gelatin components will differ from the non-collagen components from which they are derived.

It is in the field of photography where non-gelatin components are most significant (for a recent review see Timson *et al.*, 1966). The most interesting

components are sulphur compounds which are photographic sensitizers, and nucleic acids which are restrainers. The nucleic acids of gelatin have aroused considerable interest (e.g. Russell and Oliff, 1966; Pouradier, 1967) and are mainly present in hide gelatin. The absence of nucleic acid in bone gelatin (Russell, 1967) has been attributed to their removal during demineralization of bone. Although nucleic acids are degraded during gelatin manufacture, they retain their fundamental chemical properties.

In this chapter attention will be directed to the nature and quantity of various components present in collagenous tissues, particularly those tissues used for gelatin manufacture.

II. COMPOSITION OF MAMMALIAN TISSUE

A. Collagen and Elastin

Mammalian tissues have many things in common. For example they usually consist of cells embedded in a matrix consisting of collagen, elastin, and mucopolysaccharides. The interactions of these components give the tissue its structural properties, while the cells embedded in the matrix give the tissue its metabolic properties. The proportion of matrix present depends on the tissue function so that structural tissue (e.g. skin, bone or tendon) consists mainly of connective tissue, while tissues with a major metabolic function (e.g. liver or brain) contain little connective tissue.

The earliest attempt to estimate collagen and elastin in mammalian tissues was by Lowry *et al.* (1941). They assumed that the residue left after alkaline extraction consisted of collagen and elastin, while the residue after autoclaving consisted of elastin only. Collagen and elastin contents were then estimated by weight changes following successive extractions. A more refined procedure was adopted by Neuman and Logan (1950) who based the collagen and elastin contents on hydroxyproline determinations of preparations similar to those of Lowry, assuming collagen contains 13.4% hydroxyproline and elastin contains 2% hydroxyproline. While the former assumption is substantially true (modern literature favours a hydroxyproline value of 14.4% in collagen) the existence of hydroxyproline in elastin is not now accepted with certainty.

More recently Dahl and Persson (1963) have estimated the hydroxyproline content of several tissues by direct tissue hydrolysis, and their results can be converted to values of collagen content if one assumes that all the hydroxyproline is derived from collagen. In Table II some of these results have been collected in order to indicate what may be regarded as typical values. Since in no case did the author give precise details of the tissues used, the table should be considered only as a guide to collagen rich tissues.

TABLE II. Collagen content of mammalian tissues

Tissues	Species	Tissue composition as % of dry fat free tissue weight		Author
		Collagen	Elastin	
Skin	Porcine	81		<i>a</i>
	Bovine	89		<i>a</i>
	Human	72		<i>b</i>
Bone	Bovine	24		<i>b</i>
Tendon	Bovine	95		<i>a</i>
	Bovine	85	4.9	<i>b</i>
	Porcine	77	3.7	<i>b</i>
Aorta	Bovine	23	40	<i>b</i>
	Porcine	16	57	<i>b</i>
	Human	28	30	<i>c</i>
Muscle	Bovine	2		<i>a</i>
Intestine	Porcine	19		<i>a</i>
Lung	Porcine	15		<i>a</i>
	Bovine	18		<i>a</i>
Stomach	Porcine	23		<i>a</i>
	Bovine	23		<i>a</i>
Kidney	Porcine	3.8	0.6	<i>a</i>
	Bovine	5.2	1.6	<i>b</i>
Brain	Rat	1.3	1.4	<i>c</i>
Udder	Bovine	42		<i>a</i>
Ligamentum Nuchae	Bovine	34		<i>a</i>
Liver	Bovine	2	0	<i>b</i>
	Porcine	2	0	<i>b</i>

^a Dahl and Persson (1963).

^b Neuman and Logan (1950).

^c Lowry *et al.* (1941).

B. Other Components

Few surveys of other components found in a wide range of connective tissues have been made. However Meyer *et al.* (1956) isolated and characterized qualitatively the mucopolysaccharide from various connective tissues either by alkaline extraction or by enzymatic degradation of the tissue. These are listed in Table III.

More recently a group of structural glycoproteins has been isolated from several connective tissues by first extracting in hot trichloroacetic acid, and then in 8 M urea (Robert and Comte, 1968). These glycoproteins were prepared from tendon, cornea, cartilage, aorta and calf skin and were characterized by absence of hydroxyproline and a high content of hydroxylysine in some cases (40 residues per 100 in calf tendon glycoprotein). It has been

TABLE III. Mucopolysaccharide obtained from various connective tissues

Tissue	Major mucopolysaccharide present ^a
Cartilage	Chondroitin-4-sulphate and chondroitin-6-sulphate
Adult bone	Chondroitin-4-sulphate and chondroitin-6-sulphate
Pig skin	Dermatan sulphate and hyaluronic acid
Ligamentum nuchae	Dermatan sulphate, hyaluronic acid and chondroitin-4-sulphate
Tendon	Dermatan sulphate and chondroitin-6-sulphate
Heart valve	Dermatan sulphate and chondroitin-6-sulphate
Cornea	Keratan sulphate, chondroitin-4-sulphate and chondroitin
Aorta	Dermatan sulphate, chondroitin-4-sulphate and hyaluronic acid

^a Meyer *et al.* (1956).

suggested by Shentall *et al.* (1969) that the high hydroxylysine content is an artifact. They claim it is a new component which moves at the same rate as hydroxylysine during aminoacid chromatography. Its true composition awaits confirmation.

III. SKIN AND HIDE

A. Overall Structure

1. Histology

A complete histological study of animal skins is beyond the scope of this chapter, but excellent coverage of this subject can be found in Hides, Skins and Leather under the Microscope (1957). Figure 1 shows a schematic cross-section of ox-hide, freely drawn from diagrams in McLaughlin and Theis (1945) and Gustavson (1956). It shows the location of various components, and while this is strictly a section of ox-hide, a similar structure occurs in other mammalian skins and hides.

2. Epidermis

This consists of several layers of epidermal cells on the outer surface of the skin. The inner layers of cells are living and dividing and during the passage of time are pushed outwards from the grain layer by new cells, and away from the rich supply of nutrients present there. These cells eventually die, giving rise to an outer layer of dead cells which dries out and is eventually lost by mechanical abrasion. This layer of cells is easily removed during pretreatment operations, whether in the tannery or gelatine works, and so is of little consequence in the main processes of gelatin manufacture.

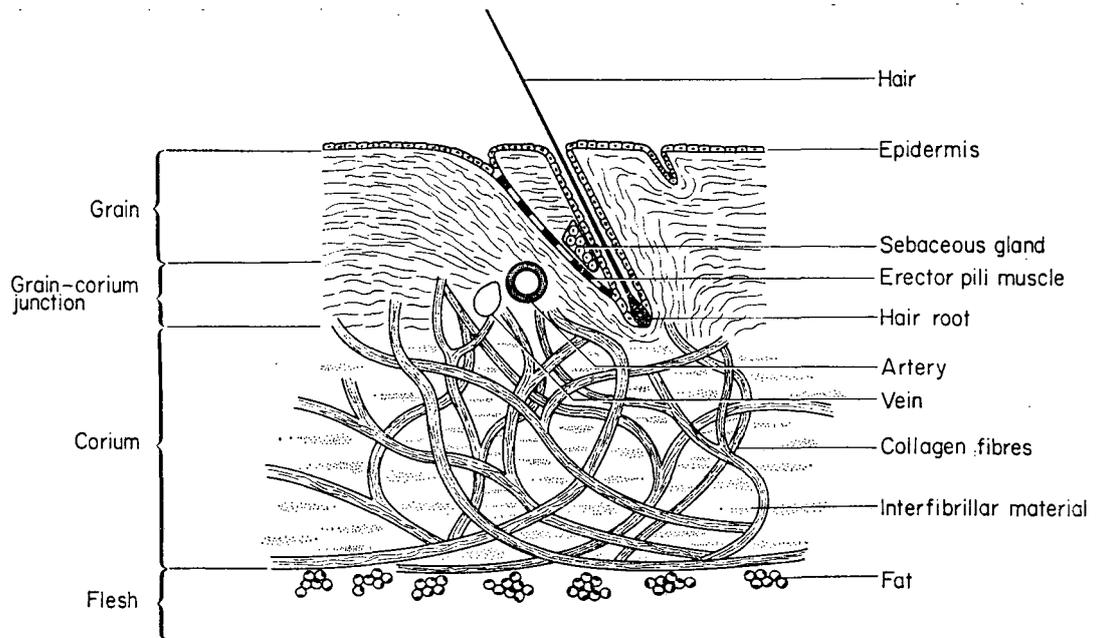


FIG. 1 Schematic cross-section of ox-hide.

3. Hair

Hair roots are located in pockets of the epidermis, and clearly hair varies a great deal according to species. The main component of hair and hair roots is keratin, a protein characterized by a high content of sulphur containing amino acids. The material is removed during pretreatment operations along with the epidermis.

4. Grain layer

This layer is rich in blood vessels, muscle and cells, most of which are difficult to remove, and so represents a possible source of contaminants in finished gelatin in the cases where the whole hide thickness is used for gelatin manufacture. The amount of collagen in this layer is less than in the corium.

5. Corium

This is the portion of the skin consisting mainly of collagen, and gives the skin its structural properties. The collagen fibrils are arranged in fibre bundles which are embedded in a network of ground substances. The fibre bundles are interwoven to give the skin its two dimensional tensile strength i.e. in directions parallel to the skin surface. Other components of this layer are elastin, reticulin and fibroblasts—the cells responsible for collagen biosynthesis. The thickness of this layer varies according to species, and

TABLE IV. Overall composition of calf skin^a

(i) Skin constituents %	(ii) Protein constituents %	
Grease 1–10	Epidermis	0.5–1.0
Water 60–65	Globular proteins	4–6
Protein 30–35	Muscle	very small
Carbohydrate 1	Elastin	Small
Mucopolysaccharide 0.5–1.0	Reticulin	Small
	Collagen	90–95
	Residue after autoclaving	0.6
	(iii) Collagen components ^b	
	Soluble in phosphate buffer pH 9.0	8.5
	Soluble in citrate buffer pH 3.7	20
	Soluble in acetic acid pH 2.8	8
	Soluble in alkali pH 12.2	2
	Residue	60

^a Bowes *et al.* (1957).

^b Middle split of corium extracted in above solutions in sequence.

gelatin is prepared commercially from species with thick corium layers. The fluid which occurs in this layer is in partial equilibrium with serum which accounts for the presence of serum proteins in skin.

6. *Subcutaneous tissue*

This is the fatty tissue, by which the skin is attached to the animal. In some cases the subcutaneous fat and the corium remain separate (e.g. ox-hide) so that the skin as obtained is almost fat free. In other cases however these two layers are intermingled (e.g. pig skin) so that the skin as obtained contains large amounts of fat.

7. *Overall composition of calf skin*

Few authors have attempted to produce a balance sheet for hide and skin, but a useful summary of components has been compiled by Bowes *et al.* (1957) for calf skin. The essential components are shown in Table IV.

B. Components of Epidermis

It has been claimed that epidermal protein is similar to, but not identical with keratin (Rudall, 1946). The outer layers of the epidermis were more heat stable than the inner layers, suggesting that as the cells age and move outwards more sulphur crosslinks are formed. This view is supported by the fact that the thermal contraction temperature is higher in the outer layers.

Epidermis was isolated in a complete sheet in a yield of 1.0 mg/sq cm of skin by soaking bovine hide in concentrated acid or by autoclaving (Hoover *et al.*, 1955). Its hydroxyproline content was too low (about 1.0%) for it to be collagen, while its cystine content was too low (0.8%) for it to be keratin. The sheet was very elastic, but as no elastin layer can be demonstrated histologically, Mellon and Korn (1956) suggested it was a condensed elastin preparation coming from the whole grain layer.

A similar material was produced at a yield of 0.7 gm/sq ft. by Bowes and Elliott (1957) although they used milder treatment conditions (extraction of skin at 60°C for 5 minutes). This yield is equivalent to 1.1 mg/sq cm very close to that obtained by Hoover *et al.* Complete amino acid composition confirmed that the sulphur containing amino acid content was less than that of keratin, and the protein was called pseudo-keratin.

By extraction of human skin in lithium bromide at room temperature Roe (1956) obtained a protein which had the same X-ray pattern as epidermal protein. This had very few sulphur crosslinks, and the author suggests that it may be a precursor of epidermal keratin, the formation of which depends on sulphur crosslinking.

C. Insoluble Collagen

The properties of collagen have been adequately covered in Chapter 1 of this volume, so that only details peculiar to skin collagen will be given here. The major part of collagen is present in the corium layer which itself is mostly collagen. Thus the preparation of pure skin collagen usually begins with mechanical isolation of the corium. In the case of small mammals (rats and mice) this involves shaving the skin to remove hair, grain layer and fat (Dickerson and John, 1964), while in the case of large mammals a splitting machine is used to prepare the corium. This layer is freed of soluble components by mincing and prolonged extraction with various solutions (Veis and Cohen, 1954; Deasy, 1959) and followed by an organic solvent extraction to remove fat and dehydrate the residue. Hydroxyproline determinations on this residue usually show it to be almost pure collagen. It is however contaminated with a small amount of elastin which cannot be removed easily. The composition of this residue of insoluble collagen is quite reproducible, and only small species differences occur. The main difference is the degree of crosslinking which also increases with age.

The amount of insoluble collagen in total skin is variable, for example in mature rats and mice it can vary between 23 and 62% of dry skin with most samples in the range 30–40% (Dickerson and John, 1964). In mature cattle and sheep skin, collagen nitrogen represents 70–80% of the total skin nitro-

TABLE V. Stratigraphic distribution of collagen through cattle hide^a

Layer	Dry wt. (mg)	Hydroxyproline (mg)	Collagen (mg) ^b	Total N (mg)
1	112	7	49	18
2	162	14	98	27
3	184	19	133	32
4	224	34	238	38
5	275	39	273	48
6	288	43	301	51
7	310	48	336	54
8	301	49	343	53
9	320	45	314	56
10	317	50	350	55
11	316	42	294	55
12	335	24	168	58
13	336	16	112	58

The figures represent weights of each component in each piece which was of uniform thickness and area.

^a Mellon *et al.* (1960).

^b Collagen calculated as hydroxyproline $\times 7.0$.

gen (Bowes and Raistrick, 1968), which probably represents 65–75% of skin dry weight (the nitrogen content of collagen is higher than the other components).

The variation in the composition of different layers of cattle skin has been studied by Mellon *et al.* (1960). They divided cattle skin into 13 layers of uniform thickness and then analysed each slice separately. Table V shows the results, and it will be noticed that an abrupt change in composition occurs at layers 4–6 which corresponds to the change from grain layer to corium. A second change is also evident at layers 12–13 where corium gives way to subcutaneous tissue.

The grain layer not only contains less collagen, but its total dry weight content is less, thus representing a more open structure. The fact that the collagen weight as calculated from the hydroxyproline content is consistently higher than the dry weight is not easily explained, but does illustrate the fact that most of the corium consists of collagen.

D. Reticulin

The relationship between reticulin and collagen has been considered by several authors (e.g. Robb-Smith, 1958 and Puchtler, 1964), most of whom consider that the chemical composition of the protein of reticulin is identical with collagen (Windrum *et al.*, 1955). This view is supported by the fact that collagenase, a collagen specific protease acts on collagen and reticulin in the same manner (Habermann, 1961) while peptides released by the action of pepsin on collagen and reticulin are identical (Berrens and van Driel, 1962). However reticulin fibres are branched, while collagen fibres are not, and distinct histological differences can be demonstrated (Melcher, 1964) but the findings of Windrum *et al.* (1955) that reticulin fibres consist of a complex of collagen, glycoprotein, and lipid are still generally accepted (Melcher, 1966). In skin this type of fibre represents only a small proportion of the total collagen, so that most of the work referred to above is based on renal cortex which is very rich in reticulin fibres.

Some recent chemical analyses of reticulin by Snellmann (1963) used rat skin reticulin, although most of his later work used reticulin from bovine spleen and lymph node. The carbohydrate component was characterized and reticulin was claimed to be a complex of 1 mole collagen, 29 moles of glycoprotein, and 89 moles of a guanosine triphosphate peptide all bound together by electrostatic and hydrogen bonds (Snellman, 1963a). The fact that no lipid was detected, while the material was found to be capable of collagen biosynthesis (Snellman, 1963b) suggests that this material is different from the renal cortex reticulin obtained by most other workers. Unfortunately there is no evidence as yet which indicates whether skin reticulin is similar to

renal cortex reticulin, lymph node reticulin, or is different material from either.

E. Elastin

The study of skin elastin has the same problems as the study of skin reticulin since it is present in skin in only small amounts. For this reason most elastin preparations are from ligamentum nuchae, a rich source of elastin (Partridge, 1962). Skin elastin is usually prepared by autoclaving, and it is then assumed that the residue is elastin. Human skin elastin has been prepared by this method (Smith *et al.*, 1963) and its amino acid composition was very similar to that of ligamentum nuchae elastin prepared by Partridge and Davis (1955). Quantitative histological techniques have been applied to human skin to demonstrate that no change in elastin content occurs during ageing (Hult and Goltz, 1965). The technique was not absolutely quantitative so that no value for the elastin content of the skin could be given.

Elastin has been prepared from the grain layer of bovine skin (Mellon and Korn, 1956) and, by slicing the skin prior to elastin determination, it was shown that elastin was distributed right through the grain layer at a quantity representing 2-4% of total nitrogen, although the elastin content was much smaller in the lower portion of the grain layer. This agrees with histological evidence that elastin content is much higher in the grain layer than in the corium. Ornes and Roddy (1960) found that elastin comprised 2.5% of cattle skin weight, and up to 86% of this could be removed by trypsin treatment.

F. Soluble Collagen

As the properties of soluble collagen have been described in detail in Chapter 1 this section will be restricted to those details which are specifically related to skin.

The procollagen of Orekhovich *et al.* (1948) was prepared by citrate buffer extraction (pH 4.0) of the residue after various skins and hides had been ground and extracted in neutral phosphate solutions. This material, soluble in acid buffers, is often called tropocollagen, although the systematic name acid soluble collagen seems more logical. The terms procollagen and tropocollagen both infer that this material is a precursor of insoluble collagen, but in fact this is not so (Harkness *et al.*, 1954) but the precursor of both acid soluble collagen and insoluble collagen is a fraction soluble either in phosphate buffer solution (pH 9.0) or neutral solution. This fraction was first known as alkali soluble collagen, but is now usually known as neutral salt soluble collagen. It should be noted that if no neutral salt extraction is carried out prior to acid extraction, both these fractions are extracted by acid solution. In no case can these extracts be called "pure soluble collagens" since non-collagenous

materials are also extracted under these conditions. Pure collagens are prepared by reprecipitation, usually in the form of fibrils, and then re-extracting in acid solutions. The properties of acid soluble and neutral salt soluble collagen have been compared closely—Kawai *et al.* (1966) and Veis *et al.* (1966) have examined their physical and chemical properties, while Davidson and Cooper (1968) have considered differences in the number of internal cross-links. They concluded that only if there were significant differences in the teleopeptides could one account for the differences in solubility properties shown by these two fractions.

The quantities of soluble collagen which can be obtained depends on the species, age of animal and the techniques used. Table VI is an attempt to collect typical quantitative data on this point.

TABLE VI. Quantities of soluble collagens extractable from various animal skins

Species	Age	Percentage of soluble collagen (based on dry skin weight)			Author
		Neutral salt soluble %	Acid soluble %	Total %	
Rabbit	4 weeks	3.6	19	22.6	<i>a</i>
	Birth	9.0	0	9.0	<i>b</i>
	12 weeks	13.0	6.5	19.5	<i>b</i>
	9 months	3.9	3.9	7.8	<i>b</i>
Rat		10	3.3	13.3	<i>c</i>
Lamb		4.8	2.1	6.9	<i>d</i>
Sheep		0.6		2.6	<i>d</i>
Sheep	15 weeks			12-18	<i>e</i>
Calf				7	<i>f</i>
Calf				100 ^g	<i>g</i>
Bull		0.03	0.50	0.53	<i>h</i>
Bull				100 ^k	<i>g</i>

^a Harkness *et al.* (1954).

^b Nimni *et al.* (1965).

^c Houck and Jacob (1960).

^d Yates (1968).

^e Bowes and Raistrick (1966).

^f Kahn and Witnauer (1969).

^g Reiss (1964).

^h Veis *et al.* (1960).

^j After 10-15 weeks extraction.

^k After 27-30 weeks extraction.

G. Serum-Like Proteins

Various proteins are found in neutral extracts of animal skins and several attempts have been made to characterize them. Unfortunately many protein species exist, and as their total content in animal skins is small, studies of individual proteins are difficult, especially in the case of smaller mammals. By extraction of rabbit skin and rat skin at pH 8.0 Humphrey *et al.* (1956 and 1957) were able to isolate several serum-like proteins, one of which was similar to serum albumin. This fraction comprised 0.5–0.7% of wet rabbit skin, corresponding to 25–30% of the total body serum albumin. Use of immunological techniques confirmed its identity as serum albumin, so confirming the findings of Harkness *et al.* (1954) who identified rabbit skin proteins as serum proteins by moving boundary electrophoresis.

More recently Adelman (1966) has examined rat skin by extraction in physiological buffer solution (phosphate), 2 M sodium chloride, and then by digestion of the residue with collagenase. The distribution of non-collagenous protein in each fraction, and their identification is shown in Table VII. The proteins specific to skin were not characterized.

TABLE VII. Identification of non-collagen proteins of rat skin^a

Fraction	Proportion of non-collagenous protein in fraction %	Identification of fraction
Physiological phosphate buffer extract	8	7 serum proteins
2 M sodium chloride extract	13	4 skin specific proteins
Collagenase digest	1	Skin specific protein
Collagenase residue	70	Keratin, elastin, cell components and non-collagenous protein (unidentified).

^a Adelman (1966).

Turning now to cattle skins, several proteins were obtained by extraction of calf skin with 10% sodium chloride (Cooper and Johnson, 1957) but the yield was very low (2.2% of total nitrogen) and only 20% of the extracted material was non-dialysable. This non-dialysable fraction was examined by moving boundary electrophoresis, and five components were separated. Hydroxyproline determination indicated a low collagen contamination (about 2.5%), although this fraction could be converted to rope-like twisted fibrils containing no hydroxyproline (Cooper and Johnson, 1956). Subsequent extraction of the skin in either phosphate buffer solution (pH 7.0)

or half saturated limewater liberated further quantities of a similar material (Cooper and Johnson, 1959). The total extractable protein comprises 0.75% of the dry skin weight, and so was rather less than the yield of protein extracted from rabbit skin (0.7% of wet skin weight), (Humphrey *et al.*, 1957). Each of these extracts was examined by moving boundary electrophoresis, and Table VIII shows the composition of each extract. Fraction A was tentatively identified as serum albumin and fraction C as β_1 -globulin.

TABLE VIII. % Composition of extracts of cattle hide by moving boundary electrophoresis^a

Component	Mobility ($\times 10^5$)	Protein extracted in		
		Sodium chloride %	phosphate buffer %	Limewater %
A	-7.4	25	28	22
B	-5.1	21	20	18
C	-3.6	21	14	41
D	-2.3	13	12	
E	-0	20	12	12
F	-1.6		14	
G	-11.1			15

^a Cooper and Johnson (1958).

In a later series of experiments (Cooper *et al.*, 1967) fresh calf skin was extracted exhaustively with sodium chloride to yield 9.3 gm protein per 100 gm dry skin, considerably more than in previous experiments. The total extract contained 5.3% polysaccharide, and 87% collagen free protein. The protein was fractionated by a variety of methods into at least 8 components, one of which was probably serum albumin. The purity of these components must be viewed with some doubt since the proportions of each as shown by DEAE-cellulose chromatography and moving boundary electrophoresis were quite different. The yield of plasma type protein isolated in the last experiment is very comparable to the yield (8-10%) consistently obtained by Bowes *et al.* (1958) by a similar extraction of hide.

Two proteins have been obtained from foetal calf skin by ammonium sulphate fractionation of an aqueous extract. One was a glycoprotein which resembled feutin in many of its properties (Bourillon and Got, 1962) while the second was similar to serum albumin (Got and Bourillon, 1962). Probably the serum albumin corresponds to that obtained by Cooper, while the glycoprotein may correspond with another of his fractions, or it may be like feutin and only occur during the foetal state, disappearing rapidly after birth.

H. Other Non-Collagen Proteins

Alkaline extraction of calf skin (about pH 12.0) was shown to separate a non-collagenous protein (Bowes *et al.*, 1958) whose hexosamine content suggested only small mucopolysaccharide contamination, and whose amino acid composition was very different from collagen. Its hydroxyproline content suggested it was contaminated with some collagen (about 5%) and it comprised about 10–15% of the hide weight (this was in addition to the 8–10% of plasma type protein previously separated by extraction at pH 9.1). A similar material could also be obtained by acid extraction after precipitation of the collagen from this extract (Bowes *et al.*, 1956).

A glycoprotein has been prepared from calf skin by first treating with hot trichloroacetic acid, and then extracting the residue in 8 M urea solution (Robert and Comte, 1968). This protein was low in glycine and hydroxyproline suggesting slight contamination of a non-collagenous protein by some collagen. By a similar method of preparation from rabbit skin, Timple *et al.* (1968) obtained proteins which were subsequently water soluble, but only extractable in strong denaturing agents, or strong alkali. The amino acid composition was comparable to that of the calf skin glycoprotein prepared by Robert and Comte (1968).

An insoluble residue was prepared from a middle corium split of steerhide by collagenase digestion (Tancous, 1969). This procedure removed collagen completely as well as soluble proteins and polysaccharide, to leave a glycoprotein structural network, comprising 2% of the dry weight of the corium split. This was readily soluble in alkali, but not in lime solution, and was also readily solubilized by trypsin digestion.

Thus proteins soluble in severe conditions have been prepared by Bowes *et al.* (alkali), Timple (alkali or urea), Robert and Comte (urea) and Tancous (alkali) all having some sugar content. While it is not suggested that these proteins are identical, it is suggested that they may be related, or may be similar degradation products of the same skin protein component.

J. Mucopolysaccharides

Mucopolysaccharides have been extracted under the name of "mucoid" from cattle hides for many years by extraction with half saturated lime solutions (McLaughlin and Theis, 1924). It is perhaps significant that it is still considered necessary to use a strongly alkaline extractant even when the tissue is finely divided or has received prior enzyme digestion (Barker *et al.*, 1969). Thus one may assume that the mucopolysaccharide fraction is not in free solution in the tissue spaces as are the serum-like proteins, but is chemically linked to the matrix by alkali labile bonds. Most of the early work on

cattle skin was done at a time when mucopolysaccharide fractionation techniques had not been developed while more recent work has concentrated on the skin of man or small mammals.

Some mucopolysaccharides were prepared from human skin in 1949 (Pearce and Watson), and the sodium hydroxide extract was fractionated into hyaluronic acid and chondroitin sulphate (about 0.06% of each by weight). More recent work (Loewi, 1961) showed that human skin also contained dermatan sulphate, but it has been shown that even more species of mucopolysaccharide are present in human skin (Barker *et al.*, 1969, 1969a). Here a mucopolysaccharide extract was separated by ion-exchange chromatography (Permutit De-Acidite FF) to give seven fractions which were identified as hyaluronic acid (two fractions), chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparin and keratin sulphate. Thus proper evaluation of the mucopolysaccharide fraction requires careful fractionation in order to separate all the species present.

The study of smaller mammals follows a similar pattern. Rabbit skin was shown to contain hyaluronic acid and chondroitin (Schiller and Dorfman, 1960). The last two authors also showed how the mucopolysaccharide content was related to the age of the rat, and found in general that the amount in each species fell with age.

A more complete extraction of rat skin by Kofoed and Bozzini (1969) followed by careful fractionation showed that rat skin contained the same six species as did human skin. The quantity of each is shown in Table IX.

Pigskin mucopolysaccharides were prepared by Loewi and Meyer (1958) and separated into hyaluronic acid, chondroitin-6-sulphate and dermatan

TABLE IX. Mucopolysaccharides isolated from rat skin^a

Mucopolysaccharide	Uronic acid	% of total
	(μg uronic acid/gm dry skin) μg	uronic acid %
Total	1184	100
Glycoprotein	48	4
Hyaluronic acid	528	44
Heparitin sulphate	58	5
Chondroitin-4-sulphate	69	6
Chondroitin-6-sulphate	68	6
Dermatan sulphate	205	17
Heparin	71	6
	1047	88

^a Kofoed and Bozzini (1969).

TABLE X. Mucopolysaccharides isolated from cattle hides^a

Cattle hide split	Mucopolysaccharide expressed in % of dry tissue		
	Total %	Dermatan sulphate %	Hyaluronic acid %
Grain split	0.410	0.177	0.226
Corium split	0.285	0.183	0.102
Flesh split	0.340	0.208	0.115

^a Meyer *et al.* (1957).

sulphate totalling 0.3% for embryonic skin and 0.2% for adult skin (assuming adult pig skin has a dry weight content of 60%). In the case of the adult skin the composition of the mucopolysaccharide fraction was dermatan sulphate 64%, hyaluronic acid 30%, and chondroitin sulphate 0.1%. By papain digestion of finely divided pig skin Cifonelli and Roden (1968) were able to isolate heparin and heparitin sulphate as well as chondroitin-4-sulphate and chondroitin-6-sulphate. Thus the total number of mucopolysaccharide species identified in pig skin is the same as from rat skin and human skin. It is noticeable that only since 1968 has the true complexity of skin mucopolysaccharides been realized.

Very little quantitative data is available for cattle skin since most authors quote values for total mucopolysaccharide content. Ox hide has been divided into three layers and the mucopolysaccharides present in each layer divided into dermatan sulphate and hyaluronic acid (Meyer *et al.*, 1957) and the results are shown in Table X. It remains to be seen if cattle skin will eventually be shown to contain the same large number of mucopolysaccharides that has been isolated from other skins.

K. Nucleic Acids

The nucleic acid content of skin, a metabolically inert tissue is very low, so that studies of skin nucleic acids are rarely made. In work by Natarajan and Bose (1965) rat skin nucleic acids were prepared by the Schmidt-Thanhouser-Schneider technique. Calculations from the data published show that the rat skin contained about 0.8% ribonucleic acid and about 0.2% deoxyribonucleic acid in dry defatted tissue.

L. Lipids

The source of skin lipids is in some doubt in that although many studies of

surface lipids have been made for several animals (Nicolaidis *et al.*, 1968; Wilkinson, 1969) the lipid which is exuded to the skin surface is probably not a true skin component. However by slicing cattle skin Bitcover and Mellon (1966) showed that lipids were present right through the skin. Lipids identified were waxes, sterol esters, triglycerides, sterols and free fatty acids, although phospholipid was almost completely absent.

M. Sialic Acids

Although sialic acids are not components of skin as such they are certainly identifiable in some skin components. Their presence in these materials may represent the presence of sialoproteins as contaminants of that particular fraction or it may be that the particular component contains sialic acid of one of its molecular constituents. Several skin constituents have been prepared by Bose (1963) and their sialic acids contents determined, along with other carbohydrates as shown in Table XI. The high sialic acid content of the goat skin proteins does suggest that a true sialoprotein may be present, as is the case with bone (Andrews and Herring, 1965), but whether this is a general property of animal skins, or just found in goat skin remains to be seen.

TABLE XI. % Carbohydrate contents of various skin components ^a

Protein	Source	NANA ^b %	NGNA ^c %	Hexose %	Hexosamine %
Insoluble collagen	Buffalo hide	0.134	0	0.59	0.09
Citrate soluble collagen	Rat skin	0.036	0		
Salt soluble collagen	Rat skin	0	0		
Elastin	Ligamentum nuchae	0.062	0		
Albumin	Goat skin	0.380	0.121	1.90	0.42
Globulin	Goat skin	0.675	0.171	2.21	0.52
Mucoid	Goat skin	2.909	0.309	6.97	1.69

^a Bose (1963).

^b N-Acetyl neuraminic acid (determined by quantitative chromatography).

^c N-Glycolyl neuraminic acid (determined by quantitative chromatography).

IV. BONE

A. Overall Composition

As organs the overall composition of bone varies considerably according to species and type. This variation is mainly due to the fact that bones contain several types of tissue, for example, marrow, cartilage, spongy

bone tissue, compact bone tissue, periosteum and blood vessels. Table XIII gives some indication of the possible variation in overall composition, while an extensive survey of many aspects of bone composition was made by Eastoe (1961).

The material usually used in research is compact bone tissue, partly because its composition is fairly reproducible, and partly because it is easily separated from other types of tissue. Possibly the best method of preparing compact bone tissue is that of Herring and Kent (1963) where bone was powdered in a mill cooled with liquid nitrogen. Values for the overall composition of compact bone tissues are given in Table XIII. More recent work (Oldroyd and Herring, 1967) shows that collagenase digestion of EDTA demineralized ox bone leaves a non-collagenous carbohydrate containing residue of 9.5% of the organic matrix, which is separable into several fractions by DEAE-cellulose chromatography. (This material is considered in greater detail later.)

TABLE XII. Variation in the composition of whole bones ^a

Water	14-44
Fat	1-27
Other organic matter	16-33
Inorganic matter	25-56

^a Range of composition of whole bones from a fresh whole skeleton of a typical animal (Gerngross and Goebel, 1933).

TABLE XIII. Composition of compact bone tissue ^a

	% by weight
Inorganic matter	
Insoluble (including up to 1% citrate)	69.7
Soluble	1.25
Organic matter	
Collagen	18.6
Mucopolysaccharide-protein complex	0.24
Resistant protein	1.02
Water	8.2
	99.9

^a Eastoe and Eastoe (1954).

A paper by Campo and Tourtellotte (1967) has given a detailed comparison of the composition of various tissues in the ox long bone. The figures given for the compact bone agree very well with those of Eastoe in Table XIII, and it seems quite clear from this work that the chemical composition of spongy and compact bone are very similar despite their very different structure.

A selection of results are found in Table XIV.

TABLE XIV. Comparison of composition of bovine compact and spongy bone ^a

	Primary spongiosa % by weight	Bone tissue Secondary spongiosa % by weight	Compact % by weight
Nitrogen	4.5	4.5	4.3
Ash	65.2	65.1	66.6
Water	6.4	6.1	5.6
Collagen	21.4	22.5	21.9
Chondroitin sulphate	0.55	0.31	0.23
Keratan sulphate	0.4	0.2	0.2
Sialic acid	0.12	0.10	0.07
Other proteins ^b	3.0	1.9	1.3

^a Campo and Tourtellotte (1967).

^b Other proteins was calculated from the difference between the collagen nitrogen plus hexosamine nitrogen and the total nitrogen.

The changes in composition of bone tissue during growth and maturation have been considered by Dickerson (1962) for human bone and by Dickerson (1962a) for pig, rat and fowl. As each animal matured the cortical bone showed an increase in the proportion of inorganic components and of collagen, while the proportion of non-collagen nitrogen fell. This change presumably represents the final stages of mineralization of the embryonic cartilage template of the rudimentary embryonic bone. Analysis of the whole bones of the animals showed that during maturation the total bone weight and fat content both increased while the proportion of water fell.

B. Inorganic Phase

Complete analysis of the inorganic phase of bone shows that many ions are present, mostly in relatively small amounts. Thus, calcium phosphate and carbonate ions constitute about 98% of the total, while the other 2% is made

up of ions such as magnesium, sodium, potassium, chloride and fluoride, plus others in amounts less than 0.01% (Eastoe, 1961).

A total of 12 trace elements were detected and measured in human bone by Becker *et al.* (1968), while a further 24 elements were shown to be absent, or present below the limits of detection by arc emission spectroscopy. Those elements present in modern bone ranged from zinc and lead present at about 50–100 p.p.m., with silver intermediate at about 25 p.p.m. The high content of strontium (130 p.p.m.) is probably not normal but due to uptake of radioactive fall-out. While inorganic ions are usually considered to be part of the inorganic phase it is quite likely that trace elements present at these levels may be associated with either phase.

There has been much dispute about the nature and structure of the main calcium phosphate component (for a recent comment see McLean, 1967). Although X-ray diffraction has proved to be a very useful technique, the differences of the lines, due in part to the small crystal size, makes accurate interpretation of the patterns difficult. There have been many contenders for the role of major component, notably carbonato-apatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$), hydroxy-apatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), tricalcium phosphate hydrate ($\text{Ca}_9(\text{PO}_4)_6\text{H}_2(\text{CH})_2$) and octa-calcium phosphate ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$). Table XV compares the overall composition of these with that found for bone.

Various people have maintained that each of these components represents the structure of the inorganic phase of bone. Hydroxy-apatite is usually regarded as the main component (Samachson, 1968), although carbonato-apatite has been suggested again (Newesely, 1963; Hayek, 1967) since it has become possible to prepare it synthetically. Tricalcium phosphate hydrate is still considered to be a major component by Dallemagne (1964) while

TABLE XV. Composition of various calcium phosphates

	%Ca	%PO ₄	%CO ₃	Ca: PO ₄ molar ratios
Bone ^a	36.9	48.9	5.15	
Bone (adjusting sum of above figures to 100%)	40.0	54.3	5.7	10.7:6
Bone calcium phosphate (assuming calcium carbonate forms a separate phase)	40.0	60.0		9.5:6
Carbonato-apatite	39.3	56.1	4.6	10:6
Hydroxy-apatite	39.8	56.6		10:6
Tricalcium phosphate hydrate	37.3	59.0		9:6
Octa-calcium phosphate	35.9	63.9		8:6

^a Eastoe (1961).

octacalcium phosphate is suggested by Brown *et al.* (1962) and it is claimed that X-ray analysis cannot differentiate between this material and hydroxy-apatite.

The presence of other ions (especially carbonate and fluoride) makes the situation difficult to resolve, especially as the crystallographic differences between these materials are quite small. Other possibilities are that changes in structure occur after mineralization (Newesley, 1966) or that local variations in composition occur within the crystal lattice (Newesley and Hayek, 1963). Finally the known variation in structure and composition of apatites may account for some of the confusion associated with studies of the inorganic phase of bone (Winand, 1965; McConnell, 1965; Rowles, 1965).

A novel suggestion is that some of the calcium phosphate is non-crystalline (up to 40%), Termine and Posner, 1967, 1967a) an idea based on X-ray, electron spin resonance and infra-red evidence. This suggestion has been criticized by McConnell (1967) who claimed that the experimental results of Termine and Posner should be explained differently. Amorphous calcium phosphate preparations are unstable and slowly form crystals via the solution phase, but the anhydrous preparations are stabilized *in vitro* by magnesium and carbonate ions, both of which are present in bone (Eanes *et al.*, 1966). Other work by Termine and Posner (1966) suggests that amorphous calcium phosphate may be an intermediate in the formation of hydroxy-apatite, while Brown (1965) has proposed that octacalcium phosphate may be a similar intermediate. The precipitation *in vitro* of amorphous calcium phosphates and their stabilization by magnesium ions has also been studied by Bachra *et al.* (1965).

The spatial arrangement of the micro-crystals is unique, each crystal having one dimension longer than the other two with this longer dimension always parallel to the fibre direction (Engström and Finean, 1953; Carlström *et al.*, 1955). Their original dimensions have recently been modified and current evidence based on X-ray analysis and electron microscopy favours dimensions of $30\text{--}40 \times 7.5 \times 7.5$ nm (Engström, 1966). On this basis two micro-crystals fit into each 65 nm unit of the collagen fibre. This crystal size may help to account for the diffuse bands found in X-ray patterns, and since the apatite unit cell has dimensions of 0.914×0.768 nm each crystal can contain only about 1200 unit cells of which almost half will be on the surface of the crystal. Thus there is a strong possibility that surface defects or surface absorption of other ions will play an important part in determining the overall structure and composition of the inorganic phase.

Another important ion occurring in bone is the citrate ion, which apparently is present even before birth (Gedalia *et al.*, 1967) and can account for up to 1% by weight of whole bone tissue. After ethylene diamine extraction of the organic phase (Taylor, 1960) most of the citrate remains bound to the inor-

ganic phase. Following acid decalcification, however, no citrate was lost into the acid liquors, although EDTA decalcification solubilized up to half of it. This suggests that the citrate may be divided between the organic and inorganic phases and that some exists in the (calcium citrate) form rather than (citrate) or its undissociated molecule. Since the citrate ion is larger than the phosphate ion it seems unlikely that citrate will replace phosphate in the hydroxy-apatite lattice, although this might be possible on the crystal surface.

In contrast with Taylor's findings, Leaver and his associates have demonstrated the presence of citric acid in the acid demineralization liquors of bone and dentine (Hartles and Leaver, 1960; Leaver *et al.*, 1960). The citric acid is not bound to the peptides (Leaver *et al.*, 1965) and more recently other organic acids (lactic acid in particular) have been found along with the citric acid (Leaver and Shuttleworth, 1967).

C. Collagen

In view of the rather special nature and properties of bone collagen (ossein) this section will deal with those properties of hard tissue collagens which differ significantly from the properties of soft collagens. It should be borne in mind that the overall amino-composition of these two classes of collagen is very similar (Eastoe, 1955; Tristram and Smith, 1963).

Although the extraction of soluble collagen from bone was claimed by Araya *et al.* (1961) no quantitative data were presented, and indeed doubt has been cast on the existence of any soluble collagen in bone (Piez, 1963). Mills and Bavetta (1966) found that as calcification of the immature cartilaginous bone occurred in the rat there was a marked fall in the amount of extractable collagen. This suggests that the collagen becomes crosslinked and insolubilized as calcification proceeds, so that mature bone contains little or no extractable collagen.

Glimcher *et al.* (1965) have recently solubilized collagen from decalcified chicken bone by a procedure involving cooling to -70°C after which treatment up to 30% of the collagen was soluble in dilute acetic acid. This soluble material could be reprecipitated as native type fibrils. Alternatively if the demineralized material was extracted in solutions of hydrogen bond breakers (Glimcher and Katz, 1965) under such conditions that covalent bonds would not be broken, up to 80% was soluble. A large proportion of the solubilized protein had a similar sedimentation coefficient to the α -components prepared from soluble hide collagen and smaller proportions had sedimentation coefficients comparable to the β - and γ -components from hide collagens. Thus it appears that covalent crosslinks in bone collagen are intermolecular in nature, and that the triple helix assembly is maintained by hydrogen bonds only (Glimcher and Katz, 1965).

This latter conclusion is not supported by the findings of Miller *et al.* (1967) who when using similar starting material and similar methods were only able to solubilize 17% of the collagen by hydrogen bond breakers. However properties of the solubilized material which they quote agree quite well with those quoted by Glimcher.

Veis and Schlueter (1963) considered crosslinking from the point of view of the swelling properties of the insoluble collagen matrix. They found that when treated under the same conditions, whole hide swelled to seven times its original volume although demineralized dentine did not swell at all. Also the demineralized dentine was far less easily solubilized in hot water (60°C), and was more difficult to denature using urea. Further work by the same authors was concentrated on the covalently bound phosphate found in demineralized dentine (Veis and Schlueter, 1964). This could not represent residual calcium phosphate since the calcium content was negligible by comparison. The dentine collagen contained 4 moles of bound phosphate per 100,000 g of collagen and when the collagen was solubilized by sodium metaperiodate one of the products was a phosphate-rich peptide (Schlueter and Veis, 1964). By comparison, hide contained neither covalently bound phosphate or the phosphate-rich peptide. Subsequent work (Veis and Perry, 1967) characterized the phosphate-rich peptide as an ultracentrifugally homogeneous species (MW 38,000) containing 34 phosphate groups per molecule and large amounts of serine and aspartic acid.

On the basis of these swelling experiments these authors have proposed phosphate and carbohydrate crosslinks in dentine collagen (Schlueter and Veis, 1964) although it is not claimed that the phosphate-rich peptide itself is involved in crosslinking (Veis and Perry, 1967).

Comparable experiments have been carried out by Courts (1963) using demineralized ox bone alongside other soft tissues (skin and ox tendon). No swelling was detected at any pH value with demineralized bone although the other tissues showed significant swelling in the range pH 2.0–6.0 and above pH 10.0. More recently McKernan and Dailly (1966) have confirmed these results of Courts, and shown that the content of bound phosphorus was much less in demineralized bone (0.006% HPO_3 or 0.07 moles/100,000 g) than in demineralized dentine (0.4% HPO_3 or 5 moles/100,000 g) and they suggest that in bone the amount of bound phosphate is probably too small to account for crosslinking.

Glimcher and his associates have also made a study of covalently bound phosphate in relation to collagen. Skin collagens were found to contain covalently bound phosphate (1–3 moles per mole of collagen or 0.3–1 mole per 100,000 g) and collagenase digests of these collagens contain peptides rich in phosphate, serine and carbohydrate (Glimcher *et al.*, 1964) More recently using α -chains of bone collagen solubilized by the freezing technique Fran-

cois *et al.* (1967) found that bone collagen also contains covalently bound phosphate (2 moles/mole collagen or 0.7 moles/100,000 g). It is suggested that this covalently bound phosphorus could act as a nucleating site for calcification. (See G. Calcification.)

The non-covalent association of phosphorus with collagen has also been examined in some detail. Using soft tissue collagens Glimcher and Krane (1964) found that under physiological conditions 150–170 moles of phosphate were associated with 1 mole of collagen (or 50–57 moles/100,000 g) much greater than the amount of covalently bound phosphate. While Glimcher and his associates have not yet carried out parallel experiments on hard tissue collagens, Mobbs (1966) has studied the association with a solubilized dental collagen (“dental eucollagen”). He found 148 moles of phosphate were associated per mole of eucollagen (at a molecular weight of 217,000 this corresponds to 68 moles per 100,000 g), a similar result to that for soft tissue collagens. This association was inhibited by reaction of the protein with 1-fluoro-2,4-dinitrobenzene which suggests that this association is related to the presence of free ϵ -amino groups.

Another important factor is the availability of an ϵ -amino groups in bone collagen. Solomons and Irving (1958) found that demineralization of bone frees the ϵ -amino groups making them available to 1-fluoro-2,4-dinitrobenzene (FDNB). This was interpreted in terms of the ϵ -amino groups being involved in collagen-inorganic matrix bonding, and that as inorganic ions are removed, the amino groups become free to react. Glimcher (1960) however, considered that the inorganic component simply acts as a diffusion barrier, so preventing access of FDNB to the reacting sites. Solomons and Neuman (1960) have found that the reaction of demineralized dentine and bone depends on the conditions of the FDNB reaction—if the FDNB is present when demineralization takes place, all the ϵ -amino groups react. If not, the demineralized matrix loses its reactivity towards FDNB, demineralized bone becomes unreactive after several days, while demineralized dentine becomes unreactive after several weeks. Later, Wuthier *et al.* (1964) concluded that only 50% of the ϵ -amino groups are involved in associations with the mineral phase, while Glimcher *et al.* (1965a) found other evidence to support the idea that ϵ -amino groups are involved in calcification. Here recalcification of decalcified bone was interfered with after treatment with two reagents which modify amino groups (FDNB and carbobenzoxy) but not after treatment with other amino modifying reagents.

D. Mucopolysaccharides

These materials were first studied by Hawk and Gies (1901) who prepared “osseomuroid” by extraction of demineralized ox bone with half-saturated

lime water. Hisamura (1938) divided "osseomucoid" into two fractions, and later Eastoe and Eastoe (1954) studied the overall composition of this material. Rogers (1951) extracted a carbohydrate component, probably chondroitin sulphate, from ossein by heating at 90°C and then digesting with trypsin, while Glegg and Eiding (1955) isolated two components, one of which was probably chondroitin sulphate, after ethanol fractionation of alkali extracts of demineralized bone.

In 1958 Dische *et al.* prepared a whole series of polysaccharides from bone where decalcification by EDTA solubilized some 35% of the total protein along with some polysaccharide (acidic mucopolysaccharide and a polysaccharide containing hexose and hexosamine). The EDTA residue was extracted with alkaline ethanol when most of the residual protein dissolved and the solution contained polysaccharide material: (i) Non-dialysable material containing hexosamine and (ii) dialysable material containing no hexosamine. The alkaline ethanol residue was mostly water soluble and the small amount of polysaccharide material contained significant amounts of fucose. Unfortunately all these methods of preparation are sufficiently severe to degrade the more labile components, for example the sialic acid which has been found in compact bone (Castellani *et al.*, 1960).

In general the more delicate techniques of Herring and his associates are more likely to produce native products. For example by demineralization of bone powder (powdered in such a way that the temperature was kept below 25°C) with EDTA at pH 7.4 and then dialysing away the EDTA and other inorganic ions, a solution containing carbohydrate components was left (Herring and Kent, 1963). This extract was then fractionated by precipitation, chromatography and electrophoresis into several components with included (i) a material rich in hydroxyproline, (ii) a mucoprotein, (iii) a sialoprotein, (iv) chondroitin sulphate. In an alternative fractionation scheme (Herring, 1964), the electrophoretic step was eliminated and similar components were prepared in a fairly pure state. The chondroitin sulphate from this procedure was split into 3 fractions by DEAE chromatography, and these were electrophoretically homogeneous (Herring, 1968) although their composition varied (Table XVI). Whether these are individual molecular species, or complex mixture of chondroitin sulphate and sialoprotein is uncertain.

The sialoprotein has been prepared in a substantially pure form by the method already described and also by acetic acid and phosphate buffer extraction of whole bone (Andrews and Herring, 1965). The composition of a preparation which was homogeneous by ultracentrifugation, by moving boundary electrophoresis, and by cellulose acetate electrophoresis is given in Table XVII (Andrews and Herring, 1965; Williams and Peacocke, 1965; Andrews *et al.*, 1967). Its protein component contains no hydroxyproline or hydroxylysine (indicating absence of collagen) but it contained large amounts

TABLE XVI. Composition of chondroitin sulphate fractions^a

	D ₁	D ₂	D ₃
Total N	6.2	5.5	2.7
Total amino acids	27.5	21.7	1.80
Uronic acid (carbozole)	17.1	22.1	30.1
Galactosamine	13.9	17.9	25.1
Glucosamine	1.7	1.2	0.1
Hexose (Anthrone)	5.2	4.2	0.9
Sialic acid (Thiocarbituric acid)	12.1	6.9	0.05
Sulphate	8.5	10.9	16.9

^a Herring (1968).

of glutamic and aspartic acids. Its binding properties with calcium, yttrium and thorium are of particular interest (Peacocke and Williams, 1966) as are its binding properties with plutonium and americium (Chipperfield and Taylor, 1968).

TABLE XVII. Properties and chemical composition of bone sialoprotein^a

	% w/w	mole/mole
N-acetyl neuraminic acid	18.1	13.7
N-glycolyl neuraminic acid	2.1	1.5
Galactose	8.2	10.5
Mannose	2.5	3.1
Glucosamine	4.6	5.9
Galactosamine	4.6	5.9
Zinc	1.4	5.0
Phosphate	1.4	3.4
Protein	51.8	
	Molecular weight 23,000	
	Molecular weight of carbohydrate portion 9220	

^a Andrews *et al.* (1967).

The EDTA solubilization technique has been applied to rabbit bone by Burckard *et al.* (1966) with similar results. The carbohydrates in the EDTA solution were fractionated as shown in Table XVIII and although the identification of the various components is somewhat doubtful, the table gives some idea of the number of components and their relative proportions.

Perhaps the most delicate method of isolating the carbohydrate-containing components is that of Oldroyd and Herring (1967). Here bone powder was

TABLE XVIII. Composition of non-collagenous fraction of organic matrix of rabbit bone^a

	% by weight
γ -globulin	14
Serum albumin	21
α_1 -glycoprotein	29
Sialoprotein	24
Acid mucopolysaccharide	12

^a Non-dialysable residue after EDTA demineralization (Burckard *et al.*, 1966).

dialysed against EDTA, the non-dialysable material was digested with collagenase, and the low molecular weight peptide material was dialysed away. The residue represented 9.5% of the organic matrix and chromatography on DEAE-cellulose revealed 5 major components some of which were identified with the fractions prepared by earlier methods (see above) but detailed comment must await further publications.

E. Peptide Components

These components have been obtained from bone and dentine by Lever and his associates, beginning with a description of a peptide-citrate complex (Hartles and Leaver 1960; Leaver *et al.*, 1960), while more recently the citric acid has been separated from the peptide material; (Leaver *et al.*, 1965).

TABLE XIX. Distribution of nitrogen in fractions of acid soluble nitrogen^a

	Ox bone %	Human dentine %
Acid soluble nitrogen as a percentage of total nitrogen	4.3	3.7
Distribution of nitrogen in subfractions		
Acid reacting materials	14.8	14.4
Large M wt. material (MW 5,000)	37.8	27.4
Peptides MW > 750	7.5	16.4
MW < 750	3.7	10.9
Unaccounted for	36.2	30.9

^a Leaver and Shuttleworth (1967).

The tissue was demineralized using hydrochloric acid and the peptide material (termed Acid Soluble Nitrogen) precipitated by adjustment to pH 11.0 (Leaver and Shuttleworth, 1967). Separation on Sephadex G-25 revealed four main fractions and the two major fractions were refractionated. Table XIX compares the distribution of nitrogen in the various fractions, with a brief description of what the fractions are. It is perhaps significant that none of the fractions contain large amounts of hydroxyproline or hydroxylysine, indicating an absence of collagen or collagen degradation products. A more detailed fractionation scheme was applied to the peptide material from ox bone (Leaver and Shuttleworth, 1968) and in this case some fractions (MW 5000) did contain hydroxyproline and hydroxylysine.

F. Cellular Components

Bone tissue has an unusually low cell content with three types of cell present (Doty, 1966; Baud, 1966). The osteoblast secretes either collagen or a collagen precursor, and may also store glycogen. As the matrix matures the osteoblast is converted into an osteocyte. This is metabolically less active than the osteoblast (does not synthesize collagen) and has a less well characterized function. Evidence of metabolism is found in its Golgi apparatus, lysosomes, mitochondria, and vesicles which may well contain collagenases and acid phosphates. The osteoclast is usually considered to arise from a separate source and is principally concerned with bone resorption which takes place along its ruffled border and in fact bone crystals have been reported in vesicles inside the cell. It is metabolically active, having ribosomes and mitochondria, and is characterized by many nuclei and lysosomes.

Bone tissue also contains nerves and blood vessels, which together with cells must contribute to many minor components, such as lipids (0.5% of organic matter) found by Leach (1958) and Sakai and Cruess (1967), the resistant protein (5.2% of organic matter) reported by Eastoe and Eastoe (1954) and a small amount of nucleic acid (Russell, 1967).

Among the better documented enzymes present are the phosphatases (Jeffrey, 1964), since phosphatase activity is very high in bone tissue. Many of the usual metabolic enzymes have been detected histochemically (Pearse, 1966) after EDTA decalcification (acid decalcification damages the bone cells).

G. Calcification

It is generally held that collagen fibrils are first formed and subsequently calcified. Bone salts are in equilibrium with the extracellular fluids and indeed if powdered bone is left in water, the concentration of calcium and phosphate in solution rises until the ion product is approximately equal to that found in

body fluids (MacGregor and Nordin, 1960). The agreement is particularly close when account is taken of the effect of the bicarbonate ion (MacGregor and Nordin, 1962) which is usually present in tissue fluids. However, the ion product in body fluids is only about one half of the ion product which is necessary to precipitate calcium phosphate from a solution of inorganic ions only (MacGregor and Nordin, 1960). Two suggestions have been put forward to explain how calcification occurs in the body. The booster theory suggests that the local calcium and phosphate concentrations are raised in the vicinity of calcification although this theory is now generally discarded. The target theory which is widely accepted suggests that specific sites on the organic matrix can act as crystal nuclei, so allowing precipitation to occur at a lower concentration.

The specific binding of phosphate to collagen (Glimcher and Krane, 1964) and of calcium to sialoprotein (Peacocke and Williams, 1966) can be considered to support either theory. The binding action can be thought to raise the local ion concentration, and thus support the booster theory, or the bound ions can be thought of as acting as nucleators, and so favour target theory. More likely is that the real mechanism lies somewhere between these two extremes.

Calcification *in vivo* has been studied by implantation of collagen or collagenous tissues. Strips of rat tail tendon calcify under these conditions (Mergenhagen *et al.*, 1960). However, when soluble rabbit skin collagen is converted to reconstituted fibrils and then to a heat precipitated gel and finally implanted, calcification occurs when the fibrils had previously been suspended in a phosphate containing solution but not when suspended in sodium chloride solution. When rat tail tendon was implanted into the anterior chamber of the eye, calcification occurred when the tendon had received prior treatment in calcium chloride solution, but not when the prior treatment was in phosphate buffer solution (Urist and Adams, 1966) i.e. the opposite situation. However, when decalcified bone was implanted (Urist, 1965) new bone was formed around it, and simple calcification of the organic matrix did not occur.

In vitro experiments have yielded much useful information on calcification and have the advantage of being more easily controlled than the *in vivo* experiments. Comparison of the results is difficult, because of the many different ways used to express the results and because few workers include carbonate or fluoride ions in their media, ions which have a significant effect on calcium phosphate precipitation (Bachra, 1963; Larsen, 1966; Szot and Geisler, 1967). Most collagens will become calcified in solutions containing calcium and phosphate ions at concentrations higher than those found in the body, but lower than those necessary for spontaneous precipitation of calcium phosphate. However, some conformation requirements are necessary

for the collagen (Glimcher *et al.*, 1957; Bachra *et al.*, 1959; Bachra and Sobel, 1969) although these requirements are not fully understood. Furthermore, this property is not restricted to hard tissue collagen.

Demineralized bone and dentine calcify in synthetic solutions but still only at concentrations greater than physiological ones (Strates and Neuman, 1958; Solomons *et al.*, 1960). The only exception to this finding appears to be a tendon collagen preparation which calcified at physiological concentration (Fleisch and Neuman, 1961) but the reason for this special property is unknown.

Inhibition of calcification has recently attracted some attention. Addition of either urine (Fleisch and Bisaz, 1962) or plasma ultrafiltrate (Fleisch *et al.*, 1966) inhibited *in vitro* calcification. This was found to be due to the pyrophosphate ions and confirmed by the fact that hydroxy-apatite crystals, when coated with pyrophosphate ions cannot act as nuclei for further precipitation (Fleisch *et al.*, 1966a). This mechanism also appears to operate in culture (Fleisch and Bisaz, 1965). The effect of phosphatase may provide a reason why collagens in the body do not all calcify. Since bone phosphatase activity is very high, it may destroy local pyrophosphate and so allow calcification to occur (Fleisch and Bisaz, 1963).

Collagen binds phosphate ions, and as already mentioned, covalently-bound phosphate has been found in hard tissue collagens. The discovery of protein phosphokinases in many connective tissues fits in with the idea that collagen phosphorylation is a first step in calcification (Krane and Glimcher, 1965). This enzyme is found to phosphorylate protein-bound serine (Glimcher *et al.*, 1965b) using ATP as its source of phosphate.

The fact that these enzymes occur in all connective tissue does not necessarily detract from this idea since soft tissue collagens could contain an inhibitor or hard tissue collagens an activator so that the enzyme would only function in hard tissues. However there is at present no evidence for this.

Several other hard tissue components have been claimed as nucleators in calcification, but it seems likely that although these components might influence calcification, they are not responsible for the initial nucleation. Substances in this category are mucopolysaccharides (Weatherall *et al.*, 1964) and elastin and muco-proteins (Sobel, 1966), while electrochemical mechanisms (Digby, 1966) are probably more relevant to crystal growth than crystal nucleation.

V. TENDON

Tendon differs from skin particularly in the alignment of the collagen fibres, so that instead of forming an intertwined mesh as in skin, the fibres lie parallel, giving the tissue great longitudinal strength. Although young rat tail

tendon is very frequently used as a source of soluble collagen, the tissue is not a typical tendon tissue. For example it can be completely solubilized in water after treatment in 0.5M sodium dihydrogen orthophosphate (Dumitru and Garrett, 1957), while under identical conditions beef tendon was not solubilized at all. Also while most collagen tissues demonstrate shrinkage phenomena, young (2 month old) rat tail tendon does not, although the phenomena does occur in adult (2 year old) rat tail tendon (Lawson *et al.*, 1966). The solubility of rat tail tendon in acid buffers falls as the age increases, as in skin collagens, so for example up to 3 months old, 100% was solubilized, while at 12 months old only 20–30% was soluble (Butzow and Eichhorn, 1968). Thus these properties taken together suggest rat tail tendon represents an atypical mammalian collagen tissue, particularly young rat tail tendon.

Adult human or bovine tendon represents a more mature and highly cross-linked material. A typical composition has been determined by Loeven (1965) who showed that the residue after sodium chloride washing of achilles tendon consisted of protein (99%) and carbohydrate (2.01%) while the hydroxyproline content of 10.26% would correspond to only 75% of collagen. A very careful preparative procedure for adult bovine achilles tendon collagen was described by Harding and Wesley (1968) and an examination of their results shows that the tendon contained 40% of soluble collagen expressed as a percentage of total tendon dry weight. The residue after elastase treatment to remove traces of elastin then contained 13.7–14.1% hydroxyproline corresponding to 95–98% collagen.

Most studies of soluble tendon collagen use young rat tail tendon and extract the soluble collagen in either acid buffer solutions or in water by the procedure of Dumitru and Garrett (1957). The soluble collagen produced in this way appears to be typical soluble collagen, and this also applies to soluble collagen prepared from chicken leg tendon (Sakai *et al.*, 1967). This preparation had a denaturation temperature of 38°C, an intrinsic viscosity of 12.5 dl/g and a molecular weight of 340,000 all quite typical values for a soluble collagen although probably indicating some dimerization.

Some studies have been made of tendon mucopolysaccharide by Meyer *et al.* (1957) who found that calf and pig achilles tendon contained a total of 0.5% mucopolysaccharides (based on dry defatted tissue weight). This is similar to the quantity found in skin and hide. It consisted of chondroitin-6-sulphate and dermatan sulphate in equal amounts, along with a small amount of hyaluronic acid. No doubt modern fractionation techniques would reveal the presence of other mucopolysaccharide species as has occurred with skin. Apart from Meyer's work little has been published on tendon mucopolysaccharides except that Banga and Balo (1960) were able to isolate mucoid material from tendon by treatment with collagen mucoproteinase. This enzyme solubilized a fraction, designated mucoid₂, comprising about 16% of the

tendon dry weight, but it contained about 70% of collagen. Purification left only 0.5% of the 16% so the material represents only a small proportion of the original tissue. Another material, mucoid₁ was obtained in a yield of about 5% from the same tissue (Banga and Balo, 1957) but the relationship of these fractions to the more classical type of mucopolysaccharide of tendon, or of any other connective tissue is difficult to ascertain.

VI. CARTILAGE

Cartilage contains about half its weight of protein. A small amount of this tissue is present in gelatin raw materials. As a source of gelatin, cartilage tissue is unimportant, although its high carbohydrate content would contribute significantly to carbohydrate and mucoid components of gelatin.

The composition of calf articular and epiphyseal plate cartilage was studied by Campo and Tourtellotte (1967) and the results are found in Table XX.

TABLE XX. Composition of calf articular and epiphyseal plate cartilage^a

	Articular cartilage		Epiphyseal plate cartilage	
	% of total tissue	% of dry ash free tissue	% of total tissue	% of dry ash free tissue
Ash	5.8		23.4	
Water	6.8		7.2	
Collagen	55.1	63.7	39.0	56.2
Chondroitin sulphate	22.1	25.3	23.5	33.9
Keratan sulphate	3.2	3.7	3.0	4.4
Other protein	8.4	9.6	10.2	14.7

^a Campo and Tourtellotte (1967).

Collagen of human articular and costal cartilage has been studied by Miller *et al.* (1969) who found that neither tissue yielded soluble collagen during acid extraction, but extraction with guanidine hydrochloride solution solubilized a small amount of collagen (about 1%) together with larger amounts of non-collagenous material. The amino-acid composition of the insoluble collagen was typical of mammalian collagens, except that the hydroxylysine content was higher than usual (13 residues/1000 compared with 7 residues/1000 for skin collagen).

Cartilage mucopolysaccharides were isolated free of protein using sodium hydroxide treatment by Meyer *et al.* (1956). They identified only chondroitin-4-sulphate and chondroitin-6-sulphate, although more recent work has identified keratan sulphate and chondroitin in addition (Matthews and

Glasgow, 1966). Here it was shown that the actual composition was very dependent on age, type and species of tissue. In most tissues only the chondroitin sulphates and keratan sulphate were present, as was found in calf cartilage by Campo and Tourtellotte (1967).

These preparations of mucopolysaccharide were substantially free of protein, but several preparations of protein-polysaccharide complexes of cartilage have been reported recently, following a mild extraction of the homogenized tissue. For example Pedrini (1969) found two electrophoretically separable protein polysaccharide components in human costal and intervertebral disc cartilage and in bovine nasal septum cartilage. Pig articular cartilage has yielded similar complexes (Brandt and Muir, 1969) and here the composition and molecular weight of the complex were age dependent. Human articular and costal cartilages were found to contain protein-polysaccharide-collagen complexes (Steven, 1969). The collagen could be removed after treatment with cetyltrimethylammonium bromide, which suggests that the collagen was bound only by ionic forces. Finally a recent publication (Campo *et al.*, 1969) has shown by fractionation of the protein polysaccharide material that many separate components are present. It appears that these have a variable composition and that the results of fractionation are dependent on the methods employed.

Lipids present in cartilage have received scant attention apart from histochemical experiments of Tinacci and Cioni (1960), although Zambotti *et al.* (1962) have claimed that the lipid content of epiphyseal cartilage of new born pigs was 6.2%, while nasal cartilage from the same animal contained only 0.68%.

VII. OTHER MAMMALIAN COLLAGEN TISSUES

Mammalian tissues rich in collagen have been described in some detail in Sections III to VI, but Table I shows that many other tissues also contain collagen in smaller amounts. Some of these collagens are quite similar to the usual skin collagens, while others have properties peculiar to the particular tissue. Much attention has been paid to the uterus and both soluble collagen (Kao *et al.*, 1967) and insoluble collagen (Kao *et al.*, 1968) from this source have been characterized.

Changes in uterine collagen content during and after pregnancy have also been studied in great detail (e.g. Woessner and Brewer, 1963). Kidney basement membranes also appear to contain collagen (e.g. Kefalides, 1968) and this collagen is characterized by a particularly high hydroxylysine content (40 residues/1000). On the other hand other membrane tissue, intestinal submucosa, contains collagen which is similar in properties to bovine hide collagen (Scaria and Barat, 1965).

Of other organs examined, the eye contains collagen in the lens capsule (Fukushi and Spiro, 1969, 1969a) and in the cornea (Bosmann and Jackson, 1968). It appears that the fibre structure of corneal collagen is quite different from usual tissues, and this may be related to more specialized functions of the eye. It appears from Table I that blood vessels contain substantial amounts of collagen as well as more usually accepted elastin. Changes in the collagen content of rat aorta have been studied by Hanzlik (1965), while changes in the collagen and elastin contents occurring when human veins become varicose have been noted (Svejcar *et al.*, 1963). Finally muscle connective tissue contains collagen of which the soluble collagen has been characterized (McLain, 1969), while the insoluble collagen was found by Mohr and Bendall (1969) to have similar properties to the tendon collagen prepared from the ends of the same muscle.

VIII. NON-MAMMALIAN COLLAGENS

A. Fish Collagens

Fish collagens did have an economic value as a source of fish glue, but this is no longer made to any great extent. Soluble collagen from fish swim bladder (ichthyocol) was perhaps the first soluble collagen to be fully characterized (Boedtker and Doty, 1955, 1966) probably because of its high degree of solubility. On the other hand sturgeon swim bladder collagen (isinglass) is still used commercially for fining alcoholic beverages (Leach and Barrett, 1967, 1967a). (This use is discussed in some detail in Chapter 12.) Cod fish skin soluble collagen has also been extensively studied, and this is unusual in that the 3 α -chains are all different (Piez, 1964). In general, fish collagens have a lower imino acid content than mammalian collagens (Pikkarainen, 1968) and this is thought to be the reason for the lower denaturation temperature of these materials. This in turn appears to be related to the body temperature of the species.

Shark skin soluble collagen has been prepared and as in the case of bony fishes described above, this has a low denaturation temperature (Lewis and Piez, 1964), although in other respects it is similar to vertebrate collagens (Kubota and Kimura, 1967). Perhaps the most interesting fish collagen so far discovered is elastoidin, a protein prepared from shark fin. This collagen has an unusually high tyrosine content (7.52%), as well as a high cysteine content (0.18%) (Kimura and Kubota, 1966). Both these amino acids are thought to be involved in crosslinking, although this could not occur with mammalian collagens since cysteine is absent and tyrosine substantially absent from these proteins. Elastoidin is also unusual in having a lower molecular weight than

most other soluble collagens—150,000 as compared with 300,000 in most other cases (Saha *et al.*, 1964).

B. Invertebrate Collagens

The presence of collagen and collagen-like proteins has been shown in many invertebrates (Piez and Gross, 1959) as well as in monocellular organisms (Nordwig *et al.*, 1969). In some cases the protein lacks one or more of the properties usually considered essential for classification as a true collagen. Perhaps the collagen which has been most studied is from earthworms of various species. These soluble collagens contain sulphur crosslinks (McBride and Harrington, 1967) while their molecular weight is unusually high (900,000). The collagen tissue of earthworm cuticle is also unusual in that it does not contain any fibrils (Maser and Rice, 1962). Although the total imino acid content falls within the usual mammalian range, most of this is hydroxyproline (Mayer and Rice, 1963). An interesting suggestion has recently been made by McBride and Harrington (1967a) and by Ramachandran *et al.* (1968) who claim that earthworm cuticle collagen is a single polypeptide chain which is folded back on itself to form a triple helix. In mammalian collagens of course the triple helix contains three distinct polypeptide chains.

REFERENCES

- Adelmann, B. C., Marquardt, H. and Kuhn, K. (1966). In "Biochimie et Physiologie du Tissu Conjunctif" (Proc. Int. Symp. Lyon, 1965) (P. Comte, ed.) pp. 355-60.
- Andrews, A. T. de B. and Herring, G. M. (1965). *Biochim. Biophys. Acta* **101**, 239-41.
- Andrews, A. T. de B., Herring, G. M. and Kent, P. W. (1967). *Biochem. J.* **104**, 705-15.
- Araya, S., Saito, S., Nakanishi, I. S. and Kawanishi, Y. (1961) *Nature* **192**, 758-9.
- Armes, J. N. (1966). *J. Phot. Sci.* **14**, 143-8.
- Bachra, B. N. (1963). *Ann. New York Acad. Sci.* **109**, 251-5.
- Bachra, B. N. and Sobel, A. E. (1959). *Arch. Biochem. Biophys.* **85**, 9-18.
- Bachra, B. N., Sobel, A. E., and Stanford, J. W. (1959). *Arch. Biochem. Biophys.* **84**, 79-95.
- Bachra, B. N., Trautz, O. R. and Simon, S. L. (1965). *Arch. Oral. Biol.* **10**, 731-8.
- Banga, I. and Balo, J. (1957). In "Connective Tissue" (R. E. Tunbridge, ed.) pp. 254-263, Blackwell, Oxford.
- Banga, I. and Balo, J. (1960). *Biochem. J.* **74**, 388-93.
- Barker, S. A., Kennedy, J. F. and Somers, P. J. (1969). *Carbohydr. Res.* **10**, 57-63.
- Barker, S. A., Kennedy, J. F. and Cruikshank, C. N. D. (1969a). *Carbohydr. Res.* **10**, 65-70.
- Baud, C. A. (1966). In "Proc. 4th Europ. Symp. Calcified Tissues, Leiden", The Netherlands, pp. 4-6.

2. STRUCTURE AND COMPOSITION OF COLLAGEN CONTAINING TISSUES 67

- Becker, R. O., Spadaro, J. A. and Berg, E. W. (1968). *J. Bone and Joint Surgery* **50A**, 326-34.
- Berrens, L. and van Driel, L. M. J. (1962). *Naturwiss.* **49**, 608.
- Bitcover, E. H. and Mellón, E. F. (1966). *J. Amer. Leather Chemists Assoc.* **61**, 338-50.
- Blake, J. N. and Plaster, F. H. (1950). *J. Soc. Leather Trades Chemists* **34**, 177-86.
- Boedtker, H. and Doty, P. (1966). *J. Amer. Chem. Soc.* **77**, 248-9.
- Boedtker, H. and Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 4267-80.
- Bose, S. M. (1963). *Biochim. Biophys. Acta* **74**, 265-74.
- Bosmann, H. B. and Jackson, J. J. (1968) *Biochim. Biophys. Acta* **170**, 6-14.
- Bourrillon, R. and Got, R. (1962). *Biochim. Biophys. Acta* **58**, 63-73.
- Bowes, J. H. and Elliott, R. G. (1957). *J. Soc. Leath. Trades Chemists* **41**, 87-93.
- Bowes, J. H. and Raistrick, A. S. (1966). In "Biochimie et Physiologie du Tissu Conjonctif" (Proc. Int. Symp. Lyon. 1965) (P. Comte, ed.) pp. 295-302.
- Bowes, J. H. and Raistrick, A. S. (1968). *J. Amer. Leather Chemists Assoc.* **63**, 192-209.
- Bowes, J. H., Elliott, R. G. and Moss, J. A. (1956). *Biochem. J.* **63**, 1P.
- Bowes, J. H. Elliott, R. G. and Moss, J. A. (1957). *J. Soc. Leather Trades Chemists* **41**, 249-66.
- Bowes, J. H., Elliott, R. G. and Moss, J. A. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 71-5. Pergamon Press, London.
- Brandt, K. D. and Muir, H. (1969). *Biochem. J.* **114**, 871-6.
- Brown, W. E. (1965). In "Tooth Enamel" (M. V. Stack and R. W. Fearnhead, eds.) pp. 11-14, John Wright, Bristol.
- Brown, W. E., Smith, J. P., Lehr, J. R. and Frazier, A. W. (1962). *Nature* **196**, 1050-4.
- Burckard, J., Havez, R. and Dautrevaux, M. (1966). *Bull. Soc. Chim. Biol.* **48**, 851-61.
- Butzow, J. J. and Eichhorn, G. L. (1968). *Biochim. Biophys. Acta* **154**, 208-19.
- Campo, R. D. and Tourtellotte, C. D. (1967). *Biochim. Biophys. Acta* **141**, 614-24.
- Campo, R. D., Tourtellotte, C. D. and Bielen, R. J. (1969). *Biochim. Biophys. Acta* **177**, 501-11.
- Carlström, D., Engström, A. and Finean, J. B. (1955). In "Fibrous Proteins and their Biological Significance" (R. Brown and J. F. Danielli, eds), pp. 85-8, Cambridge.
- Castellani, A. A., Ferri, G., Bolognani, L. and Graziano, V. (1960). *Nature* **185**, 37.
- Chipperfield, A. R. and Taylor, D. M. (1968). *Nature* **219**, 609-10.
- Cifonelli, J. A. and Roden, L. (1968). *Biochim. Biophys. Acta* **165**, 553-4.
- Cooper, D. R. and Johnson, P. (1956). *Biochim. Biophys. Acta* **20**, 411-12.
- Cooper, D. R. and Johnson, P. (1957). *Biochim. Biophys. Acta* **26**, 317-29.
- Cooper, D. R. and Johnson, P. (1958). *Biochim. Biophys. Acta* **30**, 590-7.
- Cooper, D. R., Russell, A. E. and Davidson, R. T. (1967). *J. Amer. Leather Chemists Assoc.* **62**, 423-36.
- Courts, A. (1959). *Biochem. J.* **73**, 600-3.
- Courts, A. (1963). *J. Soc. Leather Trades Chemists* **47**, 213-20.
- Dahl, O. and Persson, K. (1963). *Acta Chem. Scand.* **17**, 2499-502.
- Dallemagne, M. J. (1964). In "Bone and Tooth" (H. J. J. Blackwood, ed.) pp. 171-4. Pergamon, Oxford.
- Davidson, R. J. and Cooper, D. R. (1968). *Nature* **217**, 168-9.

- Deasy, C. (1959). *J. Amer. Leather Chemists Assoc.* **54**, 246-58.
- Dickerson, J. W. T. (1962). *Biochem. J.* **82**, 56-61.
- Dickerson, J. W. T. (1962a). *Biochem. J.* **82**, 47-55.
- Dickerson, J. W. T. and John, P. M. V. (1964). *Biochem. J.*, **92**, 364-8.
- Digby, P. S. B. (1966). *Nature* **212**, 1250-2.
- Dische, Z. Danilczenko, A. and Zelmanis, G. (1958). In "Chemistry and Biology of Mucopolysaccharides" (G. E. W. Wolstenholme and M. O'Connor, eds.) pp. 116-39, Churchill, London.
- Doty, S. B. (1966) Birth Defects (Original Article Series) **2**, 45-9.
- Dumitru, E. T. and Garrett, R. R. (1957). *Arch. Biochem. Biophys.* **66**, 245-7.
- Eanes, E. D., Harper, R. A., Gillessen, I. and Posner, A. S. (1966). In Proc. 4th Europ. Symp. Calcif. Tissues, Lieden, The Netherlands, p. 24.
- Eastoe, J. E. (1955). *Biochem. J.* **61**, 589-600.
- Eastoe, J. E. (1961). In "The Biochemists Handbook" (C. J. Long, E. J. King and W. M. Sperry, eds.) pp. 715-20, Spon, London.
- Eastoe, J. E. and Eastoe, B. (1954). *Biochem. J.* **57**, 453-9.
- Engström, A. (1966) In "Proc. 4th Cong. Int. Opt. Rayon X-Microanal.", Orsay, France, 1965, pp. 630-5.
- Engström, A. and Finean, J. B. (1953), *Nature* **171**, 564.
- Fleisch, H. and Bisaz, S. (1962). *Amer. J. Physiol.* **203**, 671-5.
- Fleisch, H. and Bisaz, S. (1964). In "Bone and Tooth" (H. J. J. Blackwood, eds.) pp. 249-56, Pergamon, Oxford.
- Fleisch, H. and Bisaz, S. (1965) In "Structure and Function of Connective and Skeletal Tissue" (G. R. Tristram and S. Fitton-Jackson, eds), pp. 347-55, Butterworths, London.
- Fleisch, H. and Neuman, W. F. (1961). *Amer. J. Physiol.* **200**, 1296-300.
- Fleisch, H., Bisaz, S., Russell, R. G. G. and Straumann, F. (1966). In "Proc. 4th Europ. Symp. Calcif. Tissue", Leiden, The Netherlands, pp. 31-2.
- Fleisch, H. Russell, R. G. G. and Straumann, F. (1966a). *Nature* **212**, 901-3.
- Francois, C. J. and Glimcher, M. J. (1967). *Biochim. Biophys. Acta* **133**, 91-6.
- Francois, C. J. and Glimcher, J. and Krane, S. M. (1967). *Nature* **214**, 621.
- Fukushi, S. and Spiro, R. G. (1969). *J. Biol. Chem.* **244**, 2041-8.
- Fukushi, S. and Spiro, R. G. (1969a). *J. Biol. Chem.* **244**, 2049-58.
- Gedalia, I., Zipkin, I., Zukerman, H., Gat, I. and Leventhal, H. (1967). *Arch. Oral Biol.* **12**, 545-9.
- Gerngross, O. and Goebel, E. (1933). "Chemie und Technologie de Leim und Gelatine Fabrikation", p. 173, Steinkopff, Dresden.
- Glegg, R. E. and Eidinger, D. (1955). *Arch. Biochim. Biophys.* **55**, 19-24.
- Glimcher, M. J. (1960). In "Calcification in Biological Systems" (R. F. Sognnaes, ed.) pp. 421-87. A.A.A.S., Washington.
- Glimcher, M. J. and Krane, S. M. (1964). *Biochemistry* **3**, 195-202.
- Glimcher, M. J. and Katz, E. P. (1965). *J. Ultrastruct. Res.* **12**, 705-29.
- Glimcher, M. J., Hodge, A. J. and Schmitt, F. O. (1957). *Proc. Nat. Acad. Sci. U.S.A.* **43**, 860-7.
- Glimcher, M. J., Francois, C. J., Richards, L. and Krane, S. M. (1964). *Biochim. Biophys. Act* **93**, 585-602.
- Glimcher, M. J., Katz, E. P. and Travis, D. F. (1965). *J. Ultrastruct. Res.* **13**, 163-71.
- Glimcher, M. J., Andrikides, A. and Kossiva, D. (1965a) In "Structure and Function of Connective and Skeletal Tissue" (G. R. Tristram and S. Fitton-Jackson, eds.) pp. 342-4. Butterworth, London.

- Glimcher, M. J., Francois, C. and Krane, S. M. (1956b). In "Structure and Function of Connective and Skeletal Tissues" (G. R. Tristräm and S. Fitton-Jackson, eds.) pp. 344-7. Butterworth, London.
- Got, R. and Bourillon, R. (1962). *Biochim. Biophys. Acta* **58**, 74-9.
- Gustavson, K. H. (1956). "The Chemistry and Reactivity of Collagen", p. 30. Academic Press, New York.
- Habermann, E. (1961). *Z. Physiol. Chem.* **324**, 232-42.
- Hanzlick, J. (1965). *Polskii Arch. Med. Wewnetrynej* **35**, 771.
- Harding, J. J. and Wesley, J. M. (1968). *Biochem. J.* **106**, 749-57.
- Harkness, R. D., Marko, A. M., Muir, H. M. and Neuberger, A. (1954). *Biochem. J.* **56**, 558-69.
- Hartles, R. L. and Leaver, A. G. (1960). *Arch. Oral. Biol.* **1**, 297-303.
- Hawk, P. B. and Gies, W. J. (1901). *Amer. J. Physiol.* **5**, 387-425.
- Hayek, E. (1967). *Klin. Wochenschr.* **45**, 857-63.
- Herring, G. M. (1964) In "Bone and Tooth" (H. J. J. Blackwood, ed.) pp. 263-8, Pergamon, Oxford.
- Herring, G. M. (1968). *Biochem. J.* **107**, 41-9.
- Herring, G. M. and Kent, P. W. (1963). *Biochem. J.* **89**, 405-14.
- Hide, Skins and Leather under the Microscope (1957) Published by British Leather Manufacturers Research Association, Egham, Surrey, England.
- Hisamura, H. (1938). *J. Biochem. (Japan)* **28**, 473-8.
- Hoover, S. R., Viola, S. J. Korn, A. H. and Mellon, E. F. (1955). *Science* **121**, 672-3.
- Houck, J. C. and Jacob, R. A. (1960). *Proc. Soc. Exptl. Biol. Med.* **105**, 324-6.
- Hult, A.-M. and Goltz, R. W. (1965). *J. Invest. Dermatol.* **44**, 408-12.
- Humphrey, J. H., Neuberger, A. and Perkins, D. J. (1956). *Biochem. J.* **64**, 2P.
- Humphrey, J. H., Neuberger, A. and Perkins, D. J. (1957). *Biochem. J.* **66**, 390-9.
- Jeffrey, G. M. (1964). In "Bone and Tooth" (H. J. J. Blackwood, ed.) pp. 299-309, Pergamon, Oxford.
- Kahn, L. D. and Witnauer, L. P. (1969). *J. Appl. Poly. Sci.* **13**, 141-8.
- Kao, K.-Y. T., Hilt, W. T. and McGavack, T. H. (1967). *Proc. Soc. Exptl. Biol. Med.* **125**, 734-8.
- Kao, K.-Y. T., Hilt, W. T., Arnett, W. M. and McGavack, T. H. (1968). *Proc. Soc. Exptl. Biol. Med.* **127**, 710-12.
- Kawai, S., Hashimoto, S., Sode, T., Miyata, T. and Nishihara, T. (1966). "Biochimie et Physiologie du Tissu Conjonctif" (Proc. Int. Symp. Lyon, 1965) (P. Comte, ed.) pp. 249-58.
- Kefalides, N. A. (1968). *Biochemistry* **7**, 3103-12.
- Kimura, S. and Kubota, K. (1966). *J. Biochem. (Japan)* **60**, 615-21.
- Kofoed, J. A. and Bozzini, C. E. (1969). *Experientia* **25**, 23-4.
- Krane, S. M. and Glimcher, M. J. (1966). In "Proc. 3rd Europ. Symp. Calcif. Tissues", Davos, Switz., 1965, p. 168.
- Kubota, M. and Kimura, S. (1967). *Nippon Suisan Gakkaishi* **33**, 338-42.
- Larsen, S. (1966) *Nature* **212**, 605.
- Lawson, N. W., Giles, W. M. and Pierce, J. A. (1966). *Nature* **212**, 720.
- Leach, A. A. (1958). *Biochem. J.* **69**, 429-32.
- Leach, A. A. (1960). *J. Appl. Chem.* **10**, 367-72.
- Leach, A. A. and Barrett, J. (1967). *J. Inst. Brew.* **73**, 246-54.
- Leach, A. A. and Barrett, J. (1967a). *J. Inst. Brew.* **73**, 376-81.
- Leaver, A. G. and Shuttleworth, C. A. (1967). *Arch. Oral. Biol.* **12**, 947-58.

- Leaver, A. G. and Shuttleworth, C. A. (1968). *Arch. Oral. Biol.* **13**, 509-25.
- Leaver, A. G., Eastoe, J. E. and Hartles, R. L. (1960). *Arch. Oral. Biol.* **2**, 120-6.
- Leaver, A. G., Shuttleworth, C. A. and Triffitt, J. T. (1965). *J. Dental Res.* **44**, 1177-8.
- Lewis, M. S. and Piez, K. A. (1964). *J. Biol. Chem.* **239**, 3336-40.
- Loeven, W. A. (1965). *Acta Physiol. Pharmacol. Neerl.* **13**, 135-59.
- Loewi, G. (1961). *Biochim. Biophys. Acta* **52**, 435-40.
- Loewi, G. and Meyer, K. (1958). *Biochim. Biophys. Acta* **27**, 453-6.
- Lowry, O. H., Gilligan, D. R. and Katersky, E. M. (1941). *J. Biol. Chem.* **139**, 795-803.
- Maser, M. D. and Rice, R. V. (1962). *Biochim. Biophys. Acta* **63**, 255-65.
- Maser, M. D. and Rice, R. V. (1963). *Biochim. Biophys. Acta* **74**, 283-94.
- Matthews, M. B. and Glasgow, S. (1966). In "Biochimie et Physiologie du Tissu Conjonctif" (Proc. Int. Symp. Lyon. 1965) (P. Comte, ed.) pp. 21-6.
- McBride, O. W. and Harrington, W. F. (1967). *Biochemistry* **6**, 1484-98.
- McBride, O. W. and Harrington, W. F. (1967). *Biochemistry* **6**, 1499-514.
- McClain, (1969). *Nature* **221**, 181-2.
- McConnell, D. (1965). *Arch. Oral. Biol.* **10**, 421-31.
- McConnell, D. (1967). *Science* **155**, 607.
- McGregor, J. and Nordin, B. E. C. (1960). *J. Biol. Chem.* **235**, 1215-8.
- McGregor, J. and Nordin, B. E. C. (1962). *J. Biol. Chem.* **237**, 2704-8.
- McKernan, W. M. and Dailly, S. D. (1966). In "Proc. 3rd Europ. Symp. Calcif. Tissues," Davos, Switz., 1965, pp. 171-4.
- McLaughlin, G. D. and Theis, E. R. (1924). *J. Amer. Leath. Chem. Assoc.* **19**, 428-441.
- McLaughlin, G. D. and Theis, E. R. (1945). "The Chemistry of Leather Manufacture", pp. 5-11, Reinhold, New York.
- McLean, F. C. (1967). *Calcif. Tiss. Res.* **1**, 1-7.
- Melcher, A. H. (1964). *J. Dent. Res.* **43**, 953.
- Melcher, A. H. (1966). In "Biochimie et Physiologie du Tissu Conjonctif" (Proc. 1st Int. Symp. Lyon, 1965) (P. Comte, ed.) pp. 337-43.
- Mellon, E. F. and Korn, A. H. (1956). *J. Amer. Leath. Chem. Assoc.* **51**, 469-79.
- Mellon, E. F., Viola, S. J. and Naghski, J. (1960). *J. Amer. Leath. Chem. Assoc.* **55**, 622-33.
- Mergenhagen, S. E., Martin, G. R., Rizzo, A., Wright, D. N. and Scott, D. B., (1960). *Biochim. Biophys. Acta.* **43**, 563-5.
- Meyer, K., Davidson, E., Linker, A. and Hoffman, P. (1956). *Biochim. Biophys. Acta* **21**, 506-18.
- Meyer, K., Hoffmann, P. and Linker, A. (1957). In "Connective Tissue" (R. E. Tunbridge, ed.) pp. 86-96, Blackwell, Oxford.
- Miller, E. J., Martin, G. R., Piez, K. A. and Powers, M. J. (1967). *J. Biol. Chem.* **242**, 5481-9.
- Miller, E. J., Van der Korst, J. K. and Sokoloff, L. (1969). *Arthritis Rheum.* **12**, 21-9.
- Mills, G. and Bavetta, L. A. (1966). *J. Gerontol.* **21**, 449-54.
- Mobbs, D. R. A. (1966). *Arch. Oral. Biol.* **11**, 1071-9.
- Mohr, V. and Bendall, J. R. (1969). *Nature* **223**, 404-5.
- Natarajan, M. and Bose, S. M. (1965). *Leather Sci.* **12**, 111-18.
- Neuman, R. E. and Logan, M. A. (1950). *J. Biol. Chem.* **186**, 549-56.
- Newesely, H. (1963). *Monatsch.* **94**, 270-80.

- Newesely, H. (1966). In "Proc. 3rd Europ. Symp. Calcif. Tiss.", Davos, Switz., 1965, pp. 136-8.
- Newesely, H. and Hayek, E. (1963). *Experientia* **19**, 459-60.
- Nicolaides, N., Fu, H. C. and Rice, G. R. (1968). *J. Invest. Dermatol.* **51**, 83-9.
- Nimni, M. E., Guia, E., de, and Bavetta, L. A. (1965). *Nature* **207**, 865-6.
- Nordwig, A., Hayduk, and Gerisch, G. (1969). *Z. Physiol. Chem.* **350**, 245-8.
- Oldroyd, D. and Herring, G. M. (1967). *Biochem. J.* **104**, 20P.
- Orekhovich, V. N., Tustanovskii, A. A., Orekhovich, K. D. and Plotinkova, N. E., (1948). *Biokhimiya* **13**, 55-60.
- Ornes, C. L. and Roddy, W. T. (1960). *J. Amer. Leath. Chem. Assoc.* **55**, 124-35.
- Partridge, S. M. (1962). *Adv. Prot. Chem.* **17**, 227-303.
- Partridge, S. M. and Davis, H. F. (1955). *Biochem. J.* **61**, 21-30.
- Peacocke, A. R. and Williams, A. P. (1966). *Nature*, **211**, 1140-1.
- Pearce, A. G. E. (1966). In "Proc. 4th Europ. Symp. Calcif. Tissues", pp. 85-6.
- Pearce, R. H. and Watson, E. M. (1949). *Canadian J. Res.* **27E**, 43-57.
- Pedrini, (1969). *J. Biol. Chem.* **244**, 1540-6.
- Piez, K. A. (1963). *Ann. New York Acad. Sci.* **109**, 256-68.
- Piez, K. A. (1964). *J. Biol. Chem.* **239**, 4315-6.
- Piez, K. A. and Gross, J. (1959). *Biochem. Biophys. Acta* **34**, 24-39.
- Pikkarainen, J. (1968). *Acta Physiol. Scand. Suppl.* **309**, 72pp.
- Pouradier, J. (1967). *Sci. Ind. Photogr.* **38**, 189-95.
- Pouradier, J. (1967). *Chimia* **21**, 560-6.
- Puchtler, H. (1964). *J. Histochem. Cytochem.* **12**, 552.
- Ramachandran, G. N., Doyle, B. B. and Blout, E. R. (1968). *Biopolymers* **6**, 1771-5.
- Reiss, W. (1964). *Leder* **15**, 233-7.
- Rudall, K. M. (1946). *Proc. Symp. Fibrous Proteins* (Soc. Dyers and Colourists) May, p. 15
- Russell, G. (1967). *J. Phot. Sci.* **15**, 236-40.
- Russell, G. and Oliff, D. L. (1966). *J. Phot. Sci.* **14**, 9-22.
- Robb-Smith, A. H. T. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 38-44, Pergamon, Oxford.
- Robert, L. and Comte, P. (1968). *Life Sci.* **7**, 493-7.
- Roe, D. A. (1956). *J. Invest. Dermatol.* **27**, 1-8.
- Rogers, H. J. (1951). *Biochem. J.* **49**, XII.
- Rowles, S. L. (1965). In "Tooth Enamel" (N. V. Stack and R. W. Fearnhead, eds), Wright, Bristol.
- Saha, N. N., Das, S., Saha, A. K. and Battacharya, S. D. (1964). *Proc. Symp. Nucl. Phys. Solid State Phys. Chandigarh, India PtB*, 601-11.
- Sakai, R., Ikeda, S. and Isemura, T. (1967). *Bull. Chem. Soc. Japan* **40**, 2890-4.
- Sakai, T. and Cruess, R. L. (1967). *Proc. Soc. Exptl. Biol. Med.* **124**, 490-3.
- Samachson, J. (1965). *Nature* **218**, 1262-3.
- Scaria, S. K. and Barat, S. K. (1965). *Leather Sci.* **12**, 391-5.
- Schiller, S. and Dorfmann, A. (1960). *Nature* **185**, 111-12.
- Schiller, S. Matthews, M. B., Jefferson, H., Ludoweig, L. and Dorfmann, A. (1954). *J. Biol. Chem.* **211**, 717-24.
- Schlueter, R. J. and Veis, A. (1964). *Biochemistry* **3**, 1657-65.
- Shentall, R. D., Happey, F., Pearson, C. H., Naylor, A. and Turner, R. L. (1969). *Biochem. J.* **114**, 17P.
- Smith, J. G., Davidson, E. A. and Hill, R. L. (1963). *Nature* **197**, 1108-9.

- Snellman, O. (1963). *Acta Chem. Scand.* **17**, 1049–56.
- Snellman, O. (1963a). *Acta Chem. Scand.* **17**, 1057–61.
- Snellman, O. (1963b). *Acta Chem. Scand.* **17**, 1062–4.
- Sobel, A. E. (1966). *Fed. Proc.* **25**, 763.
- Solomons, C. C. and Irving, J. T. (1958). *Biochem. J.* **68**, 499–503.
- Solomons, C. C. and Neuman, W. F. (1960). *J. Biol. Chem.* **235**, 2502–6.
- Solomons, C. C. Irving, J. T. and Neuman, W. F. (1960). In "Calcification in Biological Systems" (R. F. Sognaes, ed.), pp. 203–16, A.A.A.S.
- Steven, F. S., Broady, K. and Jackson, D. S. (1969). *Biochim. Biophys. Acta* **175**, 225–7.
- Strates, B. and Neuman, W. F. (1958). *Proc. Soc. Exptl. Biol. Med.* **97**, 688–91.
- Svejar, J., Prerovsky, I., Linhart, J. and Kruml, J. (1963). *Clin. Sci.* **24**, 325.
- Szot, Z. and Geisler, J. (1967). *Acta Biochem. Pol.* **14**, 111–20.
- Tancous, J. J. (1969). *J. Amer. Leath. Chem. Assoc.* **64**, 388–402.
- Taylor, T. G. (1960). *Biochim. Biophys. Acta* **39**, 148–9.
- Termine, J. D. and Posner, A. S. (1966). *Science* **153**, 1523–5.
- Termine, J. D. and Posner, A. S. (1967). *Science* **155**, 607–8.
- Termine, J. D. and Posner, A. S. (1967a). *Calcif. Tissues Res.* **1**, 8–23.
- Timple, R. W., Wolff, I. and Weisier, M. (1968). *Biochim. Biophys. Acta* **168**, 168–70.
- Timson, W., Steigmann, A., Nawn, G. and Kelly, W. (1966). *Phot. Sci. Eng.* **10**, 281–6.
- Tinacci, F. and Cioni, P. (1960). *Bull. Soc. Ital. Biol. Sper.* **20**, 1092.
- Tristram, G. R. and Smith, R. H. (1963). *Adv. Pro. Chem.* **18**, 227–318.
- Urist, M. R. (1965). *Science* **150**, 893–9.
- Urist, M. R. and Adams, J. M. (1966). *Arch. Path.* **81**, 325–42.
- Venet, A. M., Pouradier, J. and Landucci, J. M. (1957). *Bull. Soc. Chim. Fr.* 1325–29.
- Veis, A. and Cohen, J. (1954). *J. Amer. Chem. Soc.* **76**, 2476–8.
- Veis, A. and Perry, A. (1967). *Biochemistry* **6**, 2409–16.
- Veis, A. and Schlueter, R. J. (1963). *Nature* **197**, 1204.
- Veis, A. and Schlueter, R. J. (1964). *Biochemistry* **3**, 1650–7.
- Veis, A., Aresy, J. and Cohen, J. (1960). *J. Amer. Leath. Chem. Assoc.* **55**, 548–60.
- Veis, A., Waykole, P. and Anesey, J. (1966). In "Proc. 7th Int. Congr. Gerontology", Vienna, p. 21.
- Weatherall, J. A. Bailey, P. J. and Weidmann, S. M. (1964). In "Bone and Tooth" (H. J. J. Blackwood, ed.), pp. 227–30, Pergamon, Oxford.
- Wilkinson, D. I. (1969). *J. Invest. Dermatol* **52**, 339–43.
- Williams, A. P. (1961). *J. Appl. Chem.* **11**, 100–3.
- Williams, P. A. and Peacocke, A. R. (1968). *Biochim. Biophys. Acta* **101**, 327–35.
- Winand, L. (1965). In "Tooth Enamel" (M. V. Stack and R. W. Fearnhead, eds), pp. 15–19, Wright, Bristol.
- Windrum, G. M., Kent, P. W. and Eastoe, J. E. (1955). *Brit. J. Exptl. Path.* **36**, 49–59.
- Woessner, J. F. and Brewer, T. H. (1963). *Biochem. J.* **99**, 75–82.
- Wuthier, R. E., Grøn, P. and Irving, J. T. (1964). *Biochem. J.* **92**, 205–16.
- Yates, J. R. (1968). *J. Soc. Leath. Trades Chemists* **52**, 425–35.
- Zambotti, V., Cescon, I., Bonferroni, B. and Bolognani, L. (1962), *Experientia* **18**, 318–19.
- Zimkin, E. A., Devyatov, Ya, B., and Maklakov, A. G. (1965). *Zh. Prikl. Khim.* **38**, 2581–5 (English translation on pages 2318–521).

Chapter 3

Chemical Constitution of Gelatin

J. E. EASTOE

Department of Dental Science, Royal College of Surgeons of England, London

AND

A. A. LEACH

The Brewers Society, London

I	Introduction	73
	A. Nomenclature	73
	B. Concept of "Purity" in Relation to Gelatin	74
II	Influence of Collagen Source on Gelatin Composition	77
	A. Effect of Species	77
	B. Effect of Tissue	82
III	Relationship of Gelatin Composition to that of Parent Collagen	83
	A. Features of Composition which may Differ	83
	B. Effects of Methods of Manufacture on Composition	88
IV	The Main Protein Component—Gelatin	90
	A. Moisture	90
	B. Amino Acid Composition	92
	C. Side Chain Groups	93
	D. Terminal Groups	93
	E. Sequence	95
	F. "Unusual Structures"	98
V	Contaminants	100
	A. Inorganic	100
	B. Proteins and Mucosubstances	101
	References	105

I. INTRODUCTION

A. Nomenclature

When tissues which contain collagen are subjected to mildly degradative processes, usually involving treatment with alkali or acid followed or accompanied by some degree of heating in the presence of water, the systematic

fibrous structure of the collagen is broken down irreversibly. The main product of this change has characteristic properties. It forms a solution of high viscosity in water, which sets to a gel on cooling and its chemical composition is, in many respects, closely similar to that of its parent collagen.

Much confusion is avoidable by following a convention suggested by A. G. Ward in 1950 and referring to this main product of mild, but irreversible collagenolytic breakdown as *gelatin*, a derived protein which, though not homogeneous, is chemically well defined. The term *gelatine* is applied to the total product of the manufacturing process. Gelatine contains gelatin as its main constituent, together with smaller amounts of various inorganic and organic impurities. *Glues* are produced by similar processes but the raw materials are less refined and their extraction involves more drastic conditions. Consequently, glues are more coloured and cloudy and they contain more impurities and slightly less gelatin, which is considerably more degraded. *Technical gelatines* are intermediate between gelatines and glues. There are no rigid borderlines between these groups of products.

Collagenous raw materials include not only the soft tissues, skin and tendon, but also the mineralized hard tissue, bone. In the manufacture of gelatine, bones are first demineralized to produce "*ossein*", whereas bone glue is extracted directly from fully mineralized bones at a higher temperature. Fat (*grease*), which is extracted initially from the bones and calcium phosphates are important by-products.

B. Concept of "Purity" in Relation to Gelatin

The idea of purity for a homogeneous substance is straightforward because, since all its molecules are identical, any other molecules present which differ from these in any way must be regarded as impurities. For a heterogeneous material such as gelatin, the molecules of which may differ in molecular weight, overall composition and structure in relation to the ends of the protein chains, the concept of "purity" is more complicated and needs detailed consideration.

The greater part of a manufactured gelatin will be derived from the more highly crosslinked insoluble collagen, which forms the bulk of mature portions of connective tissues (Chapter 1). Fibrils of collagen may be regarded as comprising very large molecules connected together via covalent intermolecular crosslinks. These crosslinks are slowly formed as the collagen "matures". Intramolecular crosslinks between pairs of chains in the triple helix also stabilize insoluble collagen.

Two basic processes are involved in the conversion of the regular structure of collagen to the more random gelatin. The first is thermal denaturation

which results in breakdown of hydrogen and probably electrostatic bonds, so disarranging the collagen helix that the three entwined protein chains are freed from each other and pass into solution as more random coils. This process, which occurs on warming to approximately 40°C, alone is sufficient to release "parent gelatin" (collagen α chains) from newly formed collagen, which has no crosslinks and can be extracted from tissues with neutral solutions of electrolytes. Thermal denaturation alone is insufficient for the release of gelatin from more mature collagen because of the stabilizing effect of crosslinks.

The second process, which must also occur before significant amounts of gelatin can be extracted from the usual raw materials, is the (hydrolytic) breakdown of covalent bonds. The release of a gelatin fragment (molecule) from a fibrillar structure consisting of crosslinked protein chains must involve the breakage of at least one covalent bond. Those likely to be broken include peptide bonds, crosslinks, bonds in their immediate neighbourhood and perhaps the unusual bonds of the types mentioned in Section IVF.

No one bond in the collagen structure is known to be so labile that it specifically breaks first under the conditions of gelatin manufacture, although in dipeptides, bonds involving glycine are relatively weak. The indications are that several bond positions are at risk, those which actually break being determined on a probability basis, having regard to the effects of pH and temperature. The positions of the breaks determine the molecular weight, the number of polypeptide chains, the number of each kind of amino acid residue and their positions with respect to the chain ends of the gelatin molecule which is released. This random characteristic of bond breakdown is the main cause of molecular heterogeneity in gelatin. Breakage may occur at all stages of manufacture—during pretreatment, at the extraction stage and in gelatin molecules after extraction, such as of the evaporation stage when it is considered as degradation. Enzymic breakdown resulting from the action of bacterial or fungal enzymes may also occur. All the many molecular species which result have an equal claim to be considered as gelatin, provided that the molecular weight exceeds an arbitrary minimum of approximately 30,000.

Tissues used as raw materials for gelatine manufacture contain a variety of substances (Chapter 2) which are sources of organic and inorganic impurities in gelatine. Other proteins from muscle and blood, keratins, glycoproteins, mucopolysaccharides such as hyaluronic acid, keratosulphate and chondroitin sulphate, lipids, nucleic acids and other cell components may be present and some of these substances probably contribute small amounts of soluble degradation products to gelatine. Inorganic ions of "physiological" origin include sodium, potassium, calcium, magnesium, iron, chloride and phos-

phate. Impurities may also be derived from substances added, either to treat the raw material or during gelatine manufacture. Acids, alkalies and salts (and their impurities), used at the pretreatment stage and the mineral content of the water supply can influence the composition of the gelatine produced. Side reactions during manufacture involving impurities and gelatin molecules can also occur, affecting the colour, clarity and photographic properties of the product.

Despite the variable nature of the raw materials and the many sources of impurities, the manufacturing process can provide a surprisingly efficient means for obtaining a product of higher intrinsic purity than the precursor. The pretreatment stage solubilizes or disperses some adventitious constituents so that they are removed in the subsequent washing. The extraction conditions are selected specifically to solubilize gelatin and many impurities are left behind in the insoluble residue (scutch) or in the finer particles removed by filtration.

At the present time, all experimental findings point to the most carefully prepared commercial gelatins consisting almost entirely of a single family of closely related protein molecules derived from collagen. This important concept was questioned by Russell (1958) who claimed to have isolated 15–19% of a protein impurity from a high grade lime-processed gelatin. Repetition of these experiments by Leach (1960a) on a similar gelatin resulted in the isolation of only 0.36% of a protein which differed markedly from gelatin in composition (see Tables V and XII). When Leach isolated a larger fraction, accounting for 6.6% of the total gelatin, by slightly changing the conditions, its composition suggested that it consisted mainly of gelatin with comparatively little impurity protein. It appears probable that Russell's impurity preparation also consisted mainly of gelatin, which was not identified correctly because of a technical difficulty (see Eastoe, 1967).

Practical criteria of purity are difficult to establish for gelatin. A low ash content suggests absence of inorganic contaminants and total nitrogen contents of 18.0–18.1 and 18.2–18.4% (on a dry, ash-free basis) for alkali- and acid-pretreated gelatins, respectively, are necessary but not sufficient indications of reasonable purity. Amino acid analysis is, perhaps, the best general guide, the difficulty here being the choice of a suitable standard for comparison. High grade gelatin which has been further purified by ethanol fractionation (Stainsby, 1954) and deionization has been analysed (Eastoe, 1955) in one approach to this problem. Another is to use $\alpha 1$ and $\alpha 2$ collagens separated from the same tissue and species. As these differ in composition, it would be necessary to calculate a weighted mean of the amino acid values to compare with a heterogeneous gelatin derived from whole collagen. Known changes in composition, during the collagen-gelatin conversion, would need to be taken into account (see Section III).

II. INFLUENCE OF COLLAGEN SOURCE ON GELATIN COMPOSITION

The source of collagen can influence the composition of gelatin in two ways; firstly, the amino acid composition of the gelatin will be similar to that of the parent collagen and will therefore reflect directly the influence of species and type of tissue (Section II), secondly, different collagens may require variations in the nature or severity of the pretreatment which also result in differences in composition (see Section III).

A. Effect of Species

The influence of species on the amino acid composition of collagens and gelatins has been reviewed and discussed in detail by Eastoe (1967) and we shall here confine our attention to the more important conclusions.

1. Vertebrate gelatins

(a) *Mammalian*. The most remarkable feature is the similarity of composition of mammalian gelatins even though the range considered extends from whale to man. This is readily seen from data in Chapter 1 and in Table I where the more recent values for some gelatins are given. Having discussed the features of the composition of mammalian gelatins we shall compare the values for other gelatins with them.

The frequency of occurrence of the most abundant residues are glycine 1 in 3, alanine, 1 in 9 and imino acids 2 in 9 (proline, < 1 in 8; hydroxyproline, > 1 in 11). These four therefore contribute some 2 in 3 of all the residues, leaving only one third of the positions for the remaining amino acids. Dicarboxylic acids represent about 1 in 8 (free carboxyl 1 in 13 in acid processed gelatins), basic groups about 1 in 11-12 and hydroxyl 1 in 6. The protein contains hydroxylysine and hydroxyproline which are unusual; there are two forms of the latter, namely 3- and 4-hydroxyproline, the 3- form contributing only some 1 in 250-1000 residues (Ogle *et al.*, 1962). Very little tyrosine (1 in 200-1200), methionine (1 in 120-250), and histidine (1 in 250) are present.

All the amino acids commonly found in proteins occur in gelatin with the probable exception of tryptophan and cystine. Most investigators have been unable to detect tryptophan in gelatin, although Piez (1965) reported less than 1 residue per 1000 in cod skin collagen and Hoerman and Mancewitz (1964) found 0.3% in demineralized bone, possibly derived from glycoprotein. The small amount of cystine occasionally reported is probably derived from keratins and impurity proteins (Section VB). This may apply also to lanthionine sulphoxide which Zahn and Wegerle (1955) detected in a gelatin

hydrolysate. Although the sulphur-containing amino acids probably have no structural significance they affect photographic properties.

The hydroxyproline content of the whale skin gelatin (Table I) is slightly lower than that of the other gelatins, it is not known whether this is the result of the long term effect of environment or diet on the composition of the collagen.

(b) *Amphibian, reptilian and avian.* Very few analyses (Table II) exist for these types and therefore some caution must be exercised in drawing conclusions from them. The reptilian and avian gelatins have a composition very similar to those of mammalian origin, but the amphibian gelatin, represented by the toad, shows significant differences. It contains less proline and hydroxyproline and more serine and threonine than mammalian gelatins, and its

TABLE I. Composition of mammalian gelatins
(Values are given as numbers of residues per 1000 total residues)

	PI	IA	Rabbit skin ^a	Whale skin ^b	Pig skin ^a	Ox skin ^b	Ox bone ^b
Alanine	6.1	730	105.0	110.5	111.7	112.0	116.6
Glycine	6.1	0	325	326	330	333	335
Valine	6.0	1690	20.7	20.6	25.9	20.1	21.9
Leucine	6.0	2490	22.2	24.8	24.0	23.1	24.3
Isoleucine	6.0	2990	13.0	11.0	9.5	12.0	10.8
Proline	6.3	2620	132.0	128.2	131.9	129.0	124.2
Phenylalanine	5.9	2650	14.0	13.0	13.6	12.3	14.0
Tyrosine	5.6	2870	3.0	3.6	2.6	1.5	1.2
Serine	5.7	40	34.7	41.0	34.7	36.5	32.8
Threonine	-	440	20.0	24.0	17.9	16.9	18.3
Cystine	5.02	1500	0.5				
Methionine	5.7	1300	5.4	4.7	3.6	5.5	3.9
Arginine	10.8	730	47.3	50.1	49.0	46.2	48.0
Histidine	7.6	500	5.5	5.7	4.0	4.5	4.2
Lysine	9.5	1500	27.4	25.9	26.6	27.8	27.6
Aspartic acid	3.0	540	47.5	46.3	45.8	46.0	46.7
Glutamic acid	3.1	550	67.5	69.6	72.1	70.7	72.6
Hydroxyproline	-	2620	105.1	89.1	90.7	97.6	93.3
Hydroxylysine	-	1500	4.4	5.8	6.4	5.5	4.3
Tryptophan	30.0	(muito amargo)					
Amide ^c			38.4	25.6	41.5	7.5	15.7
Recovery by wt. (%)			96.9	99.7	97.2	99.8	
Total N (%)			18.1	18.6	18.3	18.1	
Recovery of N (%)			99.6	100.5	101.5	100.9	
Process			alkali ^d	?	acid	alkali	alkali
Reference			Jackson <i>et al.</i> (1958)	Eastoe (1955)	Eastoe (1961)	Eastoe (1955)	Eastoe (1955)

^a Recalculated in this form by present authors.

^b Recalculated (Eastoe and Leach, 1958).

^c No. of molecules of NH₂ released during hydrolysis per 1000 total residues.

^d Short alkali treatment.

PI médio calculado = 5,40
 Índice de amargor médio hidrolizado total = 1000
 Valor acima de 1400 res. amargo

TABLE II. Composition of amphibian, reptilian and avian gelatins^a
(Values are given as numbers of residues per 1000 total residues)

	Toad skin	Crocodile skin	Python skin	Chicken tendon
Alanine	98.0	114.0	125.0	114.6
Glycine	301	324	315	331
Valine	21.9	15.4	20.2	19.8
Leucine	28.8	20.1	25.7	23.8
Isoleucine	14.0	11.4	11.7	10.9
Proline	109.7	127.9	119.4	129.5
Phenylalanine	19.3	17.7	14.2	14.3
Tyrosine	6.1	3.3	1.8	3.4
Serine	66.3	42.1	43.6	28.6
Threonine	26.4	22.0	17.9	19.1
Methionine	8.7	6.5	6.1	6.2
Arginine	49.2	49.5	49.9	44.8
Histidine	6.5	4.7	4.7	4.5
Lysine	29.1	25.3	27.6	19.0
Aspartic acid	54.9	45.5	48.0	48.1
Glutamic acid	77.9	72.8	62.4	74.1
Hydroxyproline	77.5	92.8	102.0	98.5
Hydroxylysine	4.3	4.9	4.0	9.6
Amide ^b	53.9	25.5	22.1	40.1
Recovery by wt. (%)	93.4	99.8	82.5	98.8
Total N (%)	18.0	18.3	16.2	17.8
Recovery of N (%)	96.8	102.0	95.5	103.3
Process	acid	acid	alkali	acid

^a Leach (1957), recalculated in this form by Eastoe and Leach (1958).

^b No. of molecules of NH₃ released during hydrolysis per 1000 total residues.

overall composition is between that of cold-water fish and the lung-fish (see below). In terms of evolution the latter can be regarded as closely related to the amphibians.

(c) *Elasmobranchs and fishes*. These groups have been in existence for a very long period, during the course of which they have been subjected to a range of environmental conditions, particularly with respect to temperature. From the point of view of gelatin composition we can subdivide these aquatic vertebrates into four, namely, elasmobranchs (sharks, dogfishes and rays), cold-water fishes (cod, halibut and plaice), warm-water fishes (sturgeons, carp, coelocanths and threadfins) and finally lungfishes which can be regarded as hot-water fish as they are able to survive quite high temperatures.

The gelatins from these groups cover a wider range of composition than that exhibited by the mammalian gelatins. More analyses are available than are noted in Table III (see Eastoe, 1967), however those selected reflect fairly the general situation. The hydroxyproline and proline contents are lower than

TABLE III. Composition of elasmobranch and fish gelatins
(Values are expressed as numbers of amino acid residues per 1000 total residues)

	Shark skin ^a	Cod skin	Carp skin	Lungfish skin ^a
Alanine	119.0	107	120	126.0
Glycine	333	345	317	327
Valine	21.9	19	19	17.8
Leucine	23.9	23	25	19.8
Isoleucine	19.4	11	12	9.6
Proline	113.4	102	124	129.0
Phenylalanine	13.9	13	14	13.7
Tyrosine	1.4	3.5	3.2	0.8
Serine	44.5	69	43	42.1
Threonine	25.8	25	27	23.6
Cystine (half)		< 1	< 1	
Methionine	10.0	13	12	3.4
Arginine	50.3	51	53	53.5
Histidine	7.4	7.5	4.5	4.5
Lysine	24.3	25	27	23.5
Aspartic acid	42.6	52	47	43.9
Glutamic acid	65.8	75	74	76.5
Hydroxyproline	78.5	53	73	78.2
Hydroxylysine	4.7	6.0	4.5	6.3
Amide ^b	29.4	33	26	42.3
Recovery by wt. (%)	96.2			96.1
Total N (%)	18.2			18.2
Recovery of N (%)	99.9			101.2
Process	acid	autoclaving	autoclaving	acid
Reference	Eastoe (1957)	Piez and Gross (1960)	Piez and Gross (1960)	Eastoe (1957)

^a Recalculated in this form by Eastoe and Leach (1958).

^b No. of molecules of NH₃ released during hydrolysis per 1000 total residues.

those of mammalian materials. The lowest hydroxyproline contents are possessed by the cold-water fishes e.g. 53 residues per 1000 total residues for the cod. The reduction of hydroxyl groups from this cause is offset by the increased serine content and to a lesser extent by increases in threonine and hydroxylysine, thereby resulting in an almost constant hydroxyl content for gelatins (Leach, 1957). As mentioned in Chapters 1 and 5 the imino acid content is related to the thermal stability of the collagen molecule. Harrington and Venkateswara (1967) have found that the average length of the helical segment undergoing melting decreases with increasing imino acid content.

Fish collagens contain more methionine than mammalian gelatins although it remains a minor constituent.

2. *Invertebrate gelatins*

As might be expected from the vast range of animal types in this group the invertebrate gelatins show a greater variation in composition (see Table IV and Eastoe, 1967) than occurs within the vertebrate sub-phylum. However, the majority of the parent collagens are known to possess a fibrillar structure and to exhibit an axial periodicity of 640Å.

The glycine contents of this group are high, but show a variation from 255 to 323 residues per 1000 total residues. For the accepted collagen structure,

TABLE IV. Composition of invertebrate gelatins
(Values are expressed as numbers of amino residues per 1000 total residues)

Phylum	Cnidaria	Cnidaria	Aschel- minthes	Mollusca	Annelida	Echinoder- mata
Species	Physalia	Metridium	Ascaris lumbricoides	Helix aspersa	Lumbricus	Thyone
Tissue	Float	Body wall	Cuticle	Body wall	Cuticle	Body wall
Alanine	66	70	69	72.3	103	113
Glycine	307	311	286	321	324	306
Valine	26	34	12.6	21.5	17.1	30
Leucine	31	37	18.4	23.5	28.5	22
Isoleucine	22	23	13.8	12.1	15.3	13
Proline	83	63	280	104.1	13.1	109
Phenylalanine	11	12	7.3	9.9	5.2	8.9
Tyrosine	5.6	7.9	1.2	8.8	0	7.9
Serine	47	54	22.7	61.4	105	43
Threonine	33	39	18.0	27.7	52	35
Cystine	1.6	3.2	—	0	—	2.5
Methionine	5.8	8.8	12.1	1.2	0	2.2
Arginine	54	57	42.4	50.9	21.1	54
Histidine	1.9	5.1	8.4	2.6	0	2.8
Lysine	27	27	37.1	8.3	14.7	7.5
Aspartic acid	83	80	70	66.8	56	62
Glutamic acid	104	94	77	99.1	81	110
Hydroxyproline	61	49	23.5	99.5	165	60
Hydroxylysine	30	25	0	8.2	0	11
Amide ^a	66	71	44.3	53	97	75
Recovery by wt. (%)	85.0	87.4	87.7	94.7	—	89.4
Total N (%)	16.4	16.7	17.3	15.5	17.3	16.1
Recovery of N (%)	96.8	98.7	92.0	98.3	100	103.7
Process	short dual soak plus auto- claving	short dual soak plus auto- claving	auto- claving	short alkali	hot water	short dual soak plus auto- claving
Reference	Piez and Gross (1959)	Piez and Gross (1959)	Watson and Silvester (1959)	Williams (1960)	Watson (1958)	Piez and Gross (1959)

^a No. of molecules of NH₃ released during hydrolysis per 1000 total residues.

glycine (see Chapter 1) needs to occupy 1 in every 3 residues. It is possible that some of the invertebrate specimens are impure or that the collagen is in combination with other proteins or mucosubstances. Either view is compatible with the low nitrogen content, the low weight recovery and the presence of hexosamines in some preparations.

Hydroxyproline is present in all of the invertebrate gelatins examined but shows a wider variation (20–165 residues per 1000) than it exhibits in vertebrate gelatins. A more startling variation is that of proline which ranges from about 14 residues per 1000 for *Lumbricus* to 280 residues in *Ascaris*. Whilst the ratio of hydroxyproline to proline is approximately constant at 1:1.25 for vertebrate gelatins, in invertebrate gelatins it varies from about 10:1 for *Lumbricus* to 1:14 in *Ascaris*. The hydroxylysine content also varies widely from zero, in *Ascaris* and possibly *Lumbricus*, to higher than mammalian proportions in *Physalia* float and *Metridium*. The hydroxyl content of *Lumbricus* is remarkably high since 1 in every 3 residues possesses this group. Some invertebrate gelatins contain cystine; this may, however, be derived from non-collagenous components. Many of the amide contents are considerably higher than those of mammalian materials. Some of the ammonia may have been derived from the decomposition of amino acids during hydrolysis due to the presence of carbohydrates.

B. Effect of Tissue

No systematic study of the effect of the type of tissue on gelatin composition has been made, but the corresponding data for the collagens of human, rat and ox tissues are accumulating (see Chapter 1). In general, they are very similar in composition whether comparing the tissues from the same or different mammals. Small differences do occur of which the most notable concern hydroxylysine and lysine. Piez and Likins (1957) noted that in rat tissues high hydroxylysine was accompanied by low lysine and *vice versa*, the sum of the contents being approximately constant. The ratio of lysine to hydroxylysine was for skin, 6.0; tendon, 3.9; bone, 1.9 and dentine, 1.1. Complete analyses were not carried out upon the tissues. These ratios are not fully reflected in the composition of materials from other mammals, thus for the human the following values have been obtained, skin, 4.5 (Fleischmajer and Fishman, 1965); tendon, 2.4 (Eastoe, 1955); uterus (post menopausal and puerperal), 4.9 (Harding and Wesley, 1968); dura mater, 5.8 (Harding, 1963); bone, 8.0 (Eastoe, 1955); dentine, 2.3–3.1 (Piez, 1962; Eastoe, 1963) and renal reticulin, 1.8 (Windrum *et al.*, 1955). Values for bovine collagens skin, 3.7–3.9 (Bowes *et al.*, 1955; Piez and Likins, 1960); tendon 1.8–2.4 (Piez and Likins, 1960; Nordwig *et al.*, 1961); bone, 4.1–6.4, (Eastoe, 1955; Piez and Likins, 1960) and dentine, 1.5 (Piez and Likins, 1960). The differences in

ratios between tissues of the different mammals may result in part from variations in the purity of the tissues examined and from errors in the estimation of the amino acids. It is clear, however, that the ratio is generally lower in the dentine and tendon materials, the values for chicken tendon (2.0, Leach, 1957) and wallaby tendon (3.1, Eastoe, 1955) confirming the latter.

III. RELATIONSHIP OF GELATIN COMPOSITION TO THAT OF PARENT COLLAGEN

The composition of gelatin is similar to that of the collagen from which it has been prepared. A detailed comparison of amino acid values for ox hide collagen and gelatin shows close agreement for most components (Table V). This similarity has been substantiated for several tissues and species (Chibnall, 1946; Neuman, 1949; Eastoe, 1955, 1957; Leach, 1957), the only reservation being that pointed out by Neuman (1949) which almost certainly applies only to an atypical calf skin gelatin (considered in sub-section A2 below). Three main differences in composition between gelatin and its parent collagen may be superimposed upon this otherwise close similarity (Eastoe, 1955). In addition, some oxidation of methionine to methionine sulphoxide is considered by Pouradier and Rondeau (1967) to take place during conversion of collagen to gelatin.

A. Features of Composition which may Differ

1. Amide content

Alkaline, and to a lesser extent acid, pretreatment of raw materials causes progressive hydrolysis of the amide groups of asparagine and glutamine side chains of collagen, thereby resulting in a reduction in the amide content of the gelatin subsequently extracted. Loss of amide brings about an increase in the number of free carboxyl groups in the side chains of the gelatin and the consequent increase in negative net charge on the gelatin molecule causes a reduction in its isoionic point (Ames, 1944, 1952).

Corresponding values for amide N content and isoionic point (pI) for a selection of commercial gelatins are included in Table VI. Values of 0.66 and 0.52% amide N have been reported for native and citrate-soluble ox hide collagen, respectively (Bowes *et al.*, 1955). The highest values for the gelatins is 0.62% for two preparations from pig skin, following a very mild acid pretreatment (samples 96 and 149, Table VI). This value is very close to that for collagen, which suggests that negligible loss of amide groups had occurred and that the isoionic point, (pI 9.4) represents a maximum value, presumably

TABLE V. Composition of ox hide collagen, commercial ox hide gelatins, purified gelatin, impurity fractions from gelatin and long-limbed calf skin gelatin (from Eastoe, 1967)

	Ox hide collagen		Ox hide gelatin (Eastoe, 1955) ^a					"Impurity" fractions from ox hide gelatin II			Calf skin gelatin	
	Bowes and Kenten (1948)	Bowes <i>et al.</i> (1955)	Coml. gelatin I			Coml. gelatin II		Leach (1960a)		Leach (1960b) heat-coagulable	Neuman (1949)	Eastoe (1960) ^a
			1st extract 24h ^d	3rd extraction 24h	48h	Untreated 48h	Purified ^e 48h	'0.36%'	'6.6%'			
	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	<i>G</i>	
Total N	(18.60)	(18.60)	(18.19)	(18.15)	(18.15)	(18.11)	(18.12)	—	—	(13.5)	(17.4)	(17.76)
Alanine	9.5	10.32	11.0	10.8	10.9	11.1	11.3	10.9	11.4	5.68	8.7	11.1
Glycine	26.2	26.57	27.6	27.5	27.6	27.2	27.6	7.9	23.0	5.04	26.9	27.5
Valine	3.4	2.46	2.57	2.49	2.56	2.82	2.63	6.9	3.0	4.33	2.6	2.69
Leucine	5.6	3.73	3.41	3.23	3.35	3.41	3.36	9.6	4.7	8.82	3.1	3.42
Isoleucine	5.6	1.88	1.72	1.73	1.78	1.68	1.70	4.3	2.1	3.62	1.9	1.76
Proline	15.1	14.42	16.5	16.3	16.2	16.3	16.5	4.9	14.2	4.26	14.0	16.5
Phenylalanine	2.5 ^b	2.35	2.26	2.23	2.30	2.32	2.33	5.0	2.8	4.22	1.9	2.24
Tyrosine	1.4	0.99	0.38	0.40	0.44	0.26	0.23	2.4	0.8	2.72	0.14	0.15
Serine	3.4	4.27	4.08	4.29	4.32	4.11	4.15	5.2	3.6	2.50	2.9	4.05

Threonine	2.4	2.26	2.20	2.21	2.20	2.23	2.27	5.6	2.6	3.22	2.2	2.28
Cystine	—	—	Tr.	Tr.	Tr.	Tr.	Tr.	—	—	0.91	0.05	—
Methionine	0.8	0.97	0.86	0.98	0.88	0.81	0.82	1.6	0.3	1.53	0.85	0.65
Arginine	8.8	8.22	8.7	8.8	8.7	9.1	8.9	7.1	7.5	6.31	6.4	5.7
Histidine	0.8	0.70	0.82	0.80	0.76	0.74	0.76	1.9	0.6	1.59	0.63	0.82
Lysine	4.5	3.96	4.26	4.38	4.44	4.70	4.70	6.1	6.0	5.35	5.2	4.61
Ornithine	—	—	c	c	c	c	c	—	Tr.	—	—	2.56
Aspartic acid	6.3	6.95	6.6	6.92	6.8	6.6	6.6	12.3	6.5	7.57	6.9	6.7
Glutamic acid	11.3	11.16	11.16	11.4	11.5	11.1	11.4	15.3	12.7	12.6	12.1	11.5
Hydroxyproline	14.0	12.83	13.4	13.9	14.3	14.4	14.4	0.8	13.5	1.18	14.4	14.6
Hydroxylysine	1.3	1.15	0.91	0.95	0.87	1.07	1.04	0.3	1.0	0.15	—	1.09 ^f
Amide N ^e	(0.66)	(0.66)	(0.12)	(0.11)	(0.10)	(0.16)	(0.08)	(3.9)	(2.2)	(0.44)	—	(0.014)
Total	[117.3]	[115.19]	[118.9]	[119.3]	[119.9]	[119.95]	[120.7]	[107.1]	[116.3]	[81.6]	112.5	[119.9]
Recovery by weight (%)	—	95.97	[99.1]	[99.5]	[100.0]	[100.1]	[100.7]	—	—	—	94.6	[99.9]
Recovery of N (%)	99.0	96.97	100.2	100.6	100.7	101.7	101.8	—	—	—	99.2	100.9

^a Phenylalanine and tyrosine values recalculated by Cobbett *et al.* (1962).

^b Tristram (1953).

^c The small amount of ornithine present is included in the lysine value (see text).

^d Time of hydrolysis with 6N-HCl at 100°C.

^e All gelatins were manufactured from alkali pretreated hide.

^f By ethanol-fractionation and de-ionization.

close to that for collagen. The fall in isoionic point which accompanies loss of amide, resulting from alkaline pretreatment, is at first rapid and subsequently occurs more slowly to reach eventually a lower limiting value of pI 4.80, which corresponds to an amide content of virtually zero (e.g. limed calf skin gelatin, Table VI). Other gelatins have intermediate values which mainly (see Chapter 15, Section III F) fall on a smooth curve, relating amide content to isoionic point (Fig. 1). This has the same shape as the titration curve but is somewhat steeper in the region of pH 5 (Eastoe *et al.*, 1961). This curve will

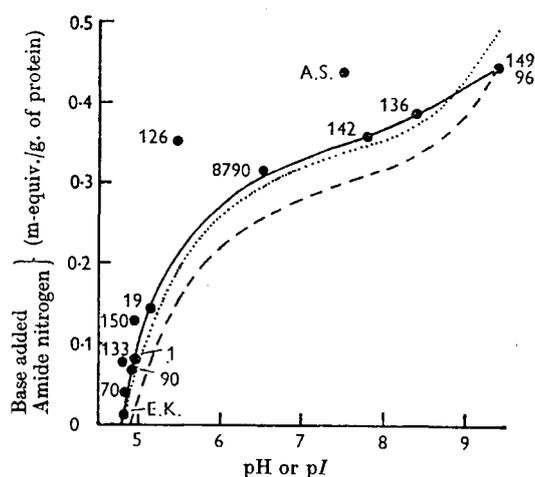


FIG. 1. Relation between amide N content and isoionic point for a range of gelatins compared with the titration curve for an alkali-processed gelatin. Amide N-pI curve (full line); titration curve for gelatin of pI 4.92 (Ketchington and Ward, 1954) (broken line); titration curve moved upwards by 0.04 mequiv./g (dotted line). Designation of gelatin is given in Table VI. (From Eastoe *et al.*, 1961.)

enable the amide content of a gelatin to be predicted if its isoionic point is known and *vice versa*. Wilkins *et al.* (1970) have shown that addition of lysine or ornithine to gelatin raises its iso-electric point.

2. Conversion of arginine side chains

Prolonged exposure of collagen or gelatin to alkali, as used in the pretreatment of the raw material, slowly brings about a reaction in which urea is split off. This results in conversion of the guanidino groups of arginine side chains to amino groups, the side chain being in effect converted to that of ornithine (the next lower homologue to lysine) which remains bound by its peptide linkages in the protein chain. This reaction is considerably slower than the

TABLE VI. Amide-nitrogen contents and isoionic points of deionized gelatins (from Eastoe *et al.*, 1961)

Designation (Fig. 1)	Type	Moisture %	Ash %	pI	Amide N (% of protein)
149	Pig skin, acid	16.6	0.016	9.4	0.615
96	Pig skin, acid	16.5	0.018	9.4	0.624
136	Pig skin, acid	16.9	0.021	8.4	0.543
142	Ossein, acid	16.9	0.037	7.8	0.498
A.S.	Ox sinew, acid	16.6	0.34	7.5	0.613
8790	—	16.1	0.073	6.5	0.442
126	Bone glue	15.4	0.13	5.47	0.493
19	Calf skin, lime-NaOH	14.7	0.015	5.15	0.200
150	Hide, limed	—	—	4.96	0.178
1	Hide, limed	15.8	0.092	4.94	0.111
90	Hide, limed	15.1	0.032	4.90	0.095
70	Sinew, lime-NaOH	16.3	0.083	4.82	0.054
E.K.	Calf skin, limed	13.5	0.03	4.82	0.018
133	Limed	15.6	0.064	4.80	0.109

hydrolysis of amide groups and proceeds to only a limited extent in the production of lime-processed gelatins by the methods currently used commercially. In Table V the composition is given for two ox hide gelatins, which can be regarded as typical products, the precursors having been limed for 3–4 months. The upper limit for the ornithine content of these was estimated as approximately 0.2% of the amino acid per 100 g of gelatin, corresponding to conversion of about 3% of the arginine (Eastoe, 1955).

Conversion is much more extensive in a type of gelatin produced by the Eastman Kodak Company by liming calf skin for 4–8 months. Hamilton and Anderson (1954) found that 34% of the arginine had been converted to ornithine. This was confirmed by Eastoe (1960) who obtained a value corresponding to 38% conversion in a complete analysis, which is compared in Table V with the data of Neuman (1949) for another sample of atypical gelatin. Lower arginine and tyrosine contents, compared with other alkali- and acid-processed gelatins are apparent in both analyses. Diminished values for these two amino acids in the calf skin gelatins led Neuman to the conclusion that gelatins show too much variation in composition to be reliable for the study of collagen composition. Clearly, gelatins prepared by using excessively vigorous alkaline pretreatment should be avoided for this purpose.

It is difficult to account for the much greater degree of arginine conversion in the calf skin gelatins solely on the basis of the somewhat longer pretreatment time. It may be that either the younger collagen is more susceptible to alkaline attack or perhaps a rather higher temperature prevails during the

pretreatment. Calf skin gelatin was used by Hitchcock (1932) for titration curve measurements and its lower guanidino and higher δ -amino content would satisfactorily account for differences in the shape of the alkaline region of the curve, compared with the curves obtained by Ames (1952b) and Kenchington and Ward (1954) for more typical alkali-processed gelatins.

3. Overall balance of amino acid composition

Superimposed on the close similarity in composition of gelatins to their parent collagens is a set of small but definite differences which conform to a clear cut pattern through a wide range of these proteins (Eastoe, 1955, 1967). Thus the gelatins are slightly richer in the abundant amino acids and relatively poorer in the rarer residues than the corresponding collagens. This was originally attributed to the processes of gelatin manufacture resulting in purification, whereby non-collagenous proteins are removed during pretreatment and extraction. In addition it now appears probable that a specific part of the collagen chain is selectively removed from the gelatin precursor during alkaline pretreatment. These features are brought out in more detail in the following discussion of effects of the various methods of manufacture on gelatin composition.

B. Effects of Methods of Manufacture on Composition

The amino acid composition of gelatins prepared by the alkaline process differs from those produced by the acid process. In general the alkali-processed gelatins possess higher hydroxyproline and lower tyrosine contents than either the acid-processed gelatins or the raw materials. There are at least three possible explanations for this; (i) the gelatins differ in purity; (ii) the acid-processed material loses peptides richer in hydroxyproline and poorer in tyrosine than gelatin during manufacture and (iii) the alkali-processed material loses peptides poorer in hydroxyproline and richer in tyrosine.

Both the acid-processed pigskin and the alkali-processed ox-skin gelatins described in Table I are of high quality. Eastoe (1955) showed that the amino acid composition of the latter is only slightly altered by purification by ethanolic fractionation and Leach (1960b) has shown that this gelatin (no. 127 in the paper) contained only a small proportion of impurities and that the pigskin gelatin (no. 149) was free of them. It would seem therefore that unless other impurities are present which have the same solution properties as gelatin, the first reason above can be ruled out. The composition of the pigskin gelatin approximates closely to that of the skin collagens, whereas the hydroxyproline and tyrosine of the alkali-processed gelatin are different, the former being higher and the latter lower. It is probable that reason (iii) represents the

true situation. This is supported by the findings of Kang *et al.* (1967) who have determined the amino acid sequences of the N-terminal ends of $\alpha 1$ and $\alpha 2$ chains of rat skin collagen. Their composition is atypical of the rest of the molecule. The very rare tyrosine residue occupies the penultimate position in both chains, valine occurs in the ninth and eleventh positions of the $\alpha 1$ chain and the seventh position in the $\alpha 2$; a leucine residue also occurs. On the other hand, these terminal regions are markedly deficient in hydroxyproline and to a lesser extent in glycine. Thus if alkali labile links occur near the N-terminal ends so that N-terminal peptides are selectively split off and lost during pretreatment, this could account for the observed differences in composition.

The amino acid data at present available are probably not sufficiently accurate and critical to prove whether this actually occurs. In order to be able to state this unequivocally further experiments are required. It is necessary to prepare both types of gelatin from the same sample of parent collagen and to examine the amino acid composition of all three. In addition all residues from the processes would need to be analysed to see if the differences could be explained in terms of particular peptide segments remaining attached to the residues. It would also be necessary to examine the treatment liquors for the presence of peptides, although in the acid-processed materials some of these are likely to remain with the gelatin.

1. *The acid process*

To prepare gelatins by the acid process the collagen is soaked in dilute acid and then extracted at about pH 4 (see Chapter 9). Many of the non-collagenous proteins and mucoproteins of the tissue are isoelectric at about this pH (see Section VB) and are therefore less soluble and more readily coagulated under the extraction conditions. The extent to which the contaminants are removed in this way depends upon the nature of the raw material and the skill of the manufacturer. It has been shown that gelatins from this process can be low in impurities or be heavily contaminated (Leach, 1960b; Williams, 1961).

The (mild) hydrolytic nature of the acid process does not appear to result in any significant removal of any part of the collagen molecule and as a consequence the nitrogen and amide contents and the amino acid composition of the gelatin do not differ greatly from that of the parent collagen. When the bulk of the amide groups remain intact the pI of the gelatin may be as high as 9.4 (see page 83). If the raw material requires more severe treatment, as for example in the manufacture of ossein, then some of the amide groups are hydrolysed and the pI of the gelatin falls, generally to the region 6-8 (Rousset, 1944; Ames, 1957, and Table VI). The removal of the impurities during the acid process results in the gelatin containing increased amounts of those

amino acids present in large amounts in collagen, i.e. glycine, proline, hydroxyproline and alanine and reduced quantities of those present in small amounts, i.e. tyrosine, phenylalanine and valine. The reduction of the last named may represent in part the extent of occurrence in the tissue of elastin, which is particularly rich in this amino acid. In contrast with the parent collagen the gelatin has detectable end groups and these are considered in Section IVD. The physico-chemical evidence indicates (see Chapter 4) that interchain links of the collagen molecule survive into the gelatin although the secondary organization has been destroyed.

2. *The alkali process*

The pretreatment of the collagen in this process consists of a prolonged soaking in alkaline solutions, usually saturated lime-water. Many of the impurities such as proteins and mucosubstances are soluble at this pH and are removed. There is a tendency for gelatins from the alkali process to be purer than those from the acid process, but again there is variation resulting from the raw material and the skill of the manufacturer.

The protein loses nitrogen during the alkaline treatment and this results from the loss of amide nitrogen and the conversion of some arginine to ornithine (see Section IIIA). Prolonged alkaline treatment yields gelatins containing less than 18.0% nitrogen, whereas the parent material contained 18.6%. As a consequence of the conversion of amide groups to carboxyl groups, the isoionic point of the gelatin is lower than that of the acid-processed materials and may be as low as pH 4.8.

In addition to the differences in amino acid composition mentioned earlier, the gelatin from this process also shows the general enrichment of certain amino acids due to the removal of impurities as described for the acid-processed material.

3. *The dual soak process*

This is in effect a combination of the acid and alkali processes (see Chapter 10). There is little published information on the composition of gelatins from this process. As the alkali stage is short it is probable that some of the interchain links and more of the amide groups remain intact than is usual in gelatins from the normal alkaline process. The other chemical features are likely to form a group intermediate between the two main types of gelatins.

4. *The autoclaving process*

Glues and technical grade gelatins prepared by autoclaving bones (without demineralization) and impure collagenous materials may contain considerable amounts of impurities (Leach, 1961; Williams, 1961), consisting largely of mucosubstances. As a consequence of this the amino acid composition can

differ significantly from that of pure collagen or gelatin (see Section VB). The impurities isolated as complexes with gelatin may represent up to 17% of the moisture-free sample (Williams, 1961). About 60% of the complex consists of gelatin (Leach, 1961). Fats form the other major impurity in these products and these are sometimes difficult to remove because of the emulsifying property of the protein. The quantity of fat will be relatively low if the bones are subjected to solvent degreasing before autoclaving.

IV. THE MAIN PROTEIN COMPONENT—GELATIN

A. Moisture

Gelatin and animal glue, as ordinarily prepared by drying a more dilute gel as far as possible in a current of warm air, still contains a substantial proportion of water. A gel exposed to air in this way slowly gains or loses water until it reaches a definite equilibrium moisture content, the value of which depends upon temperature and relative humidity. As commercially prepared for storage and use in temperate climates, the moisture content usually lies within the region 9–14% with occasional samples outside this range. Modern drying techniques (see Chapter 10) produce moisture levels at the lower end of the range. This product is known as “air dried” gelatin and when exposed to air, within the normal range of temperature and humidity, its moisture content and weight change comparatively little and slowly. For experimental work, gelatin is most conveniently weighed and handled in this form.

Sheppard *et al.* (1940) and Mason and Silcox (1943) have determined the equilibrium water contents for gelatines and glues over a wide range of relative humidity at 25 and 35°C. The moisture content of a calf skin gelatin varied from 6.5% at 10% relative humidity to 44% at 90% R.H. at pH 6.10 and 25°C (Table VII), the equilibrium moisture content rising somewhat with increased pH. Results of different investigators for this type of measurement are in approximate, but not close, agreement. There is doubt regarding whether or not a hysteresis effect occurs, whereby the previous moisture experience of a gel affects the equilibrium value eventually attained at constant temperature and humidity (Fysh, 1951). Electron microscope studies by Slonimskii *et al.* (1968) indicate that gelatin gels between 10 and 55% concentration consist of fibres of approximately 1000Å diameter.

The greater part of the water is rapidly removed when air-dried gelatin is heated *in vacuo* at a moderate temperature or exposed to the air at a somewhat higher temperature. The final stages of drying occur progressively more slowly, the water molecules being very firmly bound to the gelatin. Coulson

TABLE VII. The effect of pH on equilibrium moisture content of calf skin gelatin, dried over P_2O_5 and equilibrated at 25°C (Sheppard *et al.*, 1940)

Relative humidity %	pH	2.0	2.9	4.79	6.10	7.89	10.02
10	5	5.5	—	6.5	6.5	7	
20	7	7.5	9	9.5	9.5	9.5	
30	9	10	11.5	12	12	12	
47	13	14.5	16.5	16.5	17	17.5	
65	16	17	20	21	21.5	22	
75	20	21.5	24	26.5	27	28	
90	35.5	36	38	44	45.5	46	

(1956) pointed out that the water molecule has a special role in protein chemistry since it has the power of both giving and receiving hydrogen bonds and is sufficiently small to fit into small interstices between protein chains. According to the position which it occupies in the gel structure, it can be bound with varying degrees of firmness. Hitchcock (1931) found that air-dried gelatin reaches constant weight after two weeks *in vacuo* over $MgClO_4 \cdot 3H_2O$ at either 60 or 80°C. When dried in air at 105°C, constant weight was approached in three days, but the final weight was slightly influenced by the humidity of the air in the oven. At 110°C and above, continuous weight loss occurred *in vacuo* which suggests that decomposition of the organic constituents begins to become significant at approximately this temperature.

B. Amino Acid Composition

Except for differences described in Section III, gelatin and collagen are similar in composition. The main features of both proteins have been briefly summarized in Section IIA 1(a) (see also Eastoe and Leach, 1958; Eastoe, 1967).

Various units are available for expressing the results of amino acid analyses (Eastoe, 1967). Some of these are compared in Table VIII for a typical ox hide gelatin.

C. Side Chain Groups

Knowledge of the nature and numbers of gelatin side chain groups is obtainable by direct investigation of their reactivity with small molecules and less directly through their effects on the properties of the gelatin molecule as a whole. Almost all the information available from these sources suggests that the amino acids demonstrable by analysis of total hydrolysates of gelatin

TABLE VIII. Composition of an ox hide gelatin, expressed in various units

	G per cent amino acid	R per cent amino acid residue	N nitrogen as per cent of protein nitrogen	M gram molecules per 10 ⁵ grams protein	T residues per 1000 total residues
Alanine	11.3	9.01	9.8	126.6	114.2
Glycine	27.6	21.00	28.4	368	332
Valine	2.63	2.23	1.73	22.4	20.2
Leucine	3.36	2.90	1.99	25.7	23.2
Isoleucine	1.70	1.47	1.04	13.0	11.7
Proline	16.5	13.9	11.5	143.0	129.0
Phenylalanine	2.33	2.08	1.03	13.3	12.0
Tyrosine	0.23	0.21	0.09	1.2	1.1
Serine	4.15	3.44	3.05	39.5	35.6
Threonine	2.27	1.93	1.47	19.1	17.2
Methionine	0.82	0.72	0.43	5.6	5.1
Arginine	8.9	8.0	15.8	51.2	46.2
Histidine	0.76	0.67	1.14	4.9	4.4
Lysine	4.70	4.12	4.96	32.1	29.0
Aspartic acid	6.6	5.7	3.82	49.4	44.5
Glutamic acid	11.4	10.0	6.0	77.7	70.0
Hydroxyproline	14.4	12.4	8.5	109.9	99.1
Hydroxylysine	1.04	0.92	0.99	6.4	5.8
Amide N (0.08)					
Total	120.7	100.70	101.70	1109.0	1000.3

occur in the intact gelatin chain with all or at least a very high percentage of their side chain groups unmodified and available for reaction. The methods are generally insufficiently accurate to detect small differences. A definite exception is that a proportion of the side chain carboxyl groups of aspartic and glutamic acid residues will be present as unionizable amide groups in many gelatins. Another possible exception depends upon the existence of some γ -glutamyl linkages in gelatin (Gallop *et al.*, 1967), resulting in the replacement of γ -carboxyls by α -carboxyls as reactive groups. Recently it has been shown that an oligo-saccharide unit containing one residue each of glucose and galactose is attached to some hydroxylysine side chains in collagen (Cunningham *et al.*, 1967) which suggests possible blocking of some of the reactive groups of hydroxylysine in gelatin.

Plotting the titration curve provides a precise quantitative method for investigating the status of the intact gelatin molecule with respect to ionizable groups. Kenchington and Ward (1954) found close agreement between the numbers of various types of ionizable side chain groups calculated from the titration curve and from amino acid analysis of a lime-processed gelatin (Table IX). Almost all the ϵ -amino groups of lysine and hydroxylysine in

TABLE IX. Comparison of numbers of dissociable groups in gelatins (Kenchington and Ward, 1954)
(values are given as m-moles/g of anhydrous ash-free gelatin)

		Free carboxyl + amide	Total carboxyl	ϵ -amino including phenolic hydroxyl	Imidazole + α -amino	Imidazole ¹	Guanidino
Alkali processed ox hide	titration curve	1.23+0.03 ²	1.26	0.42	0.06	0.04	0.48 ²
	amino acid analysis	1.246+0.052	1.298	0.41 ³ +0.015 ⁴	—	0.05 ⁵	0.48
Alkali processed calf skin ⁶	titration curve	1.26	1.26	0.6	0.07	0.05	0.30 ²
Acid processed pig skin	titration curve	0.85+0.35 ²	1.20	0.42	0.06	0.04	0.49 ²

¹ Calculated from value in the previous column by subtracting 0.02 m-moles/g, i.e. the calculated value for α -amino assuming a linear polypeptide of molecular weight 60,000.

² Calculated from the titration curve and iso-ionic point.

³ Lysine + hydroxylysine + orithine.

⁴ Tyrosine.

⁵ Histidine.

⁶ Values recalculated from data of Hitchcock (1931) by Kenchington and Ward (1954).

intact gelatin in solution appear capable of reaction with O-methyl iso-urea (Eastoe and Kenchington, 1954). Both ϵ - and α -amino groups in gelatin are capable of reacting with fluorodinitrobenzene at room temperature (Courts, 1954a).

The reactivity of hydroxyl groups is discussed in Chapter 7. The high solubility and random coil behaviour of the gelatin molecule in solution suggests that there is a high degree of interaction between a substantial proportion of the large number of hydroxyl groups (which predominate over lipophilic side chains) and the aqueous environment. This suggests that hydroxyl groups are largely free and available. The ultraviolet absorption of gelatin is consistent with the presence of unmodified tyrosine and phenylalanine side chains.

D. Terminal Groups

The N-terminal groups of gelatin have been investigated by means of the fluorodinitrobenzene method by Grassmann and Hörmann (1953), Bowes and Moss (1953) and, very extensively, by Courts (1954a, 1959, 1960). The main end groups found by all these workers were glycine, alanine and aspartic acid. Serine, threonine and glutamic acid were reported in two of the three investigations. Courts (1954a) invariably found all six types of N-terminal group in ten commercial gelatins (Table X) while small amounts of valine and phenylalanine were sometimes present. Heyns and Legler (1957) reported leucine (or isoleucine), proline and lysine in addition to end groups already mentioned. Glycine almost always accounts for 50% or more of the total end groups (Courts, 1954a). Differences in the relative proportions of end groups depends more on the pretreatment received by the collagen than its source. Serine, threonine, aspartic and glutamic acid end groups predominate in alkali-processed gelatins and alanine in acid-processed ones.

The same end groups in similar proportions appear on further thermal degradation of gelatin (Courts, 1954b) which suggests that there is no fundamental difference between the bond types broken in gelatin formation and breakdown. Since native collagen contains very few detectable end groups (Bowes and Moss, 1953; Grassmann and Hörmann, 1953), breakage of peptide bonds appears essential for the irreversible reaction whereby collagen is converted to gelatin. The average chain weight, corresponding to one N-terminal group, ranges from 53,000–67,000 with a mean value of 60,000 for alkali-processed gelatins, and is somewhat higher for acid-processed ones (Courts, 1954a). On this basis it appears that alkaline treatment of collagen breaks peptide bonds in the collagen chains *in situ* thereby forming *eu collagen* (Courts, 1960). *Eu collagen* is composed of units having a number average chain weight of 60,000–65,000 still held together in the helix by hydrogen bonds. When the temperature is subsequently raised, hydrogen bond break-

TABLE X. N-terminal amino acid residues of various gelatins (Courts, 1954a)

(Results are expressed as moles per 100,000 g gelatin (moisture- and ash-free))

	Alkali-processed						Acid-processed				
	A Ossein	B Ossein	C Calf skin	D Calf skin	E Ox hide	F Sinew	G Pig skin	H Pig skin	J Sinew	K Ossein	L Isinglass
Glycine	0.95	0.98	0.83	0.80	1.27	1.00	0.74	0.59	0.96	0.72	0.25
Serine	0.24	0.23	0.19	0.19	0.24	0.23	0.10	0.05	0.04	0.06	0.05
Threonine	0.11	0.10	0.11	0.11	0.14	0.14	0.08	0.03	0.03	0.05	0.03
Alanine	0.13	0.17	0.11	0.10	0.20	0.17	0.24	0.18	0.23	0.16	Nil
Aspartic acid	0.10	0.12	0.11	0.08	0.14	0.15	0.13	0.06	0.02	0.04	0.03
Glutamic acid	0.14	0.13	0.10	0.10	0.15	0.12	0.07	0.04	0.04	0.03	0.04
Others	0.01	0.14	0.12	0.12	0.20	0.08	0.19	0.19	0.11	0.05	Nil
Total	1.68	1.87	1.57	1.50	2.34	1.89	1.55	1.14	1.43	1.11	0.40
Number-average mol. wt. $\times 10^{-3}$	60	53	64	67	43	53	65	87	70	88	250

TABLE XI. Terminal amino acids of a bone gelatin
(Heyns and Legler, 1957)

(Results are expressed as moles per 100,000 grams protein)

	N-terminal	C-terminal
Glycine	1.32	0.68
Serine	0.35	0.17
Threonine	0.12	0.13
Alanine	0.19	0.13
Aspartic acid	0.21	0.39
Glutamic acid	0.16	0.40
Valine	0.05	0.03
Phenylalanine	0.10	0.04
Leucine and isoleucine	0.15	0.03
Lysine	(0.04)	—
Proline	0.07	0.13
Hydroxyproline	—	0.15
Total	2.76	2.28

age results in chain separation and consequent loss of order in the system, as it is converted to a gelatin sol. (See also Chapter 5, VIC.) Bowes *et al.* (1955) found that conversion of acid citrate soluble collagen into gelatin by heating to 40°C for 5–10 minutes resulted in a sixfold increase in the N-terminal groups.

The carboxyl-terminal amino acids of a bone gelatin were investigated by Heyns and Legler (1957) using hydrazinolysis. They found that the proportion of acidic and β -hydroxyamino acids was approximately four times that of the non-polar substituted amino acids and imino acids, basic amino acids being absent from this position (Table XI). Joseph and Bose (1959) reported glycine, 0.5; alanine, 0.8; valine 0.2 and leucine (or isoleucine), 1.9 moles per 100,000 g of protein as C-terminal groups, determined by the thiohydantoin method in an unspecified gelatin.

E. Sequence

The sequence of amino acid residues along the polypeptide chain of gelatin constitutes its primary structure. This will also largely determine the secondary and tertiary structures, which concern the spatial arrangement taken up by the molecule in a given environment. The amino acid sequence in gelatin clearly depends almost entirely upon that of the collagen from which it was derived. It seems unlikely that any substantial rearrangement will occur during collagen-gelatin conversion. This reaction determines only the relation of the ends of the gelatin chains to the pre-existing collagen sequence. While peptide bonds break at statistically preferred points, the variety of

terminal amino acids in gelatin shows there are several such points of cleavage. Thus the sequence in different gelatin molecules will vary with respect to their ends.

Early studies of sequence in the collagen system were carried out on gelatin (Heyns *et al.*, 1951; Schroeder *et al.*, 1954) because it was then available in purified form. Since this work involved identification of di- and tripeptides from acid and alkaline partial hydrolysates, the results were valid for collagen. Most of the peptides isolated in these and related investigations did not fit the sequence $(-P-G-R-)_n$ proposed by Astbury (1940), where P represents a prolyl or hydroxyprolyl residue, G represents glycine and R, any other residue. These observations, however, formed the foundation of the current concept that non-polar regions of collagen and gelatin chains consist mainly of the sequence $(-Gly-Pro-R-)_n$ where proline (but not hydroxyproline) is attached to the glycol carbonyl group and R is any other residue, including hydroxyproline (see Chapter 1 and Hannig and Nordwig, 1967).

Gelatin is not suitable for the determination of complete or even long sequences, not only because of variations in relation to the ends of the chains but also because it is derived from all three α -chains, which differ in composition and sequence (Kang *et al.*, 1967). Methods are now available for the separation of individual α -chains from purified soluble collagens. Such molecularly homogeneous materials are now used instead of random gelatins, as starting points in sequence studies.

Schleyer (1962) found that alkaline degradation of denatured tropocollagen broke certain bonds preferentially so that two fragments, A from an $\alpha 1$ -chain and B from an $\alpha 2$ -chain could be isolated. The predominant N-terminal amino acids formed were those which occur in gelatin.

F. Unusual Structures

It is becoming apparent that various structures, linkages and groupings in addition to the long established peptide bonds and disulphide crosslinks occur occasionally in certain proteins. Several types have been suggested as occurring in collagens and gelatins. Such structures have been reviewed in detail by Harding (1965) and Gallop *et al.* (1967) and are considered briefly here.

1. "Esterlike" bonds

These were postulated by Gallop *et al.* (1959) as a result of studying the reaction of denatured soluble collagens with hydroxylamine under alkaline conditions. Each α -chain of collagen is considered to have six "esterlike" bonds, arranged in three sets of two. The distance between the bonds in each pair is not more than seven amino acid residues. Two aspartic acid residues

are involved in each pair of bonds, one contributes its α -carboxyl and the other its β -carboxyl group (Gallop *et al.*, 1967). Esterlike bonds are not considered to be crosslinks; they probably join together peptide "subunits" within a single α -chain.

Bello (1960) found that hydroxylamine reacts with acid-processed pig skin gelatin to the extent of only 0.5–1.5 moles/10⁵ g, which could be accounted for by reaction with carbohydrate. It is not clear to what extent esterlike bonds participate in gelatin structure but, assuming they are present in collagen, at least one pair could be expected in a gelatin chain of weight 50,000.

2. γ -glutamyl linkages

Dakin (1912) found that glutamyl residues in gelatin are not racemized by dilute alkali, suggesting that the γ -carboxyl groups of glutamic acid may be in peptide linkage and the α -carboxyls free. Joseph and Bose (1960) hydrolysed proteins with trypsin, which they assumed would leave γ -glutamyl linkages intact. They subjected the hydrolysates to hypobromite oxidation and obtained 1.3 and 0.14 moles/10⁵ g of peptide-bound succinic acid from buffalo hide collagen and gelatin, respectively. They considered this to represent small amounts of interchain γ -glutamyl bonds, possibly with guanidino and ϵ -amino groups rather than intrachain γ -glutamyl peptide bonds. Gallop *et al.* (1960) showed that esterified gelatin can be converted in several stages to a dinitrophenylhydroxamate, the hydrolysis products of which suggest the presence of γ -glutamyl peptide bonds in the original gelatin. Franzblau *et al.* (1963) obtained evidence that at least 30 of the 71 glutamic acid residues per 1000 total residues of ichthyocol gelatin were in γ -glutamyl peptide linkage. The possibility of α , γ interchange was considered to have been excluded under the reaction conditions used. It therefore seems possible that gelatin contains a substantial proportion of γ -glutamyl peptide linkages.

3. Aldehyde groups

It has long been known that gelatins give colour reactions for aldehydes which are of interest in relation to photographic properties (see Chapter 14). It was not clear whether the aldehyde groups were free or part of the gelatin molecule. Landucci *et al.* (1958) claimed that much of the aldehyde was in the polypeptide chain, adjacent to proline or hydroxyproline. They considered that the bond between the aldehyde residue and the next adjacent amino acid in collagen was weak and liable to be broken in the collagen-gelatin conversion. The aldehyde group thus became terminal in gelatin, although largely destroyed during alkaline pretreatment. Gallop *et al.* (1967) consider that the tropocollagen molecule contains two to three aldehyde groups, while approximately nine additional masked groups are released under conditions which break esterlike bonds. Recently Gallop *et al.* (1968) have obtained

evidence that six α -amino aldehydes (glycinal, α -alaninal, α -aspartal, lysinal, serinal and threoninal) occur as components of tropocollagen. It is based on isolation of the corresponding amino alcohols after reducing the collagen with tritiated sodium borohydride. It is not yet clear to what extent these represent temporary intermediates of biosynthesis of insoluble collagen, few of which would be expected to survive in gelatin.

Kang *et al.* (1967) have found that a specific lysine residue, five units from the N-terminal end of both α 1- and α 2-chains of rat skin collagen is oxidized to the δ -semialdehyde of α -amino adipic acid during maturation. Pairs of these aldehyde groups then undergo condensation to form interchain cross-links. The fate of these aldehyde-based crosslinks during conversion of collagen to gelatin is not entirely clear, but they may help to explain the finding of aldehyde groups in gelatin by earlier workers.

4. Carbohydrates

Several sugar-type units have been reported in collagen preparations (see Eastoe, 1967; Gallop *et al.*, 1967). Hexosamines; deoxysugars, pentoses and sialic acid are probably contained in small amounts of high molecular weight impurities which are difficult to remove completely from insoluble collagen. The hexoses, glucose and galactose, are almost invariably present in collagens and tropocollagens. Cunningham *et al.* (1967) showed that at least 75% of the hexose of soluble collagen is attached to hydroxylysine by an *O*-glycoside linkage in both the forms D-glucosyl, 1 \rightarrow 6, D-galactosyl, 1 \rightarrow *O*-hydroxylysine and D-galactosyl, 1 \rightarrow *O*-hydroxylysine. These structures may remain intact to some extent during gelatin manufacture because the bonds linking the sugar units are alkali stable.

The carbohydrates of gelatin were studied by Wood (1958) who detected glucose, probably in bound form. Venet *et al.* (1957) and Steigmann (1958a) have reported uronic acids both free and bound in polysaccharides. Pentoses occur in nucleic acids which have been shown to occur as impurities in hide gelatin by Russell and Oliff (1966). The photographic significance and analytical characterization of sugar units overlap with those of aldehydes in gelatin (Steigmann, 1958b). There is little direct information regarding the extent to which carbohydrate forms an integral part of the gelatin molecule.

V. CONTAMINANTS

A. Inorganic

1. Inorganic ions

Gelatine as ordinarily prepared contains a variety of inorganic substances, usually in ionized form, either associated with the ionized groups on the

gelatin molecule, or free. The amount is usually below 2%, but ash values up to about 5% have been recorded. Inorganic ions may be partly of physiological origin, but in high grade gelatins are probably mainly derived from water and chemicals used in manufacture. The cations, calcium, sodium, magnesium, iron, aluminium and potassium have been reported in substantial quantities, whilst copper, lead, zinc, arsenic and chromium occur in smaller amounts. Anions are derived mainly from acids used in processing, sulphate and chloride being most common, but phosphate and borate may also be present. Fluoride at levels of 1–25 p.p.m. has been shown to be present in gelatins prepared using untreated water, while gelatins where the water supply had been fluoridated to a level of 1 p.p.m. contained 29–34 p.p.m. of fluoride (Bartlett, 1961). A gelatin manufactured from skins cured with 1% sodium fluosilicate contained 160 p.p.m. of fluoride (Pektor and Navratil, 1956). Determination of ash content and conductivity provide means for approximately assessing total inorganic content. Efficient removal of inorganic contaminants can be achieved by means of mixed-bed ion-exchange resins (Janus, Kenchington and Ward, 1951), polyvalent ions such as iron and aluminium being most difficult to remove completely.

2. *Calcium sulphate granules*

Small granules of calcium sulphate have been demonstrated as occasionally occurring in the central layer of solid sheets of commercial gelatins by Eastoe and Ward (1956). These appear as florets, both single and in clusters, when the solid gelatin is swollen in a limited quantity of water and examined at a magnification of fifty or more. If sufficient water is added, the granules dissolve completely. They occur in lime-processed gelatins where sulphuric acid is used for deliming and appear to form slowly as the sheets of gelatin are dried. The granules contain almost pure calcium sulphate and gelatin, which appears to modify the crystal habit of the gypsum to produce the characteristic florets, which have rounded corners, four "petals" and a dark air space in the centre.

B. Proteins and Mucosubstances

The two classes of macromolecular contaminants have been grouped together because the amino acid compositions of the few reported examples from each class are very similar, suggesting that the proteins are derived from the mucosubstances, or alternatively that the presence of the polysaccharide component has not always been recognized. Some methods originally reported to yield proteins from gelatins were subsequently shown to isolate mucosubstances and not simple proteins (Leach, 1960b).

TABLE XII. Amino-acid composition of mucoprotein fractions¹ from gelatines, glues and related materials
(Number of residues of amino-acid per 1000 total residues)

	A	B	C	D	E	F	G	H	I	J	K
Alanine	69	96	79	150	132	118	73	138	73	76	68
Glycine	38	68	53	83	117	176	64	100	91	76	71
Valine	71	68	57	67	72	67	70	70	67	64	61
Leucine	130	122	115	106	72	114	101	82	103	97	108
Isoleucine	45	51	41	46	35		49	49	54	44	49
Proline	63	36	63	42	42	60	49	47	59	114	57
Phenylalanine	44	43	42	38	35	24	32	35	37	67	51
Tyrosine	29	28	31	14	18	29	19	9	33	41	35
Serine	53	35	59	56	60	27	62	66	67	41	66
Threonine	51	45	53	57	54	24	68	65	53	45	63
Cystine	8	7	20	N.D.	N.D.	33	9	N.F.	N.F.	N.D.	8
Methionine	12	18	9	30	trace	trace	10	4	8	14	22
Arginine	55	60	37	51	42	49	41	31	50	22	65
Histidine	22	19	23	15	13	23	29	14	17	19	25
Lysine	42	63	76	47	50	40	52	58	55	39	45
Aspartic acid	119	96	99	89	126	92	130	119	120	93	88
Glutamic acid	149	145	143	109	132	125	142	111	113	141	118

A Heat-coagulable mucoprotein from alkali-processed ossein gelatin (Leach, 1960b).

B Heat-coagulable mucoprotein from alkali-processed ox-hide gelatin (Leach, 1960b).²

C Heat-coagulable mucoprotein from acid-processed pig-skin gelatin (Leach, 1960b).

D Mucoprotein isolated by ion-exchange method, material associated with resin (Leach 1960a).²

E Mucoprotein isolated by ion-exchange method, material free of resin.²

F Adsorbed on to charcoal from alkali-processed gelatin (Maron, 1958).

G "Osseomucoid" (Eastoe and Eastoe, 1954).

H Acetic acid soluble protein from calf skin not precipitated by NaCl (Bowes *et al.*, 1957).

I Alkali-soluble protein from calf skin (Bowes *et al.*, 1957).

J Mucoprotein from cartilage (Partridge and Davis, 1958).

K Mucoprotein from bone glue (Leach, 1961).

¹ Corrected for gelatin content as described in the original papers (Leach, 1960b, 1961).

² Same parent gelatin.

Sheppard *et al.* (1931) coagulated a component, termed an albumin, by the prolonged heating of a gelatin solution acidified with acetic acid. This material contained about 14% N and was isoelectric at pH 4. From an examination of materials isolated by an almost identical procedure Leach (1960b) showed that they were mucoproteins and were similar in amino acid composition to the alkali-soluble mucoprotein fractions from cartilage (Partridge and Davis, 1958), bone (Eastoe and Eastoe, 1954) and skin (Bowes *et al.*, 1957) (see Table XII). The amounts of coagulable mucoprotein which could be isolated from gelatins varied from 0–0.9% (Sheppard *et al.*, 1931; Leach, 1960b; Williams, 1961). Williams (1961) showed that the proportions were independent of the type of process of gelatine manufacture and that it is the efficiency with which the processes are carried out which is the controlling factor.

The mucoproteins described above have gelatin and lipid complexed with them (Leach, 1960b). The N-content on a dry, ash-free basis is 12.7–13.9%. The polysaccharide component contains hexosamines, uronic acid, hexose and neuraminic acid (Leach, 1960b). The amino acid composition of the protein component is very different from that of gelatin, and after correcting for the amino acids contributed by the latter, on the basis that gelatin is present in a proportion represented by the hydroxyproline content, the following notable differences (see Table XII) emerge: (i) significantly larger amounts of tyrosine, phenylalanine, lysine, histidine, methionine, isoleucine and leucine are present, (ii) cystine and cysteic acid are present in the hydrolysates, and as cystine they constitute 0.80–2.69% of the dry, ash-free material and 8–20 residues per 1000 total residues of gelatin-free protein, and (iii) reduced amounts of glycine and proline are present.

Zimkin and Paronik (1960) isolated material which they assumed to be albuminous by degrading gelatin with dilute HCl at room temperature. It contained 3.9% of cystine and 1.2% of tryptophan. The nitrogen content was in the range 11.9–12.4%. This fraction is probably identical to that described above. The isolation procedure used here and above is dependent upon a combination of heat denaturation, formation of a complex between gelatin and mucoprotein (see Leach, 1961) and destruction of the protective effect of the gelatin.

In a study of the turbidity factor present in low grade bone glues, Leach (1961) showed that it was mucoprotein in nature and could be coagulated in large amounts from a dilute solution of the glue by adjusting it to pH 4, followed by centrifugation. The material was similar in most respects to the heat coagulable mucoproteins mentioned above. The gelatin content was higher, presumably because the gelatin had not been degraded in this case. Coagulation of the complex is optimal at pH 4, close to its isoelectric point and in this respect the complex resembles the material isolated by Maron (1958) which is mentioned later.

Williams (1961) showed that the amount of complex that could be isolated was 5.4–16.7 g and 0.08–0.99 g per 100 g dry glue for bone and hide glues, respectively. For higher grade glues the isolation procedure required modification to overcome the protective effect of the gelatin. The proportions present in the bone glues are much larger than would be expected from the analysis of compact bone carried out by Eastoe and Eastoe (1954) and the increased amounts are possibly largely derived from the spongy regions of the bone or from attached tissues.

As there was a substantial amount of material available for analysis, it was examined in detail (Leach, 1961). The amino acid composition (Table XII) was similar to that of "osseomuroid" (Eastoe and Eastoe, 1954) and to the mucosubstances described earlier. The lipid had its origin in the depot fat of the bone. The polysaccharide component was also similar to that of "osseomuroid" as described by Eastoe and Eastoe (1954) and Hisamura (1938). The presence of sulphated hexoses, acetyl hexosamines and neuraminic acid was noted. The molar ratios for some of the polysaccharide components are:

	Hexos- amine	Hexose	HSO ₄	Acetyl	Uronic Acid
Osseomuroid (Hisamura, 1938)	1.0	: 0.43	: 0.54	: 1.03	: 0.55
Turbidity factor (Leach, 1961)	1.0	: 0.70	: 0.64	: 0.76	: 0.56

About 0.3% of a protein impurity was isolated by Maron (1958) from a gelatin solution by adsorption on to activated charcoal. It possessed an isoelectric point at pH 4 and an amino acid composition very different from gelatin (see Table XII). It contained 1.2 g hydroxyproline and 6.5 g of cystine per 16 g N, and had an actual N-content of 15.3% on a dry weight basis. The material could be precipitated by adjusting its solution to pH 4.

During an examination of the behaviour of gelatins on certain ion-exchange resins Leach (1960a) isolated 0.36% of a non-adsorbed component. On hydrolysis it yielded humin and the subsequent analysis revealed hexosamines in addition to amino acids, and he concluded that it contained mucoproteins. It was similar in composition (see Table XII) to the material isolated by Maron, and later Leach (1960b) concluded that these materials were related to the acetic acid soluble protein (not precipitable by NaCl) isolated by Bowes *et al.* (1957) from calf skin. This protein also had a polysaccharide component. Thus it can be concluded that at least two types of mucoproteins are present in gelatins and glues.

The mucosubstances of gelatin contain substantial amounts of cystine and are therefore a source of photographically important compounds. Mucosubstances, particularly when present in large amounts cause turbidity in

gelatine solutions. The turbidity probably represents either denatured mucoprotein or complexes of the larger molecules of mucosubstances with the larger molecules of gelatin, the bulk of the mucosubstances remaining in solution. The mucosubstances are also responsible, via the "browning reaction" for the colour of gelatins and glues. Together with the fat they cause many of the unpleasant aromas that may occur.

The presence of mucoproteins in gelatins prepared by processes in which the raw material has undergone severe purification treatments, emphasizes the need for caution in the interpretation of the significance of the presence of carbohydrate in many collagen preparations.

REFERENCES

- Ames, W. M. (1944). *J. Soc. chem. Ind., Lond.* **63**, 200-4, 234-41, 277-80, 303-6.
 Ames, W. M. (1952a). *J. Sci. Fd Agric.* **3**, 454-63.
 Ames, W. M. (1952b). *J. Sci. Fd Agric.* **3**, 579-85.
 Ames, W. M. (1957). *J. Sci. Fd Agric.* **8**, 169-73.
 Astbury, W. T. (1940). *J. int. Soc. Leath. Trades Chem.* **24**, 69-92.
 Bartlett, J. C. (1961). *Analyst, Lond.* **86**, 200-1.
 Bello, J. (1960). *Nature, Lond.* **185**, 241.
 Bowes, J. H. and Kenten, R. H. (1948). *Biochem. J.* **43**, 358-65.
 Bowes, J. H. and Moss, J. A. (1953). *Biochem. J.* **55**, 735-41.
 Bowes, J. H., Elliott, R. G. and Moss, J. A. (1955). *Biochem. J.* **61**, 143-50.
 Bowes, J. H., Elliott, R. G. and Moss, J. A. (1957). In "Connective Tissue" (R. E. Tunbridge, ed.) pp. 264-80, Blackwell, Oxford.
 Chibnall, A. C. (1946). *J. int. Soc. Leath. Trades Chem.* **30**, 1-20.
 Coulson, C. A. (1956). *Br. Gelat. Glue Res. Ass., Res. Panel Paper No.* 28.
 Courts, A. (1954a). *Biochem. J.* **58**, 70-4.
 Courts, A. (1954b). *Biochem. J.* **58**, 74-9.
 Courts, A. (1959). *Biochem. J.* **73**, 596-600.
 Courts, A. (1960). *Biochem. J.* **74**, 238-47.
 Courts, A. and Kragh, A. M. (1960). *Analyt. Biochem.* **1**, 88-91.
 Cunningham, L. W., Ford, J. D. and Segrest, J. P. (1967). *J. biol. Chem.* **242**, 2570-1.
 Dakin, H. D. (1912). *J. biol. Chem.* **13**, 357-62.
 Eastoe, J. E. (1955). *Biochem. J.* **61**, 589-602.
 Eastoe, J. E. (1957). *Biochem. J.* **65**, 363-8.
 Eastoe, J. E. (1960). *J. appl. Chem. Lond.* **10**, 393-4.
 Eastoe, J. E. (1961). *Biochem. J.* **79**, 652-6.
 Eastoe, J. E. (1963). *Archs. oral Biol.* **8**, 633-52.
 Eastoe, J. E. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.) Vol. 1, pp. 1-72.
 Eastoe, J. E. and Eastoe, B. (1954). *Biochem. J.* **57**, 453-9.
 Eastoe, J. E. and Kenchington, A. W. (1954). *Nature, Lond.* **174**, 966-7.
 Eastoe, J. E. and Leach, A. A. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 173-8, Pergamon Press, London.
 Eastoe, J. E. and Ward, A. G. (1956). *Bull. Br. Gelat. Glue Res. Ass.* **7** (1), 11-15.
 Eastoe, J. E., Long, J. E. and Willan, A. L. D. (1961). *Biochem. J.* **78**, 51-6.

- Fleischmajer, R. and Fishman, L. (1965). *Nature, Lond.* **205**, 264-6.
- Franzblau, C., Gallop, P. M. and Seifter, S. (1963). *Biopolym.* **1**, 79-97.
- Fysh, D. (1951). *Bull. Br. Gelat. Glue Res. Ass.* **2**, (2), 19-23.
- Gallop, P. M., Seifter, S. and Meilman, E. (1959). *Nature, Lond.* **183**, 1659-61.
- Gallop, P. M., Seifter, S., Lukin, M. and Meilman, E. (1960). *J. biol. Chem.* **235**, 2619-27.
- Gallop, P. M., Blumenfeld, O. O. and Seifter, S. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.) Vol. 1, pp. 339-66.
- Gallop, P. M., Blumenfeld, O. O., Henson E. and Schneider, A. L. (1968). *Biochemistry, N. Y.* **7**, 2409-30.
- Grassmann, W. and Hörmann, H. (1953). *Hoppe-Seyler's Z. physiol. Chem.* **292**, 24-32.
- Hamilton, P. B. and Anderson, R. A. (1954). *J. biol. Chem.* **211**, 95-102.
- Hannig, K. and Nordwig, A. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.) Vol. 1, 73-101, Academic Press, New York.
- Harding, J. J. (1963). *Biochem. J.* **86**, 574-6.
- Harding, J. J. (1965). *Adv. Protein Chem.* **20**, 109-90.
- Harding, J. J. and Wesley, J. M. (1968). *Biochem. J.* **106**, 749-57.
- Harrington, W. and Venkateswara, R. N. (1967). *Conform. Biopolym. Pap. Int. Symp. Madras 2*, 513-31.
- Heyns, K. and Legler, G. (1957). *Hoppe-Seyler's Z. Physiol. Chem.* **306**, 165-72.
- Heyns, K., Anders, G. and Becker, E. (1951). *Hoppe-Seyler's Z. physiol. Chem.* **287**, 120-33.
- Hisamura, H. (1938). *J. Biochem. Tokyo* **28**, 473.
- Hitchcock, D. I. (1931). *J. gen. Physiol.* **15**, 125-38.
- Hoerman, K. C. and Mancewitz, S. A. (1964). *J. dent. Res.* **43**, 276-80.
- Jackson, D. S., Leach, A. A. and Jacobs, S. (1958). *Biochim. biophys. Acta* **27**, 418-20.
- Janus, J. W., Kenchington, A. W. and Ward, A. G. (1951). *Research, Lond.* **4**, 247-8.
- Joseph, K. T. and Bose, S. M. (1959). *Bull. cent. Leath. Res. Inst. Madras* **5**, 303-18.
- Joseph, K. T. and Bose, S. M. (1960). *Bull. cent. Leath. Res. Inst. Madras* **7**, 97-111.
- Kang, A. H., Bornstein, P. and Piez, K. A. (1967). *Biochemistry, N. Y.* **6**, 788-95.
- Kenchington, A. W. and Ward, A. G. (1954). *Biochem. J.* **58**, 202-7.
- Landucci, J. M., Pouradier, J. and Durante, M. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 62-7, Pergamon Press, London.
- Leach, A. A. (1957). *Biochem. J.* **67**, 83-7.
- Leach, A. A. (1960a). *Biochem. J.* **74**, 61-71.
- Leach, A. A. (1960b). *J. appl. Chem., Lond.* **10**, 367-72.
- Leach, A. A. (1961). *J. appl. Chem., Lond.* **11**, 10-19.
- Maron, N. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 221-4. Pergamon Press, London.
- Mason, C. M. and Silcox, H. E. (1943). *Ind. Engng. Chem. ind. Edn* **35**, 726-9.
- Neuman, R. E. (1949). *Archs Biochem.* **24**, 289-98.
- Nordwig, A., Hörmann, H., Kühn, K. and Grassmann, W. (1961). *Hoppe-Seyler's Z. physiol. Chem.* **325**, 242-50.
- Ogle, J. D., Arlinghaus, R. B. and Logan, M. A. (1962). *J. biol. Chem.* **237**, 3667-73.
- Partridge, S. M. and Davis, H. F. (1958). *Biochem. J.* **68**, 298-305.

- Pektor, V. and Navrátil, V. (1956). *Kožaářství* **6**, 149–51.
- Piez, K. A. (1962). In "Fundamentals of Keratinization" (E. O. Butcher and R. F. Sognnaes, eds) Chap. 11, American Association for the Advancement of Science, Washington.
- Piez, K. A. (1965). *Biochemistry*, N.Y. **4**, 2590–6.
- Piez, K. A. and Gross, J. (1959). *Biochim. biophys Acta* **34**, 24–39.
- Piez, K. A. and Gross, J. (1960). *J. biol. Chem.* **235**, 995–8.
- Piez, K. A. and Likins, R. C. (1957). *J. biol. Chem.* **229**, 101–9.
- Piez, K. A. and Likins, R. C. (1960). In "Calcification in Biological Systems" (R. F. Sognnaes, ed.), pp. 411–20. American Association for the Advancement of Science, Washington.
- Pouradier, J. and Rondeau, A. (1967). *Ind. Chim. Belye* **32**, 358–9.
- Rousselot, A. (1944). *C.r. hebdom. Séanc. Acad. Sci., Paris* **219**, 62–4.
- Russell, G. (1958). *Nature, Lond.* **181**, 102–3.
- Russell, G. and Oliff, D. L. (1966). *J. Photogr. Sci.* **14**, 9–22.
- Schleyer, M. (1962). *Hoppe-Seyler's Z. physiol. Chem.* **329**, 97–104.
- Schroeder, W. A., Kay, L. M., LeGette, J., Honnen, L. and Green, F. C. (1954). *J. Am. chem. Soc.* **76**, 3556–64.
- Sheppard, S. E., Hudson, J. H. and Houck, R. C. (1931). *J. Am. chem. Soc.* **53**, 760–5.
- Sheppard, S. E., Houck, R. C. and Dittmar, C. (1940). *J. Phys. Chem., Ithaca* **44**, 185–207.
- Slonimskii, G. L., Kitagorodskii, A. I., Belavtseva, V. M., Tolstoguzov, V. B. and Mal'tseva, I. I. (1968). *Vyskomol. Soedin Ser B* **10**, 640–1.
- Stainsby, G. (1954). *Discuss. Faraday Soc.* no. 18, 288–94.
- Steigmann, A. (1958a). *Sciences Inds fotogr.* **29**, 260–1.
- Steigmann, A. (1958b). *Sciences Inds fotogr.* **29**, 321–6.
- Tristram, G. R. (1953). In "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. 1, Part A, p. 181. Academic Press, New York.
- Venet, A. M., Pouradier, J. and Landucci, J. M. (1957). *Bull. Soc. chim. Fr.*, 1325–29.
- Watson, M. R. (1958). *Biochem. J.* **68**, 416–20.
- Watson, M. R. and Silvester, N. R. (1959). *Biochem. J.* **71**, 578–84.
- Wilkins, D. J., Myers, P. A. and Warren, C. A. (1970). *Advan. Exp. Med. Biol.* **7**, 217–34.
- Williams, A. P. (1960). *Biochem. J.* **74**, 304–7.
- Williams, A. P. (1961). *J. appl. Chem., Lond.* **11**, 100–3.
- Windrum, G. M., Kent, P. W. and Eastoe, J. E. (1955). *Br. J. exp. Path.* **36**, 49–59.
- Wood, H. W. (1958). *J. Photogr. Sci.* **6**, 91–6, 170–5.
- Zahn, H. and Wegerle, D. (1955). *Leder* **6**, 278–9.
- Zimkin, E. A. and Paronik, S. I. (1960). *Usp. nauch. Fotogr.* **7**, 134–6.

Chapter 4

The Physical Chemistry of Gelatin in Solution

G. STAINSBY

*Procter Department of Food and Leather Science, University of Leeds, Leeds,
England*

I Introduction	109
II Solubility	111
III Dilute Solutions	113
A. Viscosity	113
B. Molecular Weight	119
C. Optical Rotation	121
D. Aggregation	123
E. Reversion to Collagen	125
IV Concentrated Solutions	128
A. Viscosity	128
References	135

I. INTRODUCTION

During the early part of this century, when knowledge of the physical chemistry of proteins was in its infancy, many investigations were carried out using gelatin since it was easily obtainable and readily soluble in hot water. In time, however, it was realized that different samples could show quite divergent physico-chemical properties, and that in many ways gelatin was quite unlike other proteins, so interest waned. Most of this early work is now only of limited value as the term "gelatin" refers not to a group of closely similar substances but to a whole varied series of gel-forming products obtained from collagenous tissues. The series ranges from the undegraded single-chain (α -) and double chain (β -) components of soluble collagens, to highly degraded multichain structures obtained from mature tissues after rather drastic chemical and thermal treatments (Chapter 10).

Our present insight into the physico-chemical properties of the series has been gained by considering it as a class of high polymers bearing ionizable groups. (Amino-acid sequence studies have still to be completed, although the

sequence of the α 1-chain made by combining rat and calf skin collagen analyses is published by Hulme *et al.* (1973)—see Foreword.) The occurrence of several types of ionizing groups makes the acid/base behaviour of gelatin, like that of any protein, complex. An additional complication, for commercial gelatin, is that the fraction of the total number of carboxyl groups that is ionizable depends upon the method of manufacture. Thus different gelatins can have different ratios of acidic to basic groups—and hence different isoelectric points. However, the titration curves—and hence the variations of nett charge with pH—are well established (see Chapter 15). The existence of charged groups influences the interactions between neighbouring gelatin molecules in solution, between each molecule and the solvent and between different parts of the same molecule, as the polypeptide chains are flexible. The magnitude of these effects thus varies with pH, and also depends on the total ionic composition of the system (including added electrolyte and salts remaining after gelatin manufacture), so that a detailed description of the solvent as well as that of the gelatin is always necessary.

The properties of gelatins are also determined, in part, by the extent of polydispersity. A proper specification of the sample, therefore, should include not only the average molecular weight but also the distribution of molecular weights. Except for the α - and β -gelatins, which are essentially monodisperse, this information is not available. The scale of the effort needed, and the difficulties associated with the interpretation of experimental data for a complex polyampholyte such as gelatin, have no doubt been deterrents. Only rather fragmentary and poor descriptions of molecular weight distributions have been published at the present time (these are fully explained in Veis, 1964), but recent studies by Tomka *et al.* (1972) suggest that much more detailed accounts will soon be possible through the utilization of newer techniques.

One further point requires consideration regarding the physical properties of gelatins in solution. All gelatins are susceptible to thermal and enzymatic hydrolysis, when dissolved, so that the thermal history of the sample and its microbiological population are of importance. These two factors are inter-related through the temperature dependence of microbial attack. Both factors also vary with pH. Each type of enzyme present is effective over a rather restricted pH range, but thermal hydrolysis takes place at all pH values and at a rate which is increased very sharply in acid and alkaline conditions. The overall effects of pH, and of temperature, can vary in a complicated way, depending on the nature of the microbiological content, and for fundamental studies the gelatin sample needs selecting with great care or very misleading results may arise.

This chapter deals briefly with selected important aspects of the physical chemistry of gelatin solutions. The emphasis is on dilute solutions, since these yield basic information about molecular structure and properties, but more

concentrated systems are briefly considered. At very high concentrations (> 50%) fluidity is observable only when the temperature approaches 100°C, and these systems are not described at all. Technologically important aspects of solutions are encountered in Sections III and IV of this chapter.

II. SOLUBILITY

Air-dried gelatin dissolves readily in warm water once the particles have become thoroughly wetted and have had time to swell. Dissolution, therefore, follows the same pattern as that for a synthetic polymer in a good solvent. Only highly degraded gelatins dissolve in cold water to give a stable solution. Other gelatins, if in a form which promotes cold water solubility (for example, after freeze drying with water-soluble sugars), give solutions which quickly turn into the more stable condition, the gel. Ordinary gelatin, which merely swells in cold water, dissolves slowly when hydrogen bond disrupting agents (such as urea, LiBr, KSCN, etc.) are present in sufficient concentration. In these circumstances no swelling occurs.

Gelatin is freely soluble over the whole pH range. The solutions are clear and only show a haziness, or turbidity, if they are dilute and the pH is close to the isoionic point. (The salts in a commercial gelatin are usually at a high enough level to disperse the aggregates—held only by electrostatic forces—needed to give turbidity. Removal of some or all of these salts—e.g. by dialysis or ion-exchange—then encourages haziness.) Chemically modified gelatins, however, may become quite insoluble for part of the pH range (see Chapter 7). Buffers and neutral salts will not bring gelatin out of solution unless the salt is extremely soluble and has polyvalent ions (e.g. SO_4^{--}). Very high concentrations of polyhydric compounds, such as sugars, are also tolerated, but alcohols and ketonic compounds become precipitants when about one half the water has been replaced. Charged polymers can also bring gelatin out of solution if the sign of the charge is the opposite of that for the net charge on the gelatin. Thus gum arabic (negatively charged) interacts with mildly acidic solutions of gelatin (having a net positive charge) and the solubility is sharply reduced. It is in this way that different types of gelatin can act as mutual precipitants. An acid processed gelatin, negatively charged at pH 5.5, binds electrostatically to gelatin from an alkali-treated precursor, which would be negatively charged at this pH.

By suitable adjustment of the temperature and pH a coacervate, rather than a precipitate of gelatin can be formed, i.e. the gelatin becomes distributed between two liquid phases, one of which—the coacervate—is highly concentrated. Coacervation systems have the advantages that equilibrium between the two phases is reached more rapidly than for precipitates and that co-

acervation is readily reversed, to reform a single homogeneous solution. For these reasons coacervation can be made a highly reproducible process. Coacervates are usually small in volume, dense and highly viscous, so that a quick and efficient separation from the bulky main phase can be made in contrast to the difficulties that normally arise in the separation of tacky, voluminous precipitates of polymers. Gelatin coacervates have the additional advantage, for separation, that they are easily gelled by cooling. Probably the most important technical usage of gelatin coacervation at the present time is microencapsulation (see Chapter 13).

Fractionation

In physico-chemical studies coacervate-formation has been widely used to separate commercial gelatins into fractions which differ in molecular weight. (There may also be very small differences in overall chemical composition, but none has been reported as yet.) Two very different sets of conditions, chemically, have been utilized. The simplest of these was first reported by Pouradier and Venet (1950a, 1950b, 1952) and was modified by Stainsby (1954) and, further, by Veis and Cohen (1956, 1958). These authors used ethanol to produce coacervates, but isopropanol is equally effective. The methods are described in detail in Veis (1964). In quantitative studies of molecular weight distribution it is important to choose temperature, time, and pH to minimize thermal hydrolysis during the formation and separation of the coacervate, and to recover each gelatin fraction under conditions that minimize aggregate formation, and sometimes even insolubility, on drying (see Stainsby, 1954).

The second fractionation procedure was initiated by Stainsby *et al.* (1954) and makes use of the observation of Pankhurst and Smith (1947) that detergents form a series of complexes with gelatin and that the solubility of each type of complex varies with gelatin molecular weight. The complex selected for fractionation studies had a composition of approximately one anionic detergent molecule for each peptide bond. It thus presents a mainly hydrocarbon surface to the solvent, a surface radically different from that of gelatin alone. Again the precautions mentioned earlier are required for worthwhile results to be obtained and, in addition, care needs exercising to ensure that the gelatin is completely freed from detergent. The main advantage of this procedure is that the coacervate volume is so very small, thus making the method more suitable than the alcohol method for large-scale work.

Veis *et al.* (1955) have compared the two methods and concluded that the ethanol process is preferable as the fractionation can be made more selective in molecular weight. The validity of this interpretation of the results is in

some doubt, however, as the various gelatin fractions that were obtained are not easily compared.

A single-stage fractionation, by either method, yields fractions which are still very polydisperse, though refractionation of each fraction makes a marked improvement (see Stainsby, 1954). The first fraction contains not only gelatin of very high molecular weight but also most of the colour and suspended matter (such as other proteins, lipids, and polysaccharides). Subsequent fractions are correspondingly purer chemically and brighter optically.

Fractionation experiments have shown that gelatins made from alkali-pretreated collagens at low extraction temperatures contain little material of low molecular weight, as short fragments produced during pretreatment dissolve in the alkaline liquor and little further breakdown occurs during extraction and drying. Acid-process gelatins, on the other hand, contain degraded material, even in the highest quality extracts. At the opposite end of the range the molecular weight often exceeds 1×10^6 , showing that some of the links between collagen molecules can persist throughout gelatin manufacture.

III. DILUTE SOLUTIONS

A. Viscosity

The measurement of viscosity has played a predominant role throughout the history of polymer science, so it is not surprising that viscometric studies have been widely used to investigate the nature of gelatin in solution. The viscosity of any polymer solution is made up from three sources: (i) the viscosity of the solvent, (ii) the contribution of the individual, solvated polymer molecules, and (iii) a resistance to flow arising out of the mutual interactions of the polymer molecules. In order to obtain a quantity which can be related to an average size and shape for the dissolved polymer only the second of these contributions to the overall viscosity is needed. This is obtained by taking measurements over a range of concentrations, subtracting the viscosity of the solvent and, by an extrapolation procedure, eliminating the effects of mutual interaction. The extrapolated value is termed the intrinsic viscosity. In fact, it is not dimensionally a viscosity at all, but is an inverse concentration reported either as ml. g^{-1} or $\text{decilitres. g}^{-1}$. The usual extrapolation is a plot of the so-called "reduced viscosity" against concentration, this "viscosity" being defined by the expression $\eta_{\text{red}} = (\eta_{\text{solution}} - \eta_{\text{solvent}}) / \eta_{\text{solvent}} \times \text{polymer concentration}$, i.e. it is the increase in viscosity per unit concentration, expressed as a fraction of the solvent viscosity. Plots of η_{red} against c are normally straight lines, or shallow curves, and the slope

at any point is a hydrodynamic measure of the polymer interactions at that concentration.

This procedure is valid provided that the environment of the polymer is unchanged on dilution, a condition readily met for uncharged macromolecules. With gelatin, and other ionizable polymers, the condition is not easy to fulfil—the ionic strength must be held constant during dilution, so as not to change the energy of interaction between ions, and also the pH or the extent of ionization of some of the side-chain ionizing groups will vary. If this happens, then the interactions between the charged molecules will change on dilution, and the viscous contribution of each molecule may also change through shape changes arising from intramolecular effects. Thus pure (even conductivity) water is a suitable solvent for isoionic gelatin only at concentrations higher than about 1%: at the lower concentrations needed to obtain an intrinsic viscosity the pH changes towards neutrality with dilution, and even small changes in pH in these solutions give marked changes in viscosity (see Fig. 1). Buffer salts, therefore, are necessary to hold the pH constant unless a pH-stat is used when making dilutions, and neutral salts are added to keep the ionic strength high and constant. At the present time, there is no universally agreed solvent for measuring the dilute solution viscosity of gelatin, the various research groups each selecting a system which meets the above criteria and which gives plots of η_{red} vs c which are easy to extrapolate. Stainsby *et al.* for instance, have used a rather high salt content (1M NaCl) and chosen a neutral pH to minimize hydrolysis. Pouradier's group, on the other hand, utilize the isoelectric point to secure minimal polymer-polymer interactions.

Temperature also needs close control as the solvent viscosity, which is the major part of the solution viscosity, varies sharply with temperature. The contributions of the polymer, on the other hand, are not usually very sensitive to temperature. With gelatin, however, the choice of temperature is rather limited, for two reasons. Gelatin degrades in solution, and the rate increases steeply with increase in temperature, acidity or alkalinity. On the other hand, at low temperatures, gelatin molecules link to form aggregates even in dilute solutions (see page 123). The balance between these conflicting requirements is in the region of 40°C, when aggregation is usually non-existent and thermal hydrolysis is very slow. Alternatively, neutral salts such as KSCN or LiBr should be present, in moderate concentrations, to prevent aggregation. Then lower temperatures than 40°C are permitted. Systems of this kind have been used quite extensively in the U.S.A. (e.g. Boedtger and Doty, 1954).

1. *The effects of pH and added electrolyte*

Even small variations in pH can give rise to quite marked changes in reduced (or in intrinsic) viscosity, especially at low ionic strength (see Figs. 1

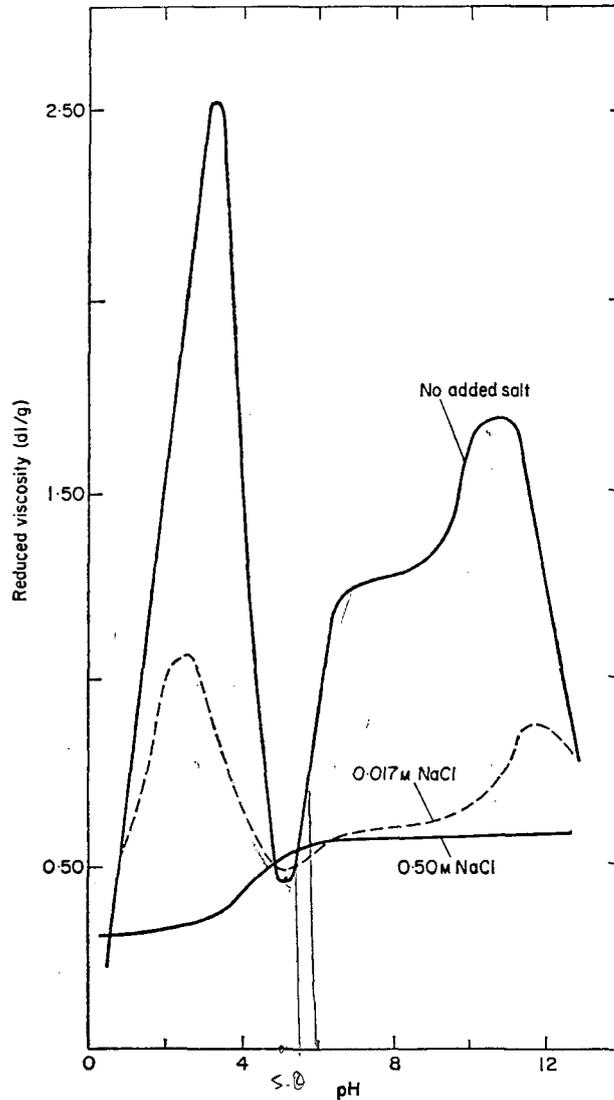


FIG. 1. The reduced viscosity of a low pI gelatin as a function of pH and salt concentration after deionization. Gelatin concentration = 0.20%. Temp. 38°C.

and 2). The reduced viscosity is a minimum at the isoionic point, and rises to a maximum at pH 3 and pH 10.5 approximately. The addition of electrolyte reduces the dependence of reduced viscosity on pH. Stainsby (1952) has

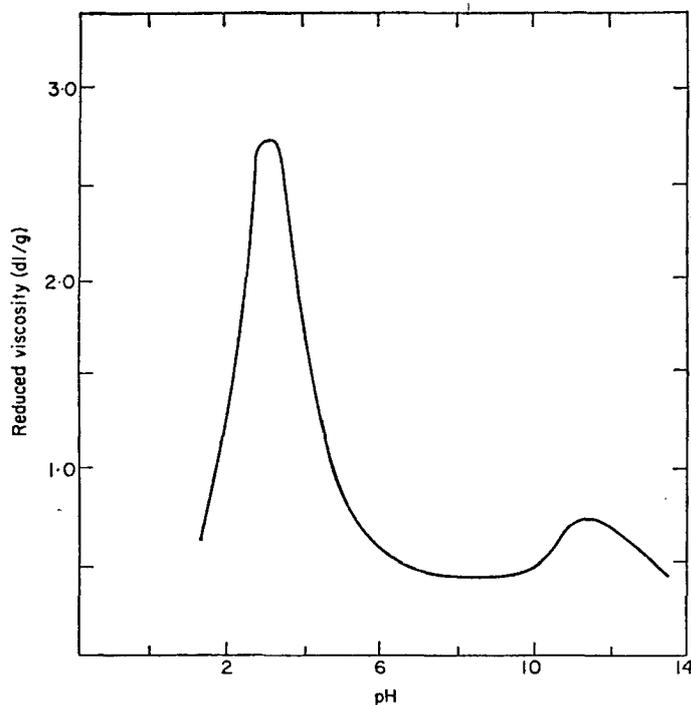


FIG. 2. Variation of reduced viscosity, at 0.20% concentration, with pH for deionized acid process pigskin gelatin. Temp. 38°C.

accounted for these results by presuming that the presence of a nett charge on a gelatin molecule gives rise to repulsive forces which are a maximum at zero ionic strength. At the isoionic point the equal numbers of positive and negative charges cause the maximum folding of the molecule. As the pH is raised or lowered the nett charge increases and the polypeptide chains unfold, giving an increase in viscosity. However, to raise or lower the pH alkali or acid must be added, and the counter ions resulting from these additions eventually become sufficiently numerous to reduce the forces between charges. At this stage the unfolding is reduced again, and viscosity falls. Addition of electrolyte similarly reduces the forces between charges. Except in the isoionic region this also leads to a fall in viscosity at any pH. At the isoionic point there is an overall attraction between the charges on each gelatin molecule: this is weakened when salts are added and a small increase in viscosity occurs.

The shape of the curve relating reduced viscosity and pH reflects the variation of nett charge with pH (though the nett charge is not usually plotted for

gelatin as it is calculable from the titration curve only when the molecular weight is known). In consequence, the curve for an acid process gelatin differs quite strikingly from that for a gelatin extracted from alkali-pretreated collagen in the range pH 6–10.

With both types of gelatin, these pH effects diminish with increasing gelatin concentration because of the increase in counter ion concentration and, with commercial samples, because of the increase in electrolyte concentration due to the inorganic ash of the sample.

2. *The effect of shear rate*

Ostwald-type viscometers have almost invariably been used to study gelatin solutions. These give non-uniform shear rates across the capillary. Only Bundenberg de Jong *et al.* (1932) appear to have investigated the dependence of viscosity on rate of shear. These authors used fairly concentrated solutions (1.93 to 16.6%) and found a negligible effect in the pH range 3.4–6.6. This is not surprising as the molecules are then in a fairly well-folded condition. Although less concentrated systems do not appear to have been investigated, it is to be expected that a significant shear dependence would only be observable when the molecules are as unfolded as possible, e.g. in very dilute solution at pH 3. The published results provide justification for the use of capillary viscometers in grading gelatins.

3. *Intrinsic viscosity and molecular weight*

As well as depending on the shape of the dissolved polymer, the intrinsic viscosity also depends on its molecular weight. For a series of chemically and structurally identical polymers the relationship has been found, empirically, to have the form $\eta_i = KM^\alpha$. The parameters K and α depend on the nature of the polymer and also on its interaction with the solvent. When the polymer is in a very compact form, as in a poor solvent, α is approximately 0.5. In better solvents α is greater than this, being typically about 0.8 to 1.0 for random coil configurations; α exceeds unity for stiff molecules, like collagen.

When the polymer sample is heterodisperse with respect to molecular weight a viscosity-average value, \bar{M}_v , is indicated by η_i . This average is identical with the weight-average value, \bar{M}_w , if $\alpha = 1$. Lower values of α make \bar{M}_v lie between \bar{M}_n and \bar{M}_w , but always closer to \bar{M}_w . It is only for monodisperse systems, such as α -gelatin or tropocollagen, that no difference exists between these average values. Gelatin fractions (see page 113) are still very polydisperse, and the results obtained using the newer methods of securing narrow distributions (e.g. gel permeation chromatography) do not appear to have been published.

Various groups of workers have determined the values of K and α for

several types of gelatin, after simple fractionation. These investigations, except for the paper by Stainsby *et al.* (1961), which was not generally available until 1969, are fully described and discussed by Veis (1964). It has to be concluded that there is no single relationship between η_i and M for all gelatins in a given solvent. Whilst some of the complexity in the results probably arises from the nature of the fractions so far used, there is still no evidence that intrinsic viscosity is uniquely related to molecular weight for gelatins of different origin, even for gelatins of the same pI. It is, therefore, dangerous to deduce a molecular weight for one gelatin, from η_i , using K and α for another. This dilemma was revealed by Pouradier and Venet (1952), who in an earlier paper (in 1950) had shown that the constants for an acid process pigskin gelatin (pI ~ 9) were substantially different from those of a low pI gelatin Kodak F-74, pI ~ 4.75 . The values of α , 0.75–0.90, reported by Pouradier and Venet are very different from those (usually 0.5–0.6) given by later workers, and there is no obvious explanation as in the early experiments—the only ones using isoionic conditions—the gelatins would be expected to be compact and tightly folded.

The results of Stainsby *et al.* (1961) make a useful start on the comprehensive and detailed survey needed to provide the information for resolving the viscosity/molecular weight relations for gelatins. Two series of gelatins were made from a single batch of ossein, one (I) after lime pretreatment and the other (II) after pretreatment with caustic soda and saturated sodium sulphate (Ward, 1953). A number of extracts were taken and each was fractionated. All the reported values of η_i and \bar{M}_w (by light-scattering) fell on a unique line which, if plotted logarithmically, gives $\alpha = 0.52$ in 0.5M saline, pH 7, 40°C. A few of the fractions (generally the first—see p. 113—and those from the 7th and 8th extracts of Series II) were too cloudy for light-scattering studies. Veis *et al.* (1960), in a limited investigation of this point using gelatins extracted mildly from unlimed purified bovine corium collagen—i.e. high pI gelatin—have reported a similar value for α , but a great deal more still remains to be done using other sources of collagen and methods of pretreatment and extraction.

Pouradier and Venet (1950) concluded also that the intrinsic viscosity of a lime pretreated gelatin was lower than that of an acid-process gelatin of the same molecular weight. Courts and Stainsby (1958), on the other hand, came to the contrary conclusion, and this is supported by Gouinlock *et al.* (1955), Veis and Cohen (1957), and by Veis *et al.* (1960), though the matter cannot be regarded as properly resolved as all the evidence so far available involves rather poor comparisons. Further work is needed and, *a priori*, it would seem unlikely that the different types of gelatin will be found to have the same relation of viscosity to molecular weight, in view of the differences in manufacture (see Chapter 10).

Molecular Weight, Size and Shape

The molecular weight of α -gelatin is now thought to be close to 90,000 (see review by Piez, 1967), but as long ago as 1950 Pouradier and Venet reported values as high as 207,000 for fractions from commercial gelatins, calculated from the osmotic pressures of isoionic solutions. Light-scattering studies (e.g. Gouinlock *et al.*, 1955) and equilibrium sedimentation experiments (e.g. Williams *et al.*, 1954) have confirmed that such high molecular weights are normal. Indeed, values of up to 10^6 have been reported (Stainsby *et al.*, 1961) for the higher fractions of some gelatins. A very full account of several of the investigations by light-scattering is given in Veis (1964), and these quite definitely establish that the gelatins were molecularly dispersed. The high molecular weights are not due, therefore, to aggregation in solution.

Courts and Stainsby (1958), by comparing the molecular weights (by light-scattering) and the average chain weight (by amino end group analysis), convincingly demonstrated that these high molecular weight fractions are multichain structures, for both lime pretreated calfskin gelatin and acid ossein gelatin. The FDNB reaction, used to determine the amino end groups, was shown not to break weak links in gelatin chains. Stainsby *et al.* (1961) have similarly demonstrated that gelatins from alkali-pretreated ossein also are multichain. It is most unlikely that polypeptide chains become linked during either main type of process of manufacture, so the multichain gelatins must retain covalent crossbonds from the original collagen. On this evidence, perhaps as many as six or seven bonds remain in low pI gelatin. Pouradier and Accary-Venet (1961) have used the titration curve to calculate the content of α -amino groups, and have concluded that there are up to four chains per molecule for a gelatin of this type.

The situation may be more complex, however, especially for acid process gelatin, for which Courts and Stainsby (1958) found the molecular weight was *lower* than the chain weight for the low viscosity fractions, and suggested that some chains do not bear terminal amino groups. Pouradier (1958) has noted a similar lack of C-terminal groups for low molecular weight acid process gelatin. It seems likely, therefore, that the end group figure provides a minimum value for the number of chains per molecule. Furthermore, the end group content itself may be too low, as the FDNB method is not very reliable for glycine, the most common N-terminal residue of gelatin. Recent studies by Grand and Stainsby (1975) give some support to this view, and suggest there are more chains per molecule than was at first thought.

The existence of highly branched molecules in gelatin samples makes it much more difficult to relate physical properties (such as viscosity, rigidity, etc.) to molecular architecture and also complicates chemical properties, such as solubility (for use in fractionation). At present these complexities

have not been unravelled, though Stainsby *et al.* (1961) suggest that intrinsic viscosity is rather insensitive to degree of branching so that viscosity and molecular weight are apparently uniquely related to one another over a very wide range.

The standard methods of polymer science have been used in attempts to obtain detailed descriptions for the sizes and shapes of gelatins in solution, but have not been very effective. The technique of light-scattering offers the best hope but, although the molecular weight can be obtained whatever the particle size, the shape can only be deduced, from the angular dependence of the scatter, if the scattering unit is a sufficiently large fraction of the wavelength of the radiation passing through the solution. When this condition is met the forward scatter is greater than the complementary backward scatter. Unfortunately, many gelatin solutions contain very small amounts of material which give intense and highly dis-symmetric scatter and it is then a major problem (see Stainsby, 1956; Tabor, 1962) to ensure that only the gelatin scatter is being measured and used to find molecular dimensions. (The extraneous scatter probably arises mostly from the clarification stage in manufacture and also from lipids and mucopolysaccharides present in the raw material, and is held in colloidal suspension by adsorbed gelatin.) Although alcohol coacervation usually concentrates most of the extraneous scatter into the least soluble fraction, tryptic digestion of the gelatin present in any solution being examined by light-scattering, so that the residual and unwanted scatter can be evaluated, is strongly to be recommended (Stainsby, 1956). The scatter then ascribed to gelatin is suspect if the residual scatter is large. Tabor (1962) has suggested that molar thiocyanate should be used as solvent in light-scattering as gelatin desorption is promoted, clarification made easier and the unwanted scatter diminished. Unfortunately these recommendations have only rarely been followed.

Weis (1964) has very usefully reviewed the situation regarding molecular dimensions of gelatins, and there seems to have been no subsequent publication. α -gelatin and some commercial gelatins (e.g. the acid process ossein gelatin used by Boedtker and Doty, 1954) give almost symmetrical scatter envelopes, and behave rather like typical synthetic aliphatic straight chain polymers (such as isobutylene) despite the partial double bond character of the peptide bonds. Fractions of calfskin gelatin, of low pI, were studied in KCNS (1M) by Gouinlock *et al.* (1955) and gave dis-symmetric scatter. Molecular dimensions were again calculated on the assumption that gelatin could be represented by a random-coil model, but this is not likely to be adequate if the molecules are multichain. The peptide chain character was taken into account, however, giving a much larger dimension for the mean end-to-end distance of the (assumed linear) chain than would be calculated for free rotations. Gelatins with a low degree of branching would be expected

to behave in solution rather like random coils, the effects of branching being undetected with the experimental techniques at present available. If the density of crosslinks is much higher, however, then the molecules should be more compact in solution than random coils. It may therefore be profitable, in future investigations, to concentrate first on a very close examination of acid process gelatins, since these appear to be more compact than alkali-precursor gelatins (Veis, 1964). It would be necessary to use only those solvents which prevent the retention of collagen-like spiral structures along parts of the polypeptide chains in order to be certain that the effects of covalent crosslinking are being revealed.

Optical Rotation

Harrington (1958) noted that the observed rotation ($[\alpha]_D^{25}$) for a warm gelatin solution is more laevorotatory than is expected on the basis of the amino-acid composition and an entirely random configuration for the polypeptide chain. He speculated that the additional laevorotation might indicate that some regions of the chain remained locked in a polyproline II-like conformation (i.e. remain collagen-like) even when the solution is well above the thermal denaturation temperature for collagen. Josse and Harrington (1964) have taken this view further, and linked the residual laevorotation with the calculated chance occurrence of sequences of the type gly-pro-pro and gly-pro-hypro. The residual rotation increases, as expected, with this probability factor and this, in turn, depends upon the amino-acid composition of the gelatin. (No allowance was made for the accumulation of imino acids in the apolar regions of each chain, in the calculations.) With a bovine hide gelatin, for instance, about 11% of all the triplets are involved in sequences of this type, provided the polypeptide chains are undegraded. The chance is halved, approximately, for codskin gelatin, due to the lower imino-acid content. At present, the calculations require confirmation by detailed sequence analyses. The figures given by Josse and Harrington (1964) need some correction, as it is not necessary to place any restriction on the location of hypro residues in triplets. For the present purposes pro and hypro are interchangeable as only pro is laid down in biosynthesis and hydroxylation takes place subsequently. When this amendment is made the proportion of imino-rich triplets rises from 11% to nearly 13% of all the triplets for undegraded bovine hide gelatin. (Putting all the imino acids in apolar regions—often taken as 65% of the chain—then raises the figure to nearly 30% imino-rich triplets.)

In addition to all the various kinds of triplets, however, there is in collagen a short telopeptide at the N-terminus and, possibly, also at the C-terminus of each chain. The composition of these appendages precludes helix forma-

tion and makes only a very minor contribution to the observed optical rotation. It is possible that these regions may only partially survive in gelatin manufacture. Their contribution to rotation, and to gelatin structure, is in any event not likely to be of major importance and has been generally ignored. It is the rest of each chain—the series of linked triplets—which needs more careful consideration. Each triplet contains nine bonds and, if no imino acids are present, only the three peptide bonds give some restriction to free rotation as a result of their partial double-bond character. Neighbouring pyrrolidine residues within the triplet increase the total number of bonds with restricted rotation to seven, and fixes the relative orientation of nine successive backbone bonds. The triplet then has the configuration of a part of a turn in the poly-L-proline II helix. Two adjacent triplets of this type, as in the sequence -gly-pro-pro-gly-pro-pro, makes two-thirds of a turn and this is thought to be the minimum requirement for conformational stability. It is from this reasoning that the link between residual collagen-like structure (gel-nucleation—Chapter 6) and the additional laevorotation stems. The presence of such stiffened regions would also be expected to affect the intrinsic viscosity and the molecular shape in solution.

Although the locked sequence, like that of poly-L-proline II, survives heating in water (below 100°C) it can be collapsed by certain salts, such as LiBr and KSCN, in high enough concentration. In this event the additional rotation vanishes, but the situation with gelatin is complicated by the observation (Harrington, 1958) that the change in rotation is much too great. The reason for excessive loss of laevorotation is still unknown.

Poly-L-proline II is stable in guanidinium chloride (GuCl) solutions. Gelatin, however, again differs as the laevorotation falls, though not as far as when LiBr is present, as Table I shows.

TABLE I. Properties of gelatin no. 127

	α_d	η_i (dl/g)
Bovine hide gelatin in 1.0 M NaCl	-144.3°	64
Bovine hide gelatin in 6 M GuCl	-104.2°	120
Similar gelatin in 8.5 M LiBr ^a	-60°	—

^a Harrington (1958).

When the residual collagen-like structure has been collapsed, through the presence of the right salt content, gelatin can be considered to be fully denatured and should behave more like a random coil. No thorough studies of this aspect are yet published, but it is of interest that the intrinsic viscosity of gelatin 127 in 6 M GuCl was almost twice the value in 1 M NaCl, at pH 7 (see Table 1). Several authors (e.g. Jirgensons, 1961; Steiner, 1964) have reported on the denaturing effect of guanidinium chloride with globular

proteins, and Nozaki and Tanford (1967) have used this salt in order to study globular proteins after first breaking disulphide crossbonds. In 6 M GuCl Tanford *et al.* (1967a) find the intrinsic viscosity, η , and the number of amino acid residues, n , to be related by the expression $\eta = 0.716 n^{0.66}$ for several globular proteins. If gelatin 127 behaves similarly in this solvent then n is calculated to be 241, i.e. the molecular weight is about 219,000, a value reasonably close to that (270,000) obtained by light-scattering.

Finally, Todd (1961) has reported small but significant differences in the values of $[\alpha_D]$ for three gelatins in solutions at 40°C. These gelatins were of closely similar viscosity (and probably of similar molecular weight but of very different rigidity factor (see Chapter 6). If the rigidity factor is related to the proportion of the locked sequences which survive in manufacture—and this view requires experimental verification—then the innate ability to form a gel should be related to the optical rotation of the sol. The subsequent parallel increase in rigidity modulus and laevorotation for cooled gels, studied by Todd, is then a measure of additional chain folding and reflects the development of regular structure from the nuclei (the locked sequences) already present in the sol (see Chapter 6).

Aggregation

Aggregation of gelatin molecules takes place in dilute solutions, which will never set to a gel, at room temperature or below. The formation of aggregates may be monitored in a qualitative manner through observation of the change in viscosity with time. Light-scattering studies, by Boedtke and Doty (1954), Beyer (1954), and Engel (1962) have been used to provide quantitative information. In this way, Boedtke and Doty (1954) were the first to show that the size of the aggregate depends not only on the temperature but also on the thermal path by which the solution is brought to the measuring temperature. Dilute solutions (0.2 g/100 ml, or less) were first clarified to remove unwanted sources of scatter, then heated for an hour to ensure a truly molecular dispersion and then quenched to 4°C and held at 4°C for one day. Some of the solutions were then, for instance, brought to 25°C and measurements taken after 18 hours at 25°C, when the scattering from small aggregates was observed. The remaining solutions were brought first to 18°C and, after 18 hours at this temperature were warmed to 25°C and held there before measurement. In these solutions the aggregates at 25°C were much larger, showing the importance of thermal history on aggregate size. Aggregates persisting at 18°C were very much larger than those which subsequently persist at 25°C.

A further, and even more important point shown by Boedtke and Doty (1954), is that the aggregates are stable to dilution at constant temperature.

Beyer (1954) confirmed that aggregates do not dissociate at constant temperature, at least over several hours. At very long times—hundreds of hours—there was some decrease in size, however. It is because of this stability that particle weights may be determined by light-scattering, from the variation of scatter intensity with concentration. Thus Boedtker and Doty (1954) were able to determine the separate effects of ionic strength and pH on aggregation at a selected temperature. For example, using a low pI ossein gelatin which had a weight-average molecular weight of 97,000 at 40°C it was shown that in 0.15 M NaCl (or in 1.0 M NaCl) at pH 5.1 and 18°C the weight-average particle weight was 5.9×10^6 , i.e. about 60 molecules per aggregate. Reduction in pH, to 3.1, led to a fall in aggregate weight to 1.65×10^6 . Deionization, leading to isoionic conditions, led to the formation of very much bigger aggregates, so that aggregation could be detected in isoionic solutions of exceptionally low concentration (0.003% gelatin).

Naturally the extent of aggregation, in a given medium, increases with gelatin concentration. At some stage, however, a complete network structure forms (i.e. the solution sets). When this happens the pattern of the light-scattering changes abruptly (see Chapter 6), but at present this transition has not been properly investigated so that the relative importance of the processes of aggregate formation and of aggregate linking cannot be evaluated.

Boedtker and Doty (1954) suggested that the best model for an aggregate was an assembly of individual gelatin molecules held together at randomly occurring "points". These "points" were thought to involve many amino acid residues and to resemble crystallites, so that the aggregate was able to resist dissociation on dilution at constant temperature. Such linked regions may now be identified with the collagen-folded zones in the gel network (see Chapter 6).

Not surprisingly, Boedtker and Doty (1954) also demonstrated by light-scattering that aggregation was prevented entirely by 0.1 M KSCN at 18°C. Unfortunately, specific ionic effects such as this have received very little attention to date, and the mechanism by which aggregation is prevented is still not understood.

Courts (1962) has studied the opposite effect—the promotion of aggregation by specific ions. By viscosity measurements it was shown that the citrate ion had a particular power to promote aggregation in dilute solutions, cooled to 20°C from 40°C, as compared with lactate and acetate ions at comparable pH and ionic strength. The viscosity increase depends quite sharply on pH, being maximal at pH 7. Using plots of relative viscosity as a function of time at 20°C Courts showed that after an initial rapid increase in viscosity over one day there was a continuing, though slower, steady development of viscosity over at least a week. With increasing time, the pH dependence becomes accentuated. The rate of growth of viscosity in the steady phase was taken as

the most useful indication of collagen-fold formation, and was found to be related to the Bloom strength of the gelatin and not to its intrinsic viscosity (or molecular weight). It would seem that these observations suggest another way of measuring the rigidity factor (see page 184) of a gelatin. The experiments provide no deeper insight, however, into the nature of this peculiar feature.

Many other chemical reagents induce linking between gelatin molecules in solution, usually through the formation of stable chemical bonds with certain side chain groups. In the presence of these reagents two kinds of bonding can often be recognized—intermolecular bonding in very dilute solutions, when different parts of the same molecule become linked and a small decrease in viscosity results, and intermolecular bonding when, through the linking of neighbouring molecules the viscosity rises with time and the system may eventually set. Formaldehyde and chrome alum are but two examples of this kind of substance. Through chemical modification of the side groups of gelatin (see Chapter 7) this effect can be extended and controlled.

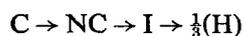
Reversion to Collagen—a Specific aggregation

Aggregation in dilute solution, under the conditions already mentioned, usually involves a partial and very limited re-forming of a collagen-like structure: total reversion of gelatin to collagen can only be achieved in special circumstances, due to the kinetic barriers which inevitably arise with all complex multichain structures. The simplest type of gelatin, the α -chain form, behaves in solution somewhat like a random-coil polymer. A collagen molecule in solution, in contrast, has a highly extended form which is maintained by co-operative non-covalent interactions between the component gelatin chains. In collagen each gelatin chain is stretched into the conformation of a poly-L-proline II helix. Such a conformation will collapse about all the α -amino residues (the bulk of the residues in gelatin) unless the structure is stabilized by interchain hydrogen bonds. The mode of stabilization is that adopted by polyglycine in the polyglycine-II structure and is possible because the glycine residues in gelatins are evenly spaced along almost the whole length of each polypeptide chain. (Other proteins, without this regular spacing for glycine, cannot utilize this method of stabilization.) Thus the structure of collagen is a composite arrangement. Neighbouring pyrrolidine residues, for steric reasons, give the conformation of a poly-L-proline II helix to a section of a gelatin chain (see above). This, with similar sections from two adjacent chains, is stabilized by a polyglycine-II structure. Additional and regular interchain hydrogen bonding at glycine residues enables the conformation of the 3-chain unit to be projected along each chain, the projection continuing across amino-acid sequences which are devoid of pyrrolidine residues (the

“band” or polar regions—see Chapter 2). The regularity of glycine in the “interband” regions, rich in pyrrolidine residues, prevents aggregation beyond the 3-chain level in each unit.

Thus, in order to regenerate the complete collagen structure the gelatin chains must come together in register and develop the specific stabilization over their entire length. The chance of this occurring is very small indeed, unless the situation is biased in favour of the collagen structure. One possible bias would be the presence of interchain covalent links to maintain registration between the chains. Highly dilute systems will also be favourable. There will be an optimal temperature, sufficiently high to encourage thermal movements of chain segments (so as to facilitate the adoption of the specific conformation) but not so high as to disrupt the rather weak interchain stabilizing forces. The optimum temperature lies several degrees below the collagen melting temperature (Beier and Engel, 1966). Solutions held in this condition are often said to be annealing. In this way the imperfect structure which is inevitably formed by chance interchain associations is improved by selectively melting out the most disordered regions and by gradually attaining more order in each unit. In addition enzymes may be used, after a period of annealing, to destroy units still containing disordered regions so that a more pure suspension of collagen may be obtained.

Kinetic studies of both fully reversible and partly reversible dilute systems have suggested that a three-step scheme is needed. (The same sequence of events is likely to be involved in the formation of a gelatin gel from a more concentrated solution. This pattern is used to account for the observations described in Chapter 6.) The kinetic scheme has been written as



where C is the largely disordered random coil state, NC is the conformation of the chain after initiation of the poly-L-proline II type of helix in sections of the chain which are particularly rich in iminoacids, I is an ordered chain intermediate having the form of a poly-L-proline II type helix along the whole chain, through growth from the nucleated regions, and $\frac{1}{3}(H)$ is the chain in the collagen state after interchain stabilization. This scheme was proposed almost simultaneously, though in somewhat differing form, by von Hippel and Harrington (1959) and Flory and Weaver (1960). During the development of these ideas the existence of small ordered regions along a gelatin chain (see page 121) was also being observed and confirmed. These regions are centres, or nuclei, from which the helix grows but at present it is not clear whether these are the only nuclei. If they are, then the first stage in this scheme is unnecessary. Attempts to determine a rate of nucleation (von Hippel and Harrington, 1961) have not been entirely satisfactory—see von Hippel (1967) for a full review. The growth phase ($NC \rightarrow I$) may be

monitored by optical rotation changes. Evidence has accumulated to favour the Flory-Weaver view that I is a transient, unstable intermediate which is transformed into the compound structure much more rapidly than it is formed. Thus the growth phase is the rate-determining step, the rate depending on the composition of the chains, their degree of crosslinking, the solvent environment and the temperature. The effects of residue sequence still require investigation, but in a qualitative way growth is expected to be slowed down as the helix spreads into regions of the chain which are rich in α -amino acids.

Using ichthyocol gelatin and calfskin parent gelatin von Hippel and Wong (1963) have shown that about 70% at most of the full collagen structure can be induced to form in very dilute solutions, as judged by optical rotation, but the melting curves are not as sharp as that for native collagen. Beier and Engel (1966), however, have convincingly demonstrated that optical rotation alone can be a misleading indication of collagen formation and that the behaviour on re-melting is a better guide, preferably supplemented by ultracentrifuge analysis and by an examination of resistance to enzymatic attack. True reformation of native collagen molecules can occur after sufficient annealing under carefully specified conditions. Otherwise this reaction is suppressed by competing and rapid formation of randomly organized aggregates. These have very high molecular weights, contain considerable pepsin-digestible non-helical parts, and have a lower thermal stability than collagen, with a broad melting profile. With increasing renaturation temperature the proportion of these aggregates diminishes, as they become no longer stable. The lowest temperature at which native molecules are formed exclusively is 26°C (in 0.25 M citrate buffer, pH 3.7) for denatured acid-soluble calfskin collagen, which contains only α and β gelatins.

Kuhn *et al.* (1964) had previously demonstrated that renaturation from α and β gelatins was improved when non-ordered aggregates were removed enzymatically. Kuhn and Zimmerman (1965) then showed that pure $\alpha 1$ components alone, from acid-soluble calfskin, could become organized first into collagen and then into SLS particles, with cross striations indistinguishable from the native collagen SLS pattern. Again, the renaturation occurs most readily upon tempering the system over a very narrow temperature range (24°–28°C in this instance). So far, no systematic study of the effects of pH or of the influences of buffer salts, such as citrate, seems to have been published.

Veis and Cohen (1956, 1958) on the other hand, had proposed that the retention of collagen crosslinks in extracted gelatins would keep the polypeptide chains in the proper register, and facilitate renaturation to the native collagen structure. After gelation and annealing, collagen fibres giving the typical banded pattern on electron micrographs were made in good yield from

a hide extract rich in γ -gelation (Veis and Cohen, 1960). This, in fact, was the first report of a successful transformation of a gelatin back to the native fibrous form of collagen. In an extension of this reasoning, Veis and Drake (1963) first deliberately added crosslinks to tropocollagen before denaturation to gelatin. When about 10 $-\text{CH}_2-$ links per collagen monomer had been added, with formaldehyde, the modified collagen melted sharply and at only a slightly higher temperature (1.4°C) than the untreated collagen, showing that the additional crossbonds had conferred very little additional stability. The presence of the additional bonds in the gelatin, however, led to an increased rate of reversion to collagen, particularly in the early stages, as judged by optical rotation only.

Veis (1967) has summarized the limited information which exists on the reversion of more normal multichain commercial gelatins, and concluded that the environment is of greater importance than for single chain gelatin, and that the overall activation energy is lower. Much more work is needed, however, before the highly complex situation—even in dilute solution—will be fully understood.

IV. CONCENTRATED SOLUTIONS

A. Viscosity

The viscosity of a concentrated solution arises mainly from the hydrodynamic interactions between the gelatin molecules, the contributions from the solvent and from the individual gelatin molecules becoming less and less important as the concentration rises, e.g. the solvent contributes less than 1% to the viscosity of a 27% solution of a high grade gelatin, and about 6% to the viscosity at 6% protein. As with dilute solutions, the main variables affecting the viscosity at a selected gelatin concentration are temperature, pH and added salts. In a concentrated solution, the charged sites on the gelatin molecules and their counterions can provide a moderately substantial ionic strength. With increasing concentration, therefore, not only are the macromolecules brought into closer proximity but the environment of each molecule (in effect, the solvent) is changed. These effects of concentration are therefore more complex and less well understood than for uncharged polymers.

1. *Temperature dependence*

As the temperature is raised above about 40°C the viscosity decreases exponentially. Croome (1953), for instance has shown that viscosity and temperature are related by an expression of the type $\text{viscosity} = A \cdot e^{K/T}$ the

term A being a function of the gelatin concentration whilst T is the absolute temperature and K is a constant.

Stainsby (1958), however, has found that the situation is rather more complex when a very wide range of samples is considered. Using 6 $\frac{2}{3}$ % solutions (as in standard testing, i.e. about 5.5% protein, depending on the moisture and ash content of the sample) the ratio viscosity at 40°C over viscosity at 60°C was found to rise steadily with increasing viscosity. This ratio is 1.39 for pure water. For very poor grade gelatins it may be as low as 1.42, whereas for high grade gelatins it reaches a value exceeding 1.61. Furthermore, with increasing gelatin concentration the ratio rises further still, but is then not so dependent on the viscosity. These results cannot be fitted to the above expression unless, for instance, K varies with temperature, gelatin concentration and viscosity. This is as expected, since K is in effect a measure of the activation energy associated with flow.

There is very little information available for highly concentrated systems. Cumper and Alexander (1952) studied the range from 15 to 48%, for a demineralized low pI gelatin, and plotted the results in the logarithmic form of the expression used by Croome. A straight line was then found, for a given concentration. Below about 43°C, however, there was an abrupt change in the slope of the graph so that the viscosity then rose more sharply with decrease in temperature. This no doubt reflects the occurrence of aggregation. Such an effect is found only rarely at the lower concentrations used in commercial testing.

When aggregation occurs the viscosity becomes time dependent, and increases continuously (see page 134). At higher temperatures the viscosity falls continuously with time, through thermal hydrolysis, and degradation of this kind can be minimized by using neutral solutions. In many applications, and especially those involving continuous production methods, gelatins having a stable viscosity are required, ideally. In practice, the rate of viscosity breakdown needs to be known and to be minimized. The rate generally increases the higher the initial viscosity so gelatins (such as acid process osseins) having inherently low viscosities are often preferred.

2. *pH dependence*

The pH effect resembles that for dilute solutions in that the viscosity is a minimum at the isoionic point and there are maxima at pH 3 and 10.5 approximately. Sheppard and Houck (1930), following the work of Bogue (1921) and Davis and Oakes (1922), showed this pH effect but found also that the shape of the viscosity-pH curve changes with the age of the solutions, particularly for acidic solutions. After 14 hours at 40°C, for example, there was no longer a maximum on the acid side of the curve. Figures 3 and 4 have been obtained using solutions which were shown by experiment to give

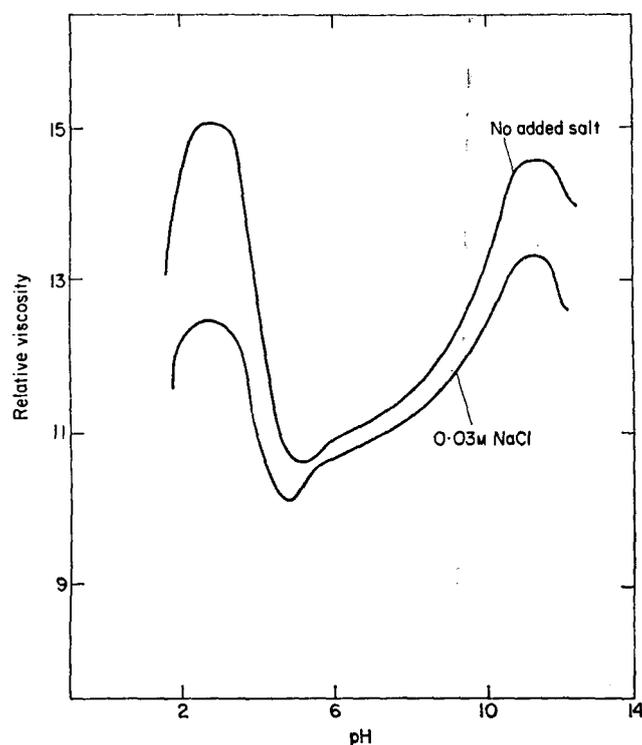


FIG. 3. Variation of viscosity with pH for low pI gelatin at $6\frac{2}{3}\%$ concentration 40°C . The gelatin was first deionised and then salt, acid and alkali added as required. The relative viscosity is taken as flow time solution over flow time solvent.

negligible hydrolysis during the determinations. The distinction between the two main types of gelatin is evident by comparing Fig. 3 and Fig. 4. As with the intrinsic viscosities the pH dependence reflects the change in net charge with pH, for each gelatin. The magnitude of the viscosity change, however, from pI to pH 4 for instance, is much less than for a dilute solution of the same gelatin.

Moreover, as the gelatin concentration is raised, the effect of pH on viscosity becomes even less marked. This is particularly noticeable for solutions on the acid side of neutrality (Fig. 4). The underlying reason for all these observations lies in the inevitably increased ionic strength of the concentrated solutions. The gelatin molecules are, in effect, added electrolytes. Furthermore, the amount of acid needed to take the solution to a chosen pH increases with gelatin concentration and the counterions, not being bound to the gelatin, add to the ionic environment. All gelatins are better buffers in the range from pH 5.5 to 3 than in the range from pH 5.5 to 10, so that the ionic strength

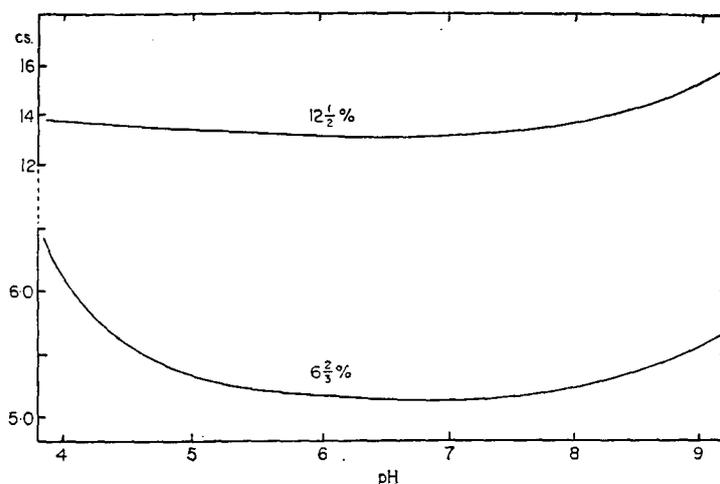


FIG. 4. Variation of viscosity with pH for acid process pigskin gelatin at 60°C. Natural pH 4-8. Measurements taken after 15 minutes at 60°C.

(arising from acid needed to reduce the pH) is greater in acidic solutions. This helps to suppress the viscosity more effectively at pH 4 than at pH 10.

3. Added electrolyte

Addition of salts decreases the viscosity of concentrated solutions of gelatin, at all values of pH (see Fig. 3, which gives the maximum effect through the use of a gelatin which had been deionized before adjusting the pH and salt content). The fall in viscosity, for a given salt content, is much less than with dilute solutions. Cumper and Alexander (1952) used a very wide range of salt concentrations. Up to an added ionic strength of 1.5 the viscosity of the gelatin solution decreased, but it then increased when more salt was added. The increase in viscosity may be due to salting-out effects, in which case the nature of the electrolyte would be important, but there seems to be no other publication on this aspect.

4. Concentration dependence

The concentration dependence of the viscosity of commercial gelatins dissolved in water has been investigated by many observers (see Ward and Saunders, 1958). All the results indicate that, to a first approximation, the viscosity is an exponential function of concentration, at least over a limited range, when $\log(\text{viscosity})$ increases linearly with gelatin content. Kragh and Langston (1959), in an extensive study of this relationship—using a wide variety of gelatins—found that the plot of $\log(\text{viscosity})$ against concentration always curved, the curvature decreasing with increasing concentration (Fig. 5). The

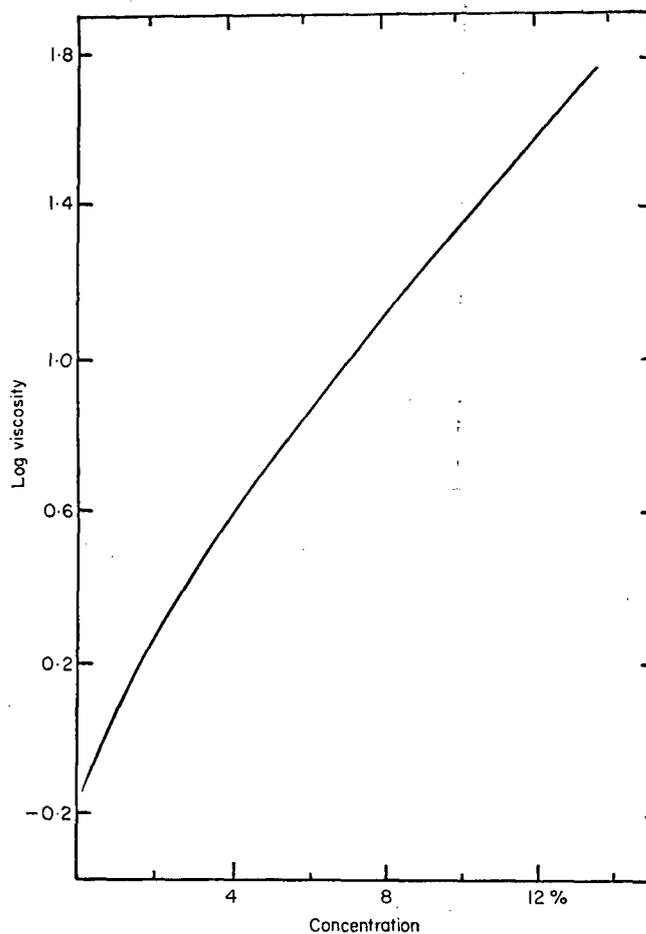


FIG. 5. Dependence of viscosity (in centistokes) on concentration for low pl gelatin at 40°C.

range of applicability of the exponential relationship, therefore, becomes larger with increasing gelatin concentration. A similar family of curves is found at 60°C. The rather complex nature of the relationship no doubt reflects the polyampholyte character of gelatin, but at present no adequate theory for the viscosity of charged macromolecules exists and the curves have only empirical significance.

For comparative purposes, in practice, it is probably the ratio of viscosity at 12½%, air dry, concentration to that at 6¼% which is most often required. This has been determined by Stainsby (1958) for a very wide variety of commercial gelatins, dissolved in distilled water. The ratio (the slope in Fig. 6)

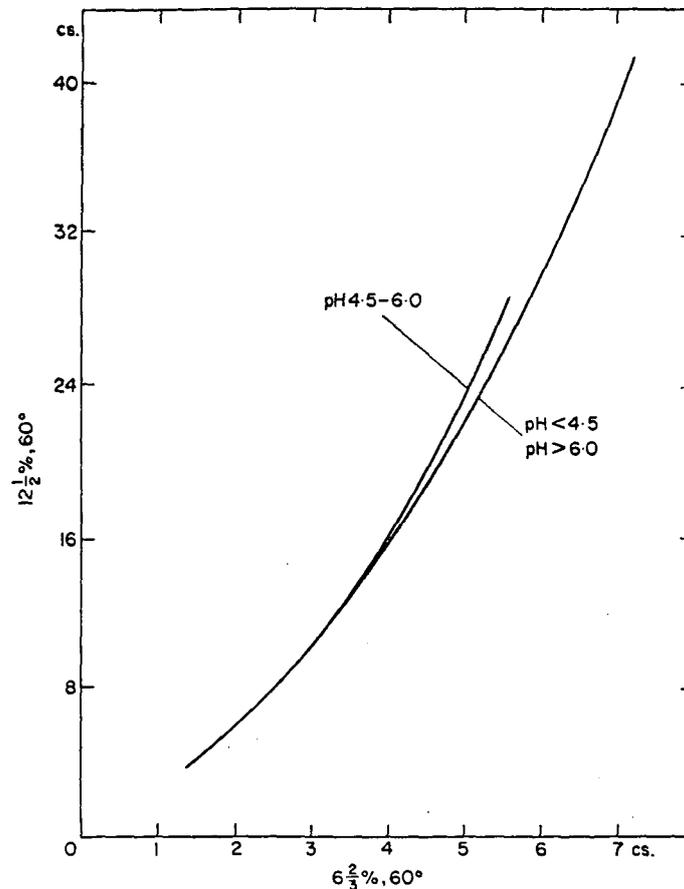


FIG. 6. The relationship of viscosity at 12½% concentration to that at 6⅔% concentration, at 60°C, for various gelatins.

increases smoothly with increase in viscosity, the precise value depending a little on pH, since the 12½% solutions show a smaller dependence of viscosity on pH than is obtained with 6⅔% solutions. (The various gelatins had different natural pH values and no adjustment of pH was made.)

For glues it is often commercial practice to use solutions at 40–50% concentrations. Wood (1968), using a range of glues, has shown that the viscosities at these high concentrations are smoothly related to the viscosities at 12½% concentration, at 60°C. At 50% concentration and 50°C, however, some high viscosity glues display visco-elasticity and the apparent “viscosity” then varies according to the method of measurement.

5. Aggregation

At temperatures above the setting temperature but below about 40°C, the viscosity can increase with time and then become non-Newtonian in character (i.e. the rate of flow depends upon the applied stress). This behaviour is due to linking together of gelatin molecules to form aggregates. It varies in extent from one gelatin sample to another and also varies with pH, salt content, gelatin concentration and temperature. Clearly it must be avoided in universal test procedures. Stainsby (1962) has indicated the main effects using viscosity ratios (e.g. $\eta_{31^\circ}/\eta_{40^\circ}$) calculated directly from flow times in a capillary viscometer (i.e. making no allowance for non-Newtonian flow). The viscosity ratio first increases quite sharply with time but then increases less and less steeply. Figure 7 shows that the extent of aggregation, as measured by the viscosity ratio after 3 hours, increases very sharply with pH over the range from 2.5 to 5.2, for a hide gelatin of low pI, and then passes through a maximum and falls slightly in the range pH 5.5 to 9.5. At higher pH values the fall is again steep but is partly due to degradation. The maximum extent of aggregation was at the natural pH of the sample (HCl or NaOH was added to give other pH values). Addition of NaCl decreases the extent of aggrega-

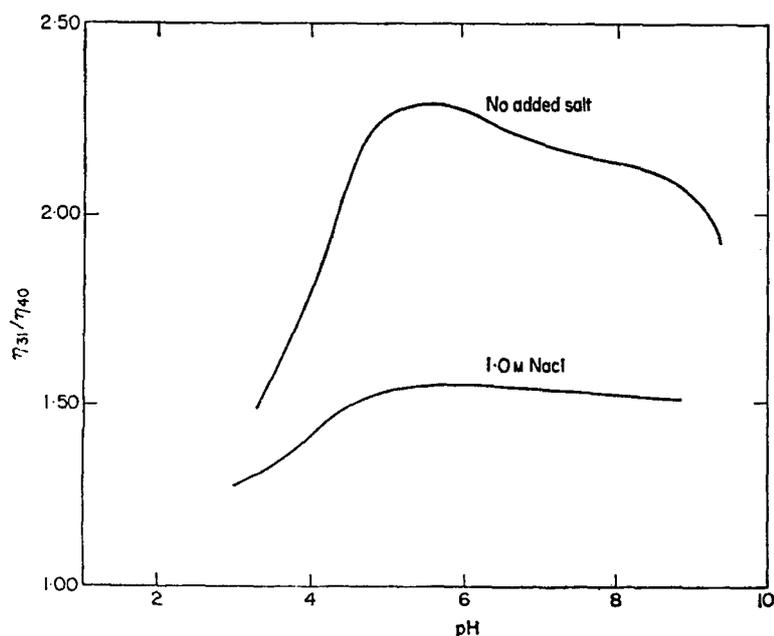


FIG. 7. Aggregation of 6.5% low pI gelatin at 31°C. Time 3 hours. (No signs of setting after 24 hours.)

tion at all pH values, the decrease being most pronounced in the region pH 5–5.5 for this gelatin.

The behaviour of an acid process pigskin gelatin was similar, the aggregate formation again being most pronounced at the natural pH (which was pH 4).

Once aggregates have been formed they do not disperse immediately on raising the temperature to 40°C, even though no aggregation occurred when the solution is cooled to and then held at 40°C. The rate of dispersion to single molecules decreases the greater the extent of aggregation, i.e. the aggregates become progressively more difficult to disrupt. After 4 hours at 31°C, for instance, dispersion was still not complete at 40°C after 1 hour. A few minutes at 60°C, however, is sufficient.

The curves shown in Fig. 7 are very similar to rigidity–pH curves, particularly for weak gels close to their melting points. If gelation involves two different processes—aggregate formation and the linking of aggregates—which overlap in time, then experiments above the normal setting point allow the formation of aggregates to be isolated from setting. Viscosimetric measurements, however, provide only a qualitative guide. Light-scattering is potentially a much better technique, but does not seem to have been used for concentrated gelatin solutions.

REFERENCES

- Beier, G. and Engel, J. (1966). *Biochemistry* **5**, 2744.
Beyer, G. L. (1954). *J. Phys. Chem.* **58**, 1050.
Boedtker, H. and Doty, P. (1954). *J. Phys. Chem.* **58**, 968.
Boedtker, H. and Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 4267.
Bogue, R. H. (1921). *J. Amer. Chem. Soc.* **43**, 764.
Bundenberg de Jong, H. G., Kruyt, H. R. and Sens, J. (1932). *Kolloid Beihefte* **36**, 429.
Courts, A. (1962). *Biochem. J.* **83**, 124.
Courts, A. and Stainsby, G. (1958). In "Recent Advances in Gelatin and Glue Research", ed. by G. Stainsby, p. 204, Pergamon Press, London.
Croome, R. J. (1953). *J. Appl. Chem.* **3**, 330.
Cumper, C. W. N. and Alexander, A. E. (1952). *Australian J. Sci. Research* **A5**, 146.
Davis, C. E. and Oakes, E. T. (1922). *J. Amer. Chem. Soc.* **44**, 464.
Engel, J. (1962). *Z. Physiol. Chem.* **328**, 94.
Flory, P. J. and Weaver, E. S. (1960). *J. Amer. Chem. Soc.* **82**, 4518.
Gouinlock, E. V., Flory, P. J. and Scheraga, H. A. (1955). *J. Polymer Sci.* **16**, 383.
Grand, R. J. A. and Stainsby, G. (1975). To be published.
Harrington, W. F. (1958). *Nature, London* **181**, 997.
Hulme, D. J. S. *et al.* (1973) *J. molec. Biol.* **79**, 137.
Jirgensons, B. (1961). *Tetrahedron* **13**, 166.
Josse, J. and Harrington, W. F. (1964). *J. molec. Biol.* **9**, 269.
Kragh, A. M. and Langston, W. B. (1959). Research Report C17, Part 3, Gelatine and Glue Research Association, London.
Kuhn, K., Engel, J., Zimmerman, B. K. and Grassman, W. (1964). *Arch. Biochem. Biophys.* **105**, 387.

- Kuhn, K. and Zimmerman, B. K. (1965). *Arch. Biochem. Biophys.* **109**, 534.
- Nozaki, Y. and Tanford, C. (1967). *J. Amer. Chem. Soc.* **89**, 736, 742.
- Pankhurst, K. G. A. and Smith, R. C. M. (1947). *Trans. Faraday Soc.* **43**, 6.
- Piez, K. A. (1967). In "Treatise on Collagen", Vol. 1, ed. by G. N. Ramachandran, p. 207, Academic Press, London.
- Pouradier, J. (1958). In "Recent Advances in Gelatin and Glue Research", ed. by G. Stainsby, p. 265, Pergamon Press, London.
- Pouradier, J. and Accary-Venet, A. M. (1961). *J. Chim. Phys.* **58**, 778.
- Pouradier, J. and Venet, A. M. (1950a). *J. Chim. Phys.* **47**, 11.
- Pouradier, J. and Venet, A. M. (1950b). *J. Chim. Phys.* **47**, 391.
- Pouradier, J. and Venet, A. M. (1952). *J. Chim. Phys.* **49**, 85 and 239.
- Sheppard, S. E. and Houck, R. C. (1930). *J. Phys. Chem.* **34**, 273.
- Stainsby, G. (1952). *Nature, London* **169**, 662.
- Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.
- Stainsby, G. (1956). *Nature, London* **177**, 745.
- Stainsby, G. (1958). Research Report C17, Part 1, Gelatine and Glue Research Association, London.
- Stainsby, G. (1962). In "Scientific Photography", ed. by H. Sauvenier, p. 253, Pergamon Press, London.
- Stainsby, G., Saunders, P. R. and Ward, A. G. (1954). *J. Polymer Sci.* **12**, 325.
- Stainsby, G., Wooton, J. W. and Ward, A. G. (1961). In "Food Science and Technology", Vol. I, ed. by J. M. Leitch. Gordon & Breach, New York, 1969.
- Steiner, R. F. (1964). *Biochem. Biophys. Acta* **79**, 51.
- Tabor, B. E. (1962). *Nature, London* **194**, 372.
- Tanford, C., Kawahara, K. and Lapanje, S. (1967). *J. Amer. Chem. Soc.* **89**, 729.
- Todd, A. (1961). *Nature, London* **191**, 567.
- Tomka, I., Spühler, A. and Bühler, K. (1972). In "Photographic Gelatin", ed. by R. J. Cox, p. 87. Academic Press, London.
- Veis, A. (1964). "The Macromolecular Chemistry of Gelatin". Academic Press, London.
- Veis, A. (1967). In "Treatise on Collagen", Vol. I, ed. by G. N. Ramachandran, p. 367, Academic Press, London.
- Veis, A., Anesey, J. and Cohen, J. (1960). *J. Amer. Leather Chem. Assoc.* **55**, 548.
- Veis, A. and Cohen, J. (1956). *J. Amer. Chem. Soc.* **78**, 6238.
- Veis, A. and Cohen, J. (1957). *J. Polymer Sci.* **26**, 113.
- Veis, A. and Cohen, J. (1958). *J. Phys. Chem.* **62**, 459.
- Veis, A. and Cohen, J. (1960). *Nature, London* **186**, 720.
- Veis, A. and Drake, M. P. (1963). *J. biol. Chem.* **238**, 2003.
- Veis, A., Eggenberger, D. N. and Cohen, J. (1955). *J. Amer. Chem. Soc.* **77**, 2368.
- von Hippel, P. H. (1967). In "Treatise on Collagen", Vol. I, ed. by G. N. Ramachandran, p. 253, Academic Press, London.
- von Hippel, P. H. and Harrington, W. F. (1959). *Biochim. Biophys. Acta* **36**, 427.
- von Hippel, P. H. and Wong, K.-Y. (1963). *Biochemistry* **2**, 1387, 1399.
- Ward, A. G. (1953). Private communication.
- Ward, A. G. and Saunders, P. R. (1958). In "Rheology, Theory and Applications", ed. F. R. Eirich, Vol. 2, p. 313, Academic Press, N.Y.
- Williams, J. W., Saunders, W. M. and Ciricelli, J. S. (1954). *J. Phys. Chem.* **58**, 774.
- Wood, P. D. (1968). Research Report A39, Gelatine and Glue Research Association, London.

Chapter 5

Relationship Between Collagen and Gelatin

P. JOHNS¹ AND A. COURTS²

Gelatin and Glue Research Association, Birmingham, England

I Introduction	138
II Preparation of Raw Material	138
III Breakage of Non-covalent Bonds	140
A. Denaturation of Soluble Collagen to Gelatin	140
B. Denaturation of Solubilized Forms of Collagen	142
C. Shrinkage	142
D. Treatment of Whole Tissue with Hydrogen Bond Breakers	144
E. Melting of Gelatin Gels	145
IV Formation of Non-covalent Bonds	146
A. Renaturation of Denatured Collagen	146
B. Formation of Fibrils	148
C. Formation of Collagen Gels	149
D. Gelation of Commercial Gelatin	150
V Mechanical Breakage of Collagen Tissue	151
VI Breakage of Intermolecular Crosslinks	151
A. Polymerized Collagen	151
B. Enzyme Solubilized Collagen	152
C. Eucollagen	153
D. Dissolution of Collagen using Thiols	155
VII Breakage of Crosslinks and of Non-covalent Bonds	156
A. Conversion to Gelatin by Conventional Processes	156
B. Preparation of Gelatin by Thermal Treatment of Collagen	156
C. Use of Acid Processes	158
D. Use of Alkali Pretreatment	161
E. Use of Autoclaving	165
F. Enzyme Methods	165
VIII Formation of Covalent Crosslinks	166
IX Breakage of Peptide Bonds	167
A. Introduction	167
B. Use of Hydrogen Peroxide	167
C. Use of Hydrazine and Hydroxylamine	168

¹ Present address: Department of Experimental Pathology, The University of Birmingham, The Medical School, Birmingham B15 2TJ.

² Present address: Imperial College of Science and Technology, London, England.

D. Use of Cyanogen Bromide	170
E. Use of Collagenase	171
F. The Effect of Radiation	171
References	173

I. INTRODUCTION

The conversion of collagen to gelatin constitutes the essential transformation which occurs in gelatin manufacture, and hence the relationship between collagen and gelatin is of critical importance in a book of this title. Since the technology of gelatin manufacture is receiving detailed attention elsewhere in this volume, it is unnecessary here to dwell in detail on the more practical aspects underlying the various stages. Hence attention will be focused on those theoretical aspects of the processes which have been clarified by recent advances made in this branch of protein science. Veis has already considered this problem in great detail in his comprehensive book *The Macromolecular Chemistry of Gelatin* (Veis, 1964). Since this was published, a certain amount of new knowledge of the interrelationship has appeared and seems ripe for discussion here. In particular structural changes and the agents causing them will be considered which are either relevant to gelatin manufacture at present, or may become relevant as new processes which are at the moment only of theoretical interest are developed to the practical stage. Thus an enzyme treatment of collagen leading to its solubilization could well be used as a process for gelatin manufacture, provided that the last traces of the enzyme could either be removed or inactivated. In addition, the section dealing with peptide bond breakage describes processes which could facilitate conventional pretreatment, or even become recognized pretreatment processes in themselves if the new technology of membrane filtration becomes economically viable.

II. PREPARATION OF RAW MATERIAL

It seems most important to consider here the treatments given to the raw material before its degradation to smaller components occurs, and to emphasize the vast difference in treatment given to raw materials in academic studies and in commercial practice. For example the cattle hide used by Veis *et al.* (1960) was washed in ice immediately after removal from the animal, the hair was removed mechanically and the pieces frozen. Subsequent treatments were all carried out at room temperature or below, under aseptic conditions which were mild enough to cause only minimal damage to the collagen. These treatments were believed to remove virtually all the non-collagenous components, while leaving the collagen intact. Although this is probably

true in the main for the non-collagenous proteins, some of the mucopolysaccharides were probably left attached to the collagen (see Chapter 2).

This must be contrasted with the treatment given to pieces of cattle hides prior to gelatin manufacture. These pieces are almost invariably obtained as by-products from tanneries. The skin can be either with hair attached, in which case preservation will have relied upon salt or, as is more usual, split pieces from unhaired and briefly limed skins are sent to the gelatin manufacturer. In the latter instance change in isoionic point due to removal of amide nitrogen by the alkali has already begun. Sulphides have been added to the liming liquors to loosen the hair and allow it to be removed easily by mechanical means. Unless the hide is carefully washed, this sulphur may remain in the gelatin as photographically active sulphur, while sulphide also causes structural changes in the collagen akin to pretreatment, due to the fact that sodium sulphide in the presence of lime produces a higher pH value than the normal (c. pH 12.6) expected for saturated calcium hydroxide. Lime treatment is useful in that it removes some of the non-collagenous components, but since at the tannery stage it is not controlled by the gelatin manufacturer, any variation in process may represent a source of considerable raw material variation.

Perhaps the most difficult hide raw material to handle is dried hide, which is sun dried under distinctly uncontrolled conditions. This material behaves as if further crosslinking of the collagen had occurred on drying, since subsequent swelling, even if assisted by detergents, never reaches the same degree regarded as normal with wet salted hides.

Pigskin on the other hand, when available for commercial use as in the U.S.A., is frozen at the slaughter house before transportation to the gelatin manufacturer. However, in order to remove the bristles the pig carcass is usually scalded in hot water after killing. Since pigs are biologically immature when slaughtered, some of the soluble pigskin collagen may be sufficiently labile to be denatured by this process. The control of the scalding temperature and time is not always exact.

Small scale experiments on bone collagen usually begin with selected ox-femur shaft from which other bone tissues (marrow, spongy bone, periosteum, etc.) have been removed mechanically. The bone is ground to a fine powder accompanied by cooling, and demineralization can then be performed in neutral solution using EDTA (Herring and Kent, 1963). Mechanical removal of the biologically active bone marrow and sponge is significant since the compact bone tissue so prepared is almost completely free of nucleic acid (bone tissue is biologically less active than bone marrow, and so has a low content of nucleic acid). In commercial preparations of bone collagen (ossein) the presence of traces of marrow probably accounts for the nucleic acid detected by Russell (1967).

Because this selective treatment is not possible in ossein manufacture, commercial ossein contains compact and spongy bone tissue, together with smaller amounts of ancillary tissues such as cartilage, marrow, and tendon. After mechanical crushing, demineralization of the bone is carried out using prolonged counter-current immersion in hydrochloric acid. This step will remove some non-collagenous material, and will convert nucleic acids to apurinic acid, a material which is very easily degraded by subsequent alkali (e.g. lime) treatment. The fact that commercial ossein is more easily prepared for conversion to gelatin by liming than is mature cattle hide may be due to the demineralization stage acting as an acid pretreatment step, as in the dual soak process (Courts, 1960).

Although tendon (sinew) is not a major raw material for gelatin manufacture, it has been used as a source of easily soluble collagen (Dumitru and Garrett, 1957) and also, in contrast, as a source of highly crosslinked mature collagen (Harding and Wesley, 1968). In the latter instance a long sequence of mild treatments was used to remove the non-collagenous material, including elastase digestion to remove most of the elastin present. Where tendon is used for gelatin manufacture it is usually obtained in the dry state (cattle tendon). It differs from dried hide in that its ability to swell is not seriously impaired by the drying conditions used in its preparation.

Collagen tissue from small mammals is of little commercial interest, although rabbit skins have been converted into high grade glues, used for instance in making violins. The convenience of handling small mammals in medical laboratories has made rat collagen in particular a widely exploited tissue for collagen structure studies. There was early recognition of the advantages of working with rat tail tendon collagen, which is almost completely soluble in pH 3.7 citrate buffer without denaturation. The low degree of crosslinking of this material reduces its resemblance to the highly cross-linked and insoluble tissue of the large mammals, as used in large scale gelatin manufacture.

III. BREAKAGE OF NON-COVALENT BONDS

A. Denaturation of Soluble Collagen to Gelatin

The simplest route from collagen to gelatin is the denaturation of soluble collagen. In this process the triple helical structure is destroyed to produce one, two or three random chain gelatin molecules. This change takes place in mild conditions either by heating at neutrality to about 40°C (Flory and Weaver, 1960) or by adding hydrogen bond breakers at room temperature or lower (Steven and Tristram, 1962). It involves breaking only the hydrogen

bonds and hydrophobic bonds which help to stabilize the collagen helix. Under these mild conditions no covalent linkages are destroyed in the times required. In more severe conditions (e.g. from 60°C upwards) some covalent bonds are broken.

The thermal denaturation process has been studied by noting changes in solution viscosity and optical rotation. The intrinsic viscosity falls from about 15 dl/g for soluble collagen to about 0.5 dl/g for the denaturation products, while the optical rotation (sodium light) falls from about -400° to about -140° during the same change (Boedtke and Doty, 1956). It has also been found that the partial specific volume rises during denaturation (Christensen and Cassel, 1967), while complex changes in the ultraviolet absorption spectra also occur (Wood, 1963; Doyle and Bello, 1968). It has proved possible to demonstrate the expected fall in molecular weight by light scattering techniques (Engel, 1962).

The thermal changes associated with denaturation have been demonstrated using differential thermal analysis (Purcell *et al.*, 1966) while the same technique has been used to calculate the entropy and enthalpy changes associated with denaturation (Privalov and Tiktopulo, 1970). These changes have been interpreted in terms of a two stage process where the first stage involves destruction of the triple helix, followed by its dissociation into small components (Engel, 1962). It has also been suggested that the water structure near the molecule changes and this plays a part in denaturation (Privalov and Tiktopulo, 1970).

The transition can be defined by a denaturation temperature (T_D) which is determined by heating aliquots of the solution of soluble collagen at a series of fixed temperatures for 30 minutes each, cooling to a fixed temperature (say 20°C) and measuring the new viscosity or optical rotation (von Hippel and Wong, 1963). By plotting the viscosity or optical rotation against the temperature of the 30 minutes heating a sharp fall is demonstrated in the denaturation region. The temperature at which 50% of this change has occurred is defined as the denaturation temperature (Doty and Nishihara, 1958).

The denaturation temperature (T_D) depends on the conditions under which the sample of a particular soluble collagen is examined. Thus at constant ionic strength, the denaturation temperature is higher at a neutral pH than at an acid pH (Dick *et al.*, 1965), while it appears that at constant pH the denaturation temperature is depressed in the presence of ionic salts (Woodlock and Harrap, 1968). The effect of organic solvents is very complex, for example methanol, ethylene glycol and propane 1:3 diol all increase the denaturation temperature while propane 1:2 diol, chlorinated alcohols and dimethyl sulphoxide all reduce it (Herbage *et al.*, 1968; Harrap, 1969).

T_D also varies according to the type of soluble collagen under consideration. It is partly related to the total imino acid content (proline + hydroxyproline)

of the collagen (von Hippel and Wong, 1963a; Rigby, 1967). For mammalian soluble collagens with a total imino content of about 225 residues/1000, T_D is about 37°C, while for fish collagens with an imino acid content of 150–200 residues/1000, T_D varies between 10°C and 30°C. However it appears that the serine and threonine content also affects the denaturation temperature (Rigby, 1967a).

The products of the denaturation of soluble collagen are now well understood since they can usually be separated into fairly homogeneous molecular species by chromatography on carboxy methyl cellulose (Piez *et al.*, 1960). However it appears that even after denaturation is complete some reassociation of the components can occur at low temperatures which persists in part in the temperature range 45–60°C (Tristram *et al.*, 1965).

B. Denaturation of Solubilized Forms of Collagen

The solubilized forms of collagen, obtained by some form of treatment of insoluble collagen, are discussed later, but mention is made here of their properties with respect to denaturation.

In the case of soluble eu collagen, its denaturation properties are analogous to those of soluble collagens except that the denaturation temperature is somewhat lower (Crosby and Stainsby, 1962; Istranov *et al.*, 1967). Thus while T_D for calf-skin soluble collagen is about 37°C at pH 7.0, T_D is about 34°C at pH 7.0 for eu collagen prepared from the same tissue.

Although it is clear that enzyme solubilized collagen can be heat denatured it appears that no actual measurements of denaturation temperature have been reported.

C. Shrinkage

Denaturation of soluble collagen is effected by mild heating (40°C) and only involves breaking non-covalent bonds. No substantial structural change is induced by heating insoluble mammalian collagen at this temperature but higher temperatures cause considerable change revealed by the physical shrinkage of the tissue. This change occurs over a small temperature range, at a temperature about 20°C higher than the denaturation temperature of the soluble collagen extracted from the same tissue (Rigby, 1967). It is interesting to note that during pretreatment of collagen prior to gelatin extraction the shrinkage temperature is reduced (Blazej and Galatik, 1966). For example when cattle skin was limed for 30 days the denaturation temperature fell from 63.8°C to 53.7°C. A pretreated collagen however still retains its hydrogen bonded helical structure, and this must be destroyed before gelatin extraction can occur. After prolonged and effective pretreatment the shrinkage temperature is about 45°C, so that shrinkage occurs during extraction of the first gelatin liquors.

In contrast to the gelatin industry which must reduce the shrinkage temperature of collagen, leather processing requires to increase it. Tanning with substances which cause chemical crosslinking (e.g. chrome complexes or glutaraldehyde) raises the shrinkage temperature, sometimes to a value greater than 100°C. The effect of vegetable tans is not as great, in agreement with the view that only secondary bonds are involved.

The methods used for measuring shrinkage temperature are varied, the simplest being to heat a piece of collagen at a uniform rate in aqueous solution and to note the temperature at which shrinkage is observed. More sophisticated methods have been devised, some of which depend on photographic recording (Vlidick and Newly, 1966), while others depend on the use of transducers to detect forces created at the shrinkage temperature (Bavinton, 1969). The latter method is often referred to as the "Isometric Tension Method" since no changes in length are allowed, but the force required to prevent shrinkage is measured.

All these methods contain some uncertainty due to the difficulty of ensuring a uniform temperature throughout the sample. By using the sensitive technique of differential thermal analysis, which requires a much smaller sample (a few milligrams) this problem can be overcome. Comparing shrinkage temperatures measured by traditional methods with those determined by DTA it was shown (Naghski *et al.*, 1966) that DTA detects satisfactorily the phase changes which cause shrinkage. Studies of epimysial and skin collagens by several techniques (McLain *et al.*, 1968) suggested that DTA is more accurate than traditional methods, and that the degree of "melting" at various temperatures in the neighbourhood of the shrinkage temperature could be determined. In particular skin collagen was found to shrink over a greater temperature range than epimysium, suggesting a greater degree of heterogeneity in the skin collagen.

Shrinkage temperatures of collagenous tissues are partly dependent on the environment, as well as on the collagen composition. As with denaturation temperature, it has been shown that the shrinkage temperature is related to the total imino acid content (Rigby, 1967). In the presence of organic solvents the shrinkage temperature changes; for example the presence of dimethyl sulphoxide increases the shrinkage temperature of human skin collagen (Russell and Winkelmann, 1966). A similar effect occurs in the presence of glycerol and ethylene glycol (Heidemann, 1964) while in the presence of alcohols, ketones and imines, the opposite occurs.

The effect of salt solutions is complex. In all cases the salt decreases the shrinkage temperature, but the extent of the fall is related to the usual lyotropic series of ions (Puett *et al.*, 1968). Indeed in the presence of lyotropic salts, as in the presence of hydrogen bond breakers of the non-ionic type (e.g. urea) the shrinkage temperature is so low that shrinkage in these solu-

tions occurs at ambient temperatures (Vlidick and Newly, 1966). Perhaps this is the best evidence that shrinkage involves disruption of those non-covalent bonds which maintain the collagen helix, involving the same changes as for soluble collagen denaturation. The loss of collagen structure on shrinkage is also demonstrated by loss of the typical collagen X-ray diffraction pattern after shrinkage in guanidine hydrochloride (Eanes and Miller, 1969). It is perhaps significant that after removal of the bond breaker from chick bone collagen the native X-ray pattern returned while in the case of rat tail tendon no native structure was recovered. This is another demonstration of differences between soft and hard tissue collagens.

The reaction of lyotropic salts on collagen has promoted a development originating at the Weizmann Institute in Israel. The shrinkage occurring when collagen fibres are treated with a hydrogen bond breaker has been harnessed and the force generated converted into energy. This direct conversion of chemical energy to mechanical energy is called a "Mechanochemical Engine" (Sussman and Katchalsky, 1970). By a pulley system a continuous band of fibres pass first into a concentrated solution of bond breaker to shrink the fibres and then into distilled water to relax the fibres. The dimensional changes produce rotary motion at the pulleys.

D. Treatment of Whole Tissue with Hydrogen Bond Breakers

Tissue as obtained usually contains a small but significant proportion of soluble collagen much of which can be removed by extraction in dilute organic acid media. The residue, by definition insoluble collagen, on treatment with hydrogen bond breakers sometimes yields appreciable quantities of soluble material, presumably previously retained as insoluble collagen by hydrogen bonds. The soluble material extracted in this way is in a denatured form and must be classed as gelatin. Thus acid extracted skin when further extracted in guanidine solution (Bornstein *et al.*, 1964) yields denatured collagen containing large amounts of β components, and in particular a β_{22} component. This must arise from a linkage between two adjacent collagen molecules since the collagen molecule contains only one α_2 chain per molecule. By related procedures Fleischmajer and Krol (1968) were able to produce a similar fraction using urea as the denaturing agent. They were able to show that the chains of the urea soluble fraction were highly crosslinked.

A more complex procedure was used by Veis *et al.* (1970). They first treated bovine corium collagen with a solution of a lyotropic salt. After this treatment a dilute acid solution caused considerable swelling, to form a product resembling "Polymerized Collagen" (q.v.), but this also solubilized 20% of the material. The soluble fraction obtained in this way was shown to be highly crosslinked.

It is perhaps significant that several authors have suggested a way of making gelatin commercially, involving a process of swelling in a hydrogen bond breaker. For example Alexandrescu and Süsser (1956) claimed increased yields over the normal liming process by soaking for 60 days in urea, and then extracting at 50–59°C without removal of the urea. More recently a high quality gelatin has been prepared from calf skin (Mitsuda *et al.*, 1967) by soaking for 1–6 days in 8–12 M urea at 35–40°C and then extracting at 80°C. However, although the yield and quality were good, the method of separating the extracted gelatin by precipitation with ethanol would certainly make the process uneconomic. It has even been claimed that gelatin can be prepared commercially from the usual materials by simply soaking in a bond breaker for up to 30 days (Grettie, 1945). While the treatment with bond breakers is of some theoretical interest it seems unlikely to be viable commercially at least until large scale dialysis and recovery units can be operated at low cost.

The results reported so far have all involved soft tissue collagens, but in 1965 Glimcher and Katz claimed that a very large proportion of young chick bone collagen could be solubilized as single chain α -components at room temperature in denaturing solutions. In particular potassium thiocyanate, guanidine thiocyanate or guanidine hydrochloride were all found to solubilize 80% of the bone collagen. Of even more interest was the claim that extraction at 100°C for 12 hours at pH 7.4 in 0.1 M sodium chloride caused complete solubilization by a similar process.

These high yields have not been confirmed by other workers. Thus Miller *et al.* (1967) applied a similar procedure to young chick bone collagen, and the guanidine treatment was only found to solubilize 14% of the collagen as against the 80% claimed by Glimcher and Katz. Again it seems unlikely that a process of this type could be used in the manufacture of ossein gelatin especially as adult cattle bone collagen is certainly more highly crosslinked than young chicken bone collagen.

E. Melting of Gelatin Gels

Gelatin gels are formed from sols when gelatin molecules are presumed partially to reform the hydrogen bonded structure of collagen, and thus the melting of gelatin gels is related to the denaturation of soluble collagens as previously discussed. But the fall in viscosity and optical rotation is rapid at or near the denaturation temperature of soluble collagen, whereas the gel rigidity changes over a wide temperature range, reducing effectively to zero at the "melting point". The optical rotation of a gel also falls steadily as the temperature of the gel rises from 0°C to the gel melting point (Todd, 1961) and no sharp changes occur. It has been considered that the change of optical rotation is a measure of the change from a helical to random coil structure

(Izmailova, 1965), but the sharp change in optical rotation which accompanies soluble collagen denaturation does not occur at the gel "melting point". The gel to sol transition is therefore perhaps due to a change in the molecular association and not solely to helix uncoiling. In the sol to gel transition Janus *et al.* (1965) infer that setting is related to crosslinking, by secondary bonds, while maturation and the further development of gel rigidity is related to the formation of the collagen helix.

Thermal changes accompanying the melting of gelatin can be measured by differential thermal analysis (Haas *et al.*, 1970). These authors detected endotherms close to the gel melting point, and also detected small exotherms just below the melting point. These exotherms were interpreted as indicating that some gel reorganization occurred just before melting. It is interesting to note that graphs of specific conductivity of gelatin gels against temperature show two breaks, one at 32°C and a second at the gel melting temperature (Rudenko and Levi, 1968), so it seems that while collagen denaturation appears to occur reasonably sharply at a well defined temperature, the melting of gelatin gels is a multistage process with many changes occurring before the physical melting point is reached.

IV. FORMATION OF NON-COVALENT BONDS

A. Renaturation of Denatured Collagen

After the denaturation of soluble collagen the components exist as random chain molecules which, at elevated temperatures or in the presence of hydrogen bond breakers, remain in an unassociated form. On cooling or removal of the hydrogen bond breaker conformational changes in the molecules occur as well as association of the molecules to re-form the collagen triple helix. There is still some dispute as to which of these processes occurs first. By studying hydrogen-deuterium exchange, Veis and Schnell (1967) were able to demonstrate that the development of hydrogen bonds occurred before conformational changes in the poly-peptide chain took place, the latter being detected by the rise in optical rotation. They propose that interchain interactions first occur, and that this is followed by formation of the collagen fold. This conflicts with earlier views (von Hippel and Harrington, 1959) that conformational changes precede the fitting together of chains involved in interchain association.

In most instances the changes in optical rotation and viscosity have been used to demonstrate renaturation—that is just those techniques which have also been used to demonstrate denaturation. The degree of renaturation can then be determined by enzyme digestion, using pepsin or trypsin to digest the

portion remaining denatured while leaving the fully renatured portion untouched. Renaturation is more rapid at lower temperatures and some non-native type associations occur which can be destroyed by heating to 22°C. Thus Grassmann (1965) recommended cycling the temperature between 4°C and 22°C to prepare the maximum amount of native type collagen. After enzyme digestion, this material showed only one peak in the ultracentrifuge and its sedimentation rate was equal to that of native collagen. Alternatively Beier and Engel (1966) maintained the temperature at 26°C to eliminate competing non-native associations and claimed that at this temperature only native molecules were formed, albeit more slowly than at lower temperatures.

The renaturation of denatured collagen can be influenced by factors other than temperature. For example it has been shown that the presence of 0.5–1.0 M urea facilitates renaturation at 15°C (Russell and Cooper, 1969). On the other hand the presence of organic solvents reduces the rate of renaturation (Russell and Cooper, 1969a), and the extent of this effect depends on solvent concentration and polarity. A reduced rate is also observed in solvents such as ethylenechlorohydrin or dimethylsulphoxide, which are known to encourage the development of the α -helix in the more common α -helix forming proteins (Kozlov *et al.*, 1969). These observations emphasize the inherent difference between the development of the α -helix in many proteins and the triple helix in collagen.

By fractionation of the products of denaturation prior to renaturation some interesting results have been obtained. Thus the rate of renaturation of β 12 molecules was independent of concentration and at low concentrations no molecular weight increase occurred (Piez and Carillo, 1964). On the other hand the renaturation of pure α_1 or α_2 chains was concentration dependent, which infers that an interchain association occurs, but in both cases the product had a much lower denaturation temperature than native collagen (20–25°C as against 38°C).

Clearly a triple helix can be assembled from three α -components, one α - and one β -component or one γ -component. It was found that γ -components renature most easily (Kühn and Zimmermann, 1965) while renaturation of three α -components is much more difficult. The third case, one α - and one β -component comes in between and so it seems that correct alignment of the chains is an important factor. By fractionating the α -components into α_1 and α_2 types it is possible to prepare collagens consisting of $(\alpha_1)_3$, $(\alpha_2)_3$ or $(\alpha_1)_2\alpha_2$, the latter representing native collagen (Tkocz and Kühn, 1969). The stability as indicated by the denaturation temperature showed $(\alpha_2)_3$ to be much less stable than $(\alpha_1)_3$ while $(\alpha_1)_2\alpha_2$ is more stable than the other combinations. Thus it appears that in some way the slight differences between α_1 and α_2 components contribute to the overall stability of the collagen molecule.

The results considered so far all involve the renaturation of the products of

denaturation of soluble collagen. This renaturation phenomenon also occurs with commercial gelatins, especially in the presence of citrate ions at 20°C (Courts, 1962). Here the viscosity rise achieved was related to the gel forming power as measured by the rigidity modulus of the gelatin in standard conditions rather than to molecular size as assessed by solution viscosity. The viscosity of the solution of renatured gelatin "molecules" approached that of native soluble collagens in favourable cases. It should be noted that the rigidity modulus is in part a measure of the ability of a gelatin to form a gel, through a hydrogen bonded structure, and the formation of hydrogen bonds also occurs in renaturation. Evidence that the chains have really assembled into collagen like molecules is provided by the preparation of collagen type fibres which are very similar in appearance to native collagen fibrils when examined by electron microscopy (Courts and Little, 1963).

B. Formation of Fibrils

Collagen molecules can assemble themselves into spatially organized structures of which the fibril is the most important. This type of association occurs extracellularly *in vivo*, but it can be induced *in vitro*, and might logically be considered as an extension of the renaturation process. Soluble collagen is normally extracted in neutral salt or dilute acid solutions. Dialysis causes the molecules to aggregate into fibrils. If the conditions are favourable the precipitated fibrils resemble native fibrils in their structure as shown by X-ray analysis and by electron microscopy they are then classified as "native type" fibrils. The special characteristic of native type fibrils is their regular 640Å spacing. However other non-native structures can be prepared by variations in the procedure resulting in different spatial arrangements (Gross *et al.*, 1954). Each form can be converted to any of the others by adjusting the conditions to first resolubilize and then to reprecipitate the molecules, so demonstrating that only weak, non-covalent bonds are involved in these structures. The form which is precipitated depends mainly on the environmental parameters existing at the time of precipitation. These parameters are pH value, ionic strength, and the presence of compounds which facilitate the precipitation of certain types of fibril (e.g. ATP, glycoprotein, or mucopolysaccharide).

As an extension of the renaturation process, Kühn *et al.* (1965) renatured $\alpha 1$ components to form a collagen molecule, $(\alpha 1)_3$, and this could be converted to SLS fibrils which were identical with SLS fibrils prepared from native soluble collagen.

A process of fibril formation is possible with commercial gelatins although native type fibrils are not produced. Courts and Little (1963) were able to prepare fibrils from renatured gelatin molecules using each of the conditions

where fibril formation of native soluble collagen occurs. By using an extract of bull hide prepared at pH 6.0 and 60°C, Veis and Cohen (1960) were able to form fibrils by heating a gel to 40°C. Under these conditions some 10% of the gelatin was precipitated in the form of fibrils. These had the native 640Å spacing and resembled fibrils prepared from neutral salt soluble collagen. Indeed this process was patented as a method of preparing commercial quantities of fibrils from gelatin (Armour and Co., 1963).

C. Formation of Collagen Gels

The collagen which constitutes the tendon in rat tail is unusual in behaviour as a component of mammalian connective tissue in that after swelling in phosphate buffer, the collagen is virtually completely soluble in cold water (Dumitru and Garrett, 1957). It is therefore most unlikely that the collagen is substantially crosslinked and it seems to be made up of pure α -chains. The same structure is likely for neutral salt soluble calfskin collagen with which Gross and Kirk (1958) have been able to demonstrate gel formation when the temperature is raised to 37°C. The gel is opaque and collagen fibrils have been detected in it.

This type of gel formation is facilitated by the presence of certain salts, particularly potassium thiocyanate (a well known lyotropic agent), but other lyotropic agents such as urea and guanidine inhibit the gel formation. Gross (1958) has demonstrated a progressive insolubilization of the gels which results from covalent crosslinks developing in the collagen.

The phenomena were studied as a function of temperature by Cassel *et al.* (1962). These authors used cold water soluble rat tail tendon collagen (Dumitru and Garrett, 1957) which was incubated at pH 7.5 at a 1% concentration at a series of temperatures ranging from 16 to 37°C. The rate of development of turbidity was used as a measure of gelation, which was much more rapid at the higher temperatures. Further by working at a fixed temperature (20°C) and varying the collagen concentration, the development of opacity was shown to occur much faster at higher concentrations. The rate was very dependent on the pH value, being a maximum at pH 5.4. At a constant pH value of 7.5 and at 23°C for various ionic strengths, the maximum rate occurred at an ionic strength of 0.6–0.8. It can be seen from these results that, apart from temperature, all the other parameters indicate a maximum rate of collagen gel formation at conditions far removed from physiological ones. Thus this process, although interesting, may perhaps only occur in *in vitro* systems so representing an artificial type of association. Also it is not clear whether the fibrils which produce the turbidity also cause the physical development of gelation, or whether gelation is due to associations which are between the fibrils themselves. If the latter is true, this type of gel

formation may be due to some partial denaturation giving the opportunity for the type of association which occurs when a gelatin solution forms a gel. There must be some doubt that hydrogen bonding as a stabilizing factor will occur at 37°C.

D. Gelation of Commercial Gelatin

The gelation of commercial gelatin is a key process in most of the uses for this protein, yet it is still imperfectly understood. There must be non-covalent molecular associations. Since hydrogen bond breaking reagents interfere or prevent gelation, hydrogen bonds must contribute to some degree to gelation. Gelation involves spatial orientation of gelation molecules, but it has been shown (Elliott, 1958) by optical rotatory dispersion that no helical structures of the α -helix type occur. From our knowledge of collagen structure with the high proportion of imino acids this is not surprising. In a range of gelatins all at standard concentration there is a relationship between the modulus of rigidity and the specific rotation of the gel (Todd, 1961). In the fully matured state the specific rotation of a high grade gelatin at 0°C approaches that of soluble collagen. This suggests that gelation involves the creation of collagen structures. Thus the gelatins showing the highest assumption of collagen structure as indicated by optical rotation were also those with the highest rigidity modulus, measured under standard conditions.

The relationship between the setting time of a sol to a gel and other parameters was studied by Janus *et al.* (1965). The time appears to be unrelated to the rigidity characteristics of the gelatin, but was more likely to be governed at least in part by the molecular weight. Molecular weight and solution viscosity of gelatins are directly related (Pouradier and Venet, 1952) but Saunders and Ward (1955) have shown that, for high molecular weight gelatins, the rigidity modulus of gels in the 0-10°C range is independent of viscosity.

Thus in brief summary, the setting of a gelatin sol to a gel depends upon the ability of molecules to aggregate while the final modulus of rigidity depends upon some factor which may be a measure of the ability of this system to reform a collagen triple helix.

It might be suggested that the gelation of commercial gelatin below the melting point and the gelation of collagen at 37°C, if the latter involves a partially denatured state, require the same random molecular association. On the other hand the fibril formation of native collagen which occurs *in vivo* in fibrogenesis and the building of fibrils in commercial gelatin solutions involves a more specific type of association.

V. MECHANICAL BREAKAGE OF COLLAGEN TISSUE

While conventional techniques for conversion of collagen to gelatin usually involves the treatment of large pieces, academic studies of collagen usually begin with the physical disintegration of the tissue. By this means reagents find easier access to the tissue and so reactions take place faster, while the degree of reaction is uniform throughout the disintegrated fragments. The disadvantage is that such small fragments present many handling problems, especially in separating solutions and residues.

It has been claimed that by prior comminution of animal hide it becomes completely extractable in dilute acid as soluble collagen (Reiss, 1964). Comminution of hide can be achieved using mechanical methods alone (Tu, 1966) or the mechanical action can be facilitated by such reagents as hydrogen peroxide (Keil and Cavanaugh, 1963), enzymes (Nichols and Oneson, 1969) or detergents (Okamura, 1969). These patents describe the preparation of collagen fibres for purposes other than gelatin conversion, but in all cases the actual comminution is achieved by mechanical means.

It is also perhaps significant that in bone handling prior to manufacture of bone glue or ossein, the bones are normally crushed mechanically into pieces typically about half an inch long.

VI. BREAKAGE OF INTERMOLECULAR CROSSLINKS

A. Polymerized Collagen

In collagenous tissue it appears that two kinds of covalent crosslinks exist—collagen to collagen crosslinks and links between collagen and the associated non-collagenous matrix. By certain techniques it is possible to destroy the latter and leave behind a very pure collagen, still retaining the collagen-collagen crosslinks. This material, called polymerized collagen can be swollen in dilute solutions of organic acids, but it does not pass into true solution as does soluble collagen. In this highly purified form it has been suggested as a useful starting material for a study of collagen crosslinks (Steven and Jackson, 1967).

Polymerized collagen was isolated from aged human achilles tendon by treating it with bacterial α -amylase (Worrall and Steven, 1966). After removal of the enzyme by washing, the collagen was swollen in dilute acetic acid. It was still highly insoluble since aqueous extraction for 1 hour at 100°C at neutral pH values gave only 2–6% in solution. By comparing the amino acid composition with that of highly purified tendon collagen it was shown to be of

high purity, and the absence of non-collagenous components was verified by electron microscopy (Steven and Jackson, 1967).

A similar material was prepared from tendon collagen by treatment with chelating agents in place of α -amylase (Steven, 1967). While EDTA was the most effective chelating agent, others such as ethylene diamine, salicylate or penicillamine could be used. In this case the interfibrillar matrix was isolated free from the collagen, suggesting that the bonds between collagen and matrix are formed by metal ions. It would also appear that this matrix is degraded by α -amylase, so explaining this alternative method of purification. Similar preparations have been made from corneal tissue (Freeman *et al.*, 1968; Bosmann and Jackson, 1968) from intervertebral disc (Steven *et al.*, 1968) and from sheepskin waste (Yates, 1968). More recently polymerized collagen has been prepared from a range of soft tissues (human bovine tendon and sclera, human skin and intestinal submucosa, and bovine cornea, aorta, and ligamentum nuchae), but perhaps significantly not from hard tissue (Grant *et al.*, 1969). This finding supports the general opinions that the intermolecular tissue structure in hard and soft tissues involves fundamentally different crosslinking systems.

B. Enzyme Solubilized Collagen

Proteolytic enzymes were used in early work to facilitate the acid solubilization of insoluble collagen. Thus an acid soluble collagen was prepared by Kühn *et al.* (1961) using acetic acid as solvent on young calf skin collagen after pepsin treatment. However mature steer hide was not so easily solubilized and Nishihara and Miyata (1962) used a process beginning with shrinkage either at 65°C or in potassium thiocyanate, followed by renaturation to leave some points for enzyme attack. After trypsin treatment the residue was dispersed in acetic acid solution in the form of a turbid aggregate which could be converted to a clear solution of molecularly dispersed collagen by pepsin treatment. This solubilized form of collagen could be reconstituted to collagen fibres and the fibres thus formed were easily converted to gelatin by heating to 60–70°C (Hikaku and Kaisha, 1963).

Many other systems have been used to solubilize collagen in a similar manner. For example trypsin alone (Nishihara, 1962), α -amylase (Nishihara, 1963), penicillium mould enzymes (Japan Leather Co., 1968) aspergillus enzymes (Nishihara, 1969), or a wide range of proteolytic enzymes from various sources (Nishihara, 1963a). In addition some methods require the presence of a detergent (Fujii, 1967) or a detergent in the presence of salts such as calcium or magnesium chloride (Japan Leather Co., 1967).

While most of these patents deal specifically with methods of solubilizing collagen for the purpose of preparing collagen fibres, collagen solutions, or

reformed collagen materials, the methods can be used to convert insoluble collagen to gelatin by subsequently denaturing the fibres. Thus the use of α -amylase (Nishihara, 1963) is specifically directed to gelatin as the end product, while gelatin was prepared after a pronase digestion in the presence of calcium chloride (Fujii, 1966). The latter gelatin had good physical properties, with an isoionic point in the range 9.15–9.4, typical of acid process pigskin gelatins. Where the raw material was calf skin the product consisted essentially of α -chains, but mature animals yielded gelatins containing significant amounts of β -components.

Although most of this work has been directed to commercial applications, the use of α -amylase was found to solubilize normal and rheumatoid joint tissue collagens (Steven, 1964). Later however Steven and his colleagues used the α -amylase technique to prepare polymerized collagen (Steven, 1965), a material already discussed.

C. Eucollagen

The transition from mature mammalian collagen to gelatin requires certain reactions at the molecular level. The following sequence is required:

1. The hydrolysis of lateral, mainly if not entirely non-peptide linkages to effect a "depolymerization" of multichain units. Some of these bonds are "acid labile" but are only slowly affected by alkali up to pH 13 although faster above this value.
2. The hydrolysis of those peptide bonds which are particularly labile and which in the main appear to involve linkages at glycine (Courts, 1954; Heyns and Legler, 1958).
3. The disorganization (melting) of the collagen triple helix by disruption of hydrogen bonding.

"Specialized" collagens such as soluble collagen and some teleostian collagens (cod skin) require only stage 3.

It was recognized however (Courts, 1961) that mature mammalian collagen subjected to stages (1) and (2) had sufficiently well defined properties to be regarded as a specialized collagen. The molecular species composing it have gelatin chain dimensions and end groups yet retain the collagen triple helix intact. It has therefore been given the separate name of eucollagen. Eucollagen is most conveniently prepared via the Ward sodium hydroxide pretreatment, originally developed as a pretreatment for gelatin preparation, and very large fractions (up to 90%) of the eucollagen can be solubilized in organic acid solutions. This solubilized material was called "soluble eucollagen" (Courts, 1961a). It has many properties in common with soluble collagen, for example high viscosity and high optical rotation, and its very high hydroxyproline content of 14.9% suggests it is a purified and modified form of collagen. On the other hand its low isionic point (pI 4.8) shows that the alkali treatment

had caused the expected loss of amide nitrogen (Crosby *et al.*, 1962). It can be prepared from hide, ossein, or tendon, or indeed any other source of collagen indicating that this material is not a product of a special kind of tissue.

Its denaturation properties have been studied and have already been discussed in Section IIIB. They confirm that the triple collagen helix structure is present in the material. Although initially it was observed that fibrils formed from soluble eu collagen by aggregation procedures were often not of native type (Crosby *et al.*, 1962), later experiments showed that native fibrils could be prepared which demonstrated the usual 640Å spacings (Higgs and Reed, 1963). Slight differences between fibrils prepared from soluble collagen and from soluble eu collagen were ascribed to different charge distributions in fibrils from the two materials, arising largely from the conversion of side-chain $-\text{CONH}_2$ groups to $-\text{COOH}$. This is consistent with the loss of amide nitrogen during the alkali pretreatment.

A Russian group working along similar lines have prepared related materials, although they used sodium chloride as the restraining salt rather than sodium sulphate (Minkin and Shestakova, 1962). More recently they have found independently of Ward (1953) that sodium sulphate is more satisfactory (Minkin *et al.*, 1963). This was confirmed by comparative tests where sodium chloride, borax, sodium sulphate, sodium thiosulphate and sodium sulphite were all tested for efficiency as the restraining salts (Steshov and Golovteeva, 1965). The properties of the solubilized material are described (Istranov *et al.*, 1967) and are quite similar to those quoted for soluble eu collagen.

Alternative methods of preparation have been proposed, the simplest being that described by Hey and Stainsby (1965), where ossein or calf skin is treated in sodium hydroxide solution at low temperature (3°C). After neutralizing the sodium hydroxide with cold citrate buffer (pH 3.1) the material was warmed to room temperature and extracted in this solution. This was claimed to give much higher yields than the sulphate process. Another alternative was less successful however (Hey and Stainsby, 1965a) where ossein was treated in alcoholic potash solutions, a reagent which is useful in the solubilization of elastin. The collagen obtained was highly degraded and the molecular weight was so small that most of it was dialysable.

An interesting procedure has been devised by Fujii (1969) by which the yield of soluble eu collagen was increased by incorporating a low concentration of methylamine or diethylamine. Up to 98% of the collagen could be solubilized, and, where amines had been incorporated, the material contained predominantly α -chains. This process has also been patented (Japan Leather Co., 1970) for the manufacture of gelatin, and it is claimed that the gelatin thus prepared is of uniform molecular weight.

Three commercial uses have been proposed for soluble eu collagen. By

reforming fibrils a leather-like material could be prepared (Ward and Stainsby, 1965), while a solution of eucollagen in commercial food acids has been used for fining beer (Courts and Johns, 1970). Another patent proposes to extrude a eucollagen dough in tubular form for sausage casings (Courts, 1965).

Attention must be drawn to another material, which has been called eucollagen, prepared from dentine (Mobbs, 1966). This was isolated from the alkali-sulphate liquors, rather than from the residue of the tissue after alkali sulphate treatment. This dentine "eucollagen" shows many properties similar to those of acid extracted soluble eucollagen, but the exact relationship between these two materials is still not clear.

D. Dissolution of Collagen Using Thiols

Another technique to solubilize collagens involves the use of thiols. In a study of the relative effectiveness of lyotropic salts as gel depressants (Courts, 1958), the high activity of thioglycolic acid was noted and it was evaluated as a reagent for solubilizing eucollagen. The reagent quantitatively dissolved eucollagen in 1 day at 18°C as a gelatin with a good gelling power. This reagent at 4 M will convert 60% of hide collagen into gelatin in 6 days at 24°C. Certain sulphidryl reactive compounds (notably thioglycollate, mercapto-ethanol, cysteamine and cysteine) reduce the tensile strength of rat tail skin at neutral pH values (Harkness and Harkness, 1966), and it was suggested that this was due to rupture of disulphide bonds present in non-collagenous components. However further work showed that insoluble rat skin collagen was solubilized in cysteamine solution at neutral pH value and low temperature (Nimni *et al.*, 1967). Cysteamine soluble collagen had many properties similar to those of neutral salt soluble collagen, and while the action appeared to be a genuine solubilizing effect, the reagents were less effective when aged tissue was used. Differences observed included a different ratio of $\alpha:\beta$ components (Nimni, 1966), while it was found that the cysteamine treated product had a higher aldehyde content, which in turn led to the development of highly crosslinked gels when the collagen was incubated at 37°C (Deshmukh and Nimni, 1968). It is interesting to note that certain of these thiols (e.g. cysteamine) are also classified as lathyrogens since they inhibit collagen crosslinking *in vivo*, so inducing a lathyrism. It has been claimed that another lathyrogen (β -amino-propionate) causes breakage of crosslinks in collagen fibrils (Fessler and Bailey, 1966). Thus it appears that this range of compounds, which includes some sulphidryl reagents and lathyrogens, either breaks specific collagen crosslinks as well as inhibiting their formation *in vivo*, or so affects the non-collagenous matrix that extraction of soluble collagen is facilitated. Any such reagents which specifically destroy collagen crosslinks may assist in the transition to gelatin.

VII. BREAKAGE OF CROSSLINKS AND OF NON-COVALENT BONDS

A. Conversion to Gelatin by Conventional Processes

While there are many processes whereby collagen can be converted to gelatin, they all have several factors in common. The intermolecular and intramolecular bonds which stabilize insoluble collagen rendering it insoluble, must be broken, and the hydrogen bonds which stabilize the collagen helix must also be broken. The latter process occurs during shrinkage of the tissue, usually due to heat. If these two processes could be made to occur in a sufficiently specific manner then gelatin would consist of a preparation of α -chains of collagen having a molecular weight of 95,000. However in conventional processing the nature of the raw materials precludes homogeneous reactivity so that during pretreatment some of the crosslinks remain intact, while some labile peptide bonds are broken. In the heating to disrupt the collagen helix, some further peptide bond breakage can occur, the extent of this being dependent on temperatures, time of heating and pH.

B. Preparation of Gelatin by Thermal Treatment of Collagen

Insoluble mature mammalian collagen can be converted to gelatin by prolonged hot water extraction alone, although the gelatin produced by this means is of little commercial value. For ossein, Manning and Schryver (1921) showed that at 90°C and at neutral pH values, up to 95% of the collagen could be extracted as a gelatin solution after about 12 hours. The actual yield was very dependent on particle size, about 3 mm diameter giving maximum yields. Where extraction was at 100°C, similar yields could be achieved in about three hours. Although the gelatin properties were not studied it may be assumed that the gelling power would be low as also would be the viscosity even by commercial standards.

A similar experiment was applied to hide collagen by Seymour-Jones (1923) where hide powder was heated at 100°C. After five hours extraction, 90% of the collagen was solubilized, but again the gelatin properties were not evaluated. More recently Ames (1944) prepared gelatin by extracting various raw materials at 80°C, and the yield and properties are shown in Table I. The yields and gel strengths confirm the view already expressed, that this type of process is of little commercial value.

A more academic approach to this problem was made by Veis *et al.* (1960, 1962), who prepared a purified bullhide corium collagen and extracted it at about its shrinkage temperature (60°C), and its isoionic pH value (pH 6.6). Under these conditions only a small proportion of the collagen was extracted

TABLE I. Gelatin prepared by extracting various collagens at 80°C at neutrality

Material	Time of extraction (hr)	Yield (%)	Gel strength	Isoelectric point
Pigskin	2	4.5	152	5.60
Ox sinew	8	11.4	115	6.75
Ossein	3½	19.3	146	5.88
Rabbit skin	2	13.7	159	5.60

(8.5%) but this contained discrete molecular components which were examined in the ultracentrifuge. The composition of the extract in terms of components designated α , β , γ and δ , is shown in Table II.

These components could be separated into relatively pure fractions by careful ethanol precipitation and the authors claim that the α -component corresponded to the α -component separable from denatured soluble collagen. The β -component, later designated γ -component, was comparable to the γ -component separable from denatured soluble collagen, and this component can be renatured to form native or SLS fibrils (Veis *et al.*, 1961). The δ -component, is probably a tetramer of the β - γ component with a high degree of crosslinking, but it is difficult to understand why no intermediate sized particles could be obtained. However, these components represent only a small fraction of the total collagen. The other 90% represents material which is more extensively crosslinked and can only be converted to gelatin after breaking the crosslinks through chemical pretreatment.

The production of bone glue is essentially a process of thermal degradation, which occurs in the normal commercial process at temperatures higher than

TABLE II. Overall composition of 60°C pH 6.6 extract, of steerhide corium collagen

Component		% of total extract	% of corium collagen	Molecular weight (i)	Molecular weight (ii)
(i)	(ii)				
α	α	27.5	2.4	135,000	
β	γ	41.3	3.5	440,000	350,000
γ	δ	21.3	1.8	1,230,000	1,440,000
		90.1	7.7		

(i) Veis *et al.* (1960); (ii) Veis *et al.* (1962).

those used by Veis *et al.* or Manning and Schryver. Whole bone is subjected to high pressure steam without any pretreatment of the bone apart from crushing and removal of extraneous material (flesh, fat, etc). During the autoclaving, during which the bone exceeds 100°C in temperature, changes in the collagen-apatite complex occur so that during subsequent extraction with water at 80°–90°C the collagen is solubilized as degraded gelatin or bone glue. This method presents a system whereby the bone collagen structure can be disrupted without prior removal of the bone salts by decalcification.

Because of its degraded form there is but little information dealing with the molecular properties of the gelatin which exists in bone glue. The physical properties of these bone glues as given by viscosity indicate that this material has suffered much more degradation than gelatins prepared by conventional liming. The low hydroxyproline content of 7.5–9.2% indicates that the gelatin content of bone glue is low (52–64%), thus a large proportion of non-gelatin components must be present (Williams, 1961).

C. Use of Acid Processes

In the acid processes used commercially, the raw materials are soaked in acid solution until the acid penetrates throughout the raw material. The period allowed is generally just sufficient to attain equilibrium, which is about one day. After decanting and adding fresh acid, extraction is carried out. The pH may then be 4.0 to 4.5. If lower, rapid adjustment after extraction is needed to prevent further degradation. Thus pretreatment, in the sense of changes occurring prior to extraction, is minimal. This type of process is substantially different from the acid extraction of limed stock since in this case some crosslinkage breakage occurs during extraction in addition to that during the lime pretreatment.

Acid processing also differs from alkaline pretreatment in that the isoionic point of the derived gelatin remains similar to that of the parent collagen. Typical isoionic points of acid processed gelatins lie in the range pH 8.0–9.4. Pigskin gelatin is invariably prepared by acid processing and so has a high isoionic point. It represents about 70% of the gelatin produced in the U.S.A. Similar isoionic points are obtained when cattle sinew or rabbit skin is acid processed (Ames, 1944). When ossein is acid processed, however, the gelatin isoionic point is much lower (pH 5.5–6.0) but this may be due to prolonged exposure to acid during demineralization. Lengthy acid pretreatment, intended to effect changes as a preparation for extraction, is not used commercially. This is a theoretically possible form of pretreatment, and it has been found (Ames, 1957) that gelatins prepared this way have properties similar in some respects to lime treatment gelatins; they have an isoelectric point of about pH 5.0, indicating loss of amide nitrogen.

There seems no reason to doubt that the high isoionic points associated with acid processed gelatins, where no alkali treatment has been given at any stage, are due to resistance to hydrolysis of the amide groups of glutamine and asparagine under acid conditions.

It has been claimed that the high isoionic point of acid processed gelatins is due to the presence of a small amount of a very basic protein (Grettie, 1965). This theory takes no account of the constancy of the isoionic point of fractions of these gelatins. The high isoionic point is a genuine property of these gelatin molecules, largely reflecting the balance of acidic and basic groups of the collagens from which they are derived.

The type of acid used appears to have a considerable effect on the products of the acid process (Reich *et al.*, 1961; Radkevich, 1966). There appears to be a relationship between the acid used, the pH which it gives and the resulting degree of swelling in the raw material on the one hand, and the yield and properties of the gelatin products on the other hand. Reich claimed that the optimum conditions were to soak the raw material overnight in acetic acid at pH 3.0, and then to wash the acid liquors away, add water and extract so that the solubilization of the collagen was effected by the acid remaining trapped in the raw material. In later work, however, the same authors used hydrochloric acid in a similar way, but this may reflect its lower price rather than any fundamental advantages.

Reich *et al.* (1962) also studied the binding of various anions to the basic groups in the raw material and its relation to the processing of pigskin. It was found that when $\frac{1}{3}$ – $\frac{1}{2}$ of the maximum anion binding capacity of the raw material had been utilized, hydrolysis of the collagen occurred readily during thermal extraction, but the gelatin was not severely degraded. It was also shown that during extraction, the acid leached into the extraction liquors was not enough to degrade extensively the gelatin already in solution. The gel forming power has been shown (Saunders and Ward, 1955) not to be affected by limited acid hydrolysis.

The effect of the age of the animal at slaughter was studied (Reich *et al.*, 1962a) and was found to be a significant factor. The raw material was treated with acetic acid as already described. Skins from pigs up to 18 months old were suitable for acid processing, while skins of pigs of 30 months old gave low yields of low quality gelatin. A similar picture emerged with cattle skin where the maximum age suitable for acid processing was 2–3 years. Thus it appears that the difference found by the gelatin industry between pigskin and cattle skin depends largely on the age of slaughter compared with the critical ages described above, rather than on any fundamental difference between the two kinds of skin. Attempts in South Africa to prepare acid process gelatins from the skins of wild pigs failed for similar reasons.

One advantage of lime pretreatment (see next section) is that the long soak in alkali removes considerable amounts of non-collagenous material from the skin. This cannot occur in the much shorter acid processing in which extractable non-collagenous components will be carried into the early liquors. This view is supported by experiments (Reich *et al.*, 1962a) where the extraction of whole pigskin was compared with that of pigskin which had been pretreated by successive extractions with water, 0.45 M sodium chloride, 10% sodium chloride and citrate buffer solution (pH 4.2). These treatments removed significant amounts of the non-collagenous components, and the resulting purified skin gave a higher yield of higher quality gelatin than the untreated skin.

A series of experiments was carried out by Küntzel *et al.* (1958) using acid as a pretreating media rather than as an aid to extraction. They pretreated cattle hide splits for up to 180 days in sulphuric acid solutions (5–20%) containing, in some cases, sodium chloride to restrict swelling, at concentrations of up to 10%. After completion of the pretreatments most of the acid was washed away and the acid remaining was neutralized to pH 7.0 with alkali. The stock was extracted for 3 hours at pH 7.0 at 65°C, and gelatins were obtained in the usual way. They had good clarity, and little tendency to foam, although they were relatively dark coloured. Where the most effective pretreatment conditions were employed, the physical properties were as good as or better than the properties of conventional lime gelatins. These optimum conditions were pretreatment for 100–150 days in 5% sulphuric acid in the presence of 5–10% sodium chloride. There seems no fundamental reason why acid pretreatment should not be employed in gelatin manufacture, although a process would not possess the ease of control provided by the limited solubility of lime and the reduction in solubility which occurs as the temperature rises.

A useful comparative study was carried out by Ames (1957) who prepared gelatin from cattle sinew by three different methods, viz.:

- (a) Conventional liming and neutral extraction
- (b) Acid pretreatment for 84 days followed by neutral extraction
- (c) A short acid soak followed by extraction in acid solution.

The properties of the acid pretreated gelatin (b) and the alkali pretreated gelatin (a) were similar, both having an isoelectric point of pH 4.8–5.0, while the acid extracted gelatin (c) was different from either, having an isoelectric point of 9.0, very similar to that of conventional acid pigskin gelatins, as would be expected from the process used.

A novel and rapid acid pretreatment process was developed for production of gelatin from a wide range of raw materials. Deutsche Gelatine Fabriken (1952) was probably the first to recognize the importance part deswelling

reagents could play in accelerated pretreatments. The raw material is heated at 80°C for 15 minutes after soaking in a solution containing sulphuric acid and sodium sulphate. Little solution takes place at this stage. After cooling the treated material is washed to remove acid and sodium sulphate and the collagen extracted under neutral conditions. This process gave high yields of high quality gelatin, but is not used commercially as far as the authors are aware. The effectiveness of the process has been verified (Ward, 1953) but it is evident that the hot pretreatment would present some difficulty in control on the large scale. The cost of this stage might also rule it out.

D. Use of Alkali Pretreatment

Lime suspension pretreatment for the manufacture of gelatin was probably the first successful method to be used for the production of high quality gelatin. In terms of yield and properties, and purity of product, it is still a very effective method for preparing gelatin from mature cattle tissue.

Although many studies of this process have been made, and many fundamental discoveries have been made, the detail of the chemical changes involved are still only partially understood. This is probably a reflection of the fact that the chemical structure of collagen crosslinks is only just becoming known (e.g. Bailey *et al.*, 1970).

It should be pointed out that while the common term "liming" has rather different meanings in the tanning and gelatin industries, both processes are relevant to gelatin production. In the tanning industry "liming" refers to a process when animal skins are treated for a short time (usually less than fourteen days, and often only one to three days) with a solution containing lime and sodium sulphide. Caustic soda and sodium sulphide may be used for the same purposes. The treatment loosens the hair and allows it to be removed by mechanical means. This tannary process has usually been applied to the cattle hides which is available for gelatin making, but not to other raw materials. "Liming" in gelatin manufacture refers to a much longer period of treatment (of the order of one to six months), when raw material is treated with a lime suspension, containing no added sulphides. This process may be accelerated by additions of sodium hydroxide, referred to as a sharpener. While there appears to be no record of the reason for lime being chosen for this purpose, any substitute would have to take account of two factors relevant to the liming process. Firstly it is very cheap and secondly the negative temperature coefficient of solubility of lime serves to control the process by reducing the alkali concentration as the temperature rises. Where, as with calf pieces, the raw material has the hair still attached, prolonged liming and washing removes the hair, but alternatively a short lime/sulphide treatment may be used.

The time of liming has a major influence on the gelatin products (Ames, 1949); this is partly because longer liming causes progressive chemical changes in the collagen, and partly because it causes destruction and removal of non-collagen components. Although limed stock is usually extracted at or near neutral pH values, extraction can be carried out at acid pH values. The extraction pH value and temperature used have a significant bearing on the yield and properties of the products, as shown in Fysh (1958). Excessive degradation in solution has to be avoided. It seems likely that after liming of cattle hide, the reduced degree of crosslinking left corresponds roughly to that of biologically younger raw material, and so the material can be rapidly converted to gelatin by acid processing. Despite the identity of the isoionic point for these gelatins with normal lime process gelatins, the structure and properties show significant differences.

The effect of the addition of sodium hydroxide to the lime suspension is to accelerate the pretreatment stage to weeks rather than months, but essentially the same physico-chemical processes are presumed to occur. In the extreme case sodium hydroxide alone can be used (Ames, 1944a) and here pretreatment is completed in a very short time, although it is accompanied by more extensive degradation and loss to the pretreatment liquors than occurs during conventional liming. Part of this extensive degradation is associated with the higher degree of swelling in sodium hydroxide solution as compared with a lime solution at the same pH value due to the substitution of Na^+ for Ca^{++} . If the degree of swelling is restrained, then the degradation is minimized, although rapid pretreatment occurs. A thorough investigation of this approach was made (Ward, 1953) which showed that sodium sulphate was the cheapest convenient salt to use. The time of alkaline treatment was easily reduced to five days and high yields of high grade gelatin obtained. While sodium thiosulphate was as effective as sodium sulphate, sodium chloride and sodium carbonate gave reduced yields. Works scale trials verified that there were no major obstacles to the use of the process on the large scale. From this work developed the studies of the preparation and properties of eucollagen (e.g. Courts, 1960). It has been applied to gelatin manufacture with apparently successful results (Babloyan, 1968).

It may be assumed, and experimental evidence of end-group analysis favours this point of view (Courts, 1960), that the various alkaline pretreatment processes are essentially similar in action in terms of chemical changes occurring during pretreatment.

Various differences occur, however, especially differences which can be attributed to shorter pretreatment times. For example, characteristics of the shortened processes are incomplete removal of non-collagenous components, fibrillation of the raw material, washing difficulties due to the fragile and swollen state of the material once the restraining salt is washed out, and

difficulties in filtration of the gelatin extracts. These effects, although important to the gelatin manufacturer, are less relevant to the study of the mechanism of pretreatment and so will not be considered in detail here.

Perhaps the most easily measured change which occurs during alkali treatment is the loss of amide nitrogen from asparagine and glutamine, with corresponding changes in the electric charge on the protein at various pHs. The isoelectric point and isoionic point of purified collagen are difficult to measure but many observers favour the value of pH 7.0 for the isoelectric point of collagen under physiological conditions (Jackson and Neuberger, 1957). This is supported by the fact that neutral extracts of four untreated tissues (pig skin, cattle sinew, cattle ossein, and rabbit skin) all had isoelectric points in the range pH 5.6–6.75 (Ames, 1944). The fall in isoelectric point during alkali treatment was related quantitatively to the loss of ammonia during conversion of amide to free acid groups (Ames, 1944b). These changes occur during cold pretreatment and not during the brief thermal extraction. This was demonstrated by Ames (1944b) who noted the ammonia formed during liming and showed that generation of ammonia during extraction is negligible. The very substantial fall in isoionic point during alkali treatment arises from the exposure of free carboxyl groups, additional to those present in collagen.

A similar effect was found when soluble collagen was treated with alkali (Zimmer and Kühn, 1963) to give "desamido-collagen". The properties of this material were similar to those of its acid soluble collagen precursor, except that its amide content and isoelectric point were lower, and it was not able to form native type fibrils. This latter deficiency was considered to be related to changed charge density along the particle surface due to the added free carboxyl groups, and suggested that when mature fibres are deamidated, their stability is impaired. In contrast soluble eucollagen extracted from mature tissue will regenerate native type fibrils but less readily than soluble collagens (p. 7). While deamidation is not regarded as the primary cause of structural change making extraction of gelatin easier arising from pre-treatment, it may have a secondary effect by reducing stability of the fibrils.

During alkali treatment changes in end groups and in the length of the polypeptide chains take place to a degree which suggests that substantial peptide bond breakage normally occurs at some stage in the transition from mature collagen to gelatin. The chain weight (i.e. "molecular" weight of the component chain) of tropo-collagen is about 95,000 and after extensive alkali treatment, corresponding to effective pretreatment, the chain weight of gelatin is 50–70,000, so the extent of hydrolysis is less than one bond per α -chain (Courts, 1960; Grassmann *et al.*, 1962). The value for number average chain weight was determined by N-terminal end group analysis, and it was evident

that about half of the end groups were glycine. Gly-gly forms a particularly weak peptide bond (Sanger, 1952) and is likely to predominate in the mild hydrolysis pretreatment conditions afforded by (a) calcium hydroxide, (b) HCl at pH 4.5 and (c) proteolytic enzymes such as papain.

Gelatin can be prepared by low temperature extraction of alkali pretreated ossein in the presence of hydrogen bond breaking reagents; such a gelatin had a chain weight of 62,000 (Courts, 1958). Thus the fall in chain weight must have occurred during the alkali treatment and not during extraction, since hydrogen bond breaking reagents at 20°C are unlikely to cause peptide bond cleavage.

The molecular weight of gelatin does not correspond to the chain weight, since a gelatin molecule can arise from parts of several constituent chains of collagen which are still covalently linked together. Gelatin molecules as highly branched chains were proposed by Courts and Stainsby (1958). This has been confirmed by Janus *et al.* (1965) who isolated gelatin fractions from commercial samples which had molecular weights greater than 1,000,000. Much of this evidence suggests that the alkali process releases multichain molecules, due to inability to hydrolyse at least some of the lateral interchain bonds effectively. The acid process, in contrast, gives a product consisting mainly of single chain molecules.

However, repeated treatment of achilles tendon collagen with a solution of sodium hydroxide and sodium sulphate was found to produce a product which corresponded closely with soluble collagen as far as behaviour after denaturation and examination in the analytical ultracentrifuge is concerned. Only α -components were detected indicating that the stronger alkali had destroyed both inter- and intramolecular crosslinks between chains without destroying the collagen triple helix (Zimmer and Kühn, 1963).

The swelling characteristics of collagen change significantly during alkali treatment so that alkali treated hide swells much more than untreated raw material (Ward, 1953; Loevern, 1954). The swelling ratio at high pH values also depends on the alkali used; in lime the degree of swelling is much less than in sodium hydroxide solution at the same pH value. (For a recent discussion see Herfeld and Schubert, 1966.) It has also been observed that sodium hydroxide is not as effective a pretreating agent as lime when both are used at the same pH value (Ames, 1944) confirming the view that efficient alkali pretreatment requires minimum swelling. Alternatively there may be some specific action of the calcium ion.

Other changes occurring during alkali treatment have been studied and in particular the number of "ester-like bonds" are found to decrease (Hörmann *et al.*, 1961). After lime treatment of cattle skin for 184 days, the number of ester-like bonds fell from 120 μ moles/g to 35 μ moles/g, but the significance of this is not obvious since the structure and chemical nature of these bonds

is not fully understood. Other changes are an increased susceptibility to digestion by trypsin and an increase in the binding capacity of chromium ions when collagen fibres are treated with lime or sodium hydroxide solutions (Okamura and Kawamura, 1967). The latter may however arise from the increase in free carboxyl groups.

Collagen fibres have been examined by electron microscopy after alkali treatment (Babloyan *et al.*, 1966), when the 640Å pattern present in native collagen fibres became more pronounced after lime or alkali sulphate treatment. In these experiments two months' treatment with a lime suspension had the same effect as one or two days' treatment with an alkali-sulphate solution. After treatment in sodium hydroxide solution (in the absence of sulphate ions) there was some loss of structure, eventually leading to complete separation of the fibrils. This evidence suggests that free sodium hydroxide solution has a degradative effect on collagen which is not shown by either lime or alkali-sodium sulphate solutions, a point which is of major practical importance when utilizing these different reagents.

E. Use of Autoclaving

The manufacture of bone glue is probably the only commercial process where high temperature pretreatment is commonly used, and even this process is fast disappearing. By treating whole bone with high pressure steam at temperatures up to 130°C, changes are induced which allow the collagenous component to be extractable in aqueous solutions at temperatures up to 100°C. The high temperature treatment modifies the chemical bonding which exists between the collagen and the bone salts and thermal shrinking is likely to have occurred. While this method of pretreatment and extraction is a cheaper process than that involving decalcification followed by pretreatment of the ossein, it has the two major disadvantages already discussed on p. 158.

Recently this type of process has been proposed for treating other collagenous tissues (Bogatyrev *et al.*, 1969). Live steam at temperatures up to 130°C was directed into a zone of raw material undergoing pulverization. The treatment time was limited to about 20 seconds, during which time the collagen was solubilized as gelatin. While this process looks promising, it cannot be fully assessed since no details of properties and composition of the product were given. Contamination with non-collagenous components is clearly one disadvantage.

F. Enzyme Methods

In the section dealing with enzyme solubilization of collagen it was shown that insoluble collagen could be solubilized by enzyme treatments and the

product heat denatured to gelatin. It is also possible to use proteolytic enzymes as a form of pretreatment in which only selected bonds are broken. The product would then require to be heated to extract the gelatin directly, with an intermediate enzyme deactivation stage. While details of such a process have not been published, it would be important in such a process to remove the last traces of the enzymes before thermal extraction. If this was not successful, the physical properties of the gelatin would be degraded by small traces of enzymes and would clearly have little practical value.

A method has been claimed (Armour, 1959) where pigskin was treated with whole living yeast, and then after washing the pigskin the gelatin was extracted in the usual way. This process appears interesting in that the enzyme system is probably non-proteolytic.

VIII. FORMATION OF COVALENT CROSSLINKS

The formation of collagen gels by incubating soluble collagen solutions at 37°C has already been discussed (Section IVC). It has been found that prolonged incubation of the gel at 37°C reduced both the rate of dissolution and the proportion of collagen which redissolved on cooling the gel. This has been interpreted in terms of the development of covalent crosslinks during incubation, a view which is supported by the fact that the matured gels are insoluble in hydrogen bond breakers. More recently (Kang *et al.*, 1970; Schiffmann and Martin, 1970) evidence for the development of chemical structures known to occur in native collagen crosslinks has been found for this type of collagen gel.

A number of factors have been shown to influence the development of crosslinking such as the presence of EDTA or the absence of oxygen (Bello and Bello, 1967), the use of lathyritic collagen (Schiffmann and Martin, 1970) or the presence of lathyrogenic agents in the collagen solution (Shimizu *et al.*, 1968). A number of these factors are probably related to the formation of aldehyde groups, together with the subsequent reaction of these groups to form covalent crosslinks. Changes in aldehyde content have been found to occur during maturation (Deshmukh and Nimni, 1969).

It is significant that these changes occurring in collagen gels are very similar to the changes thought to occur in the maturation of collagen in living tissue (Bailey *et al.*, 1970) when stabilizing crosslinks are formed. This collagen gel is a model which presents a useful *in vitro* system for studying the formation and breakage of those collagen crosslinks which are relevant to gelatin production from collagenous tissue.

IX. BREAKAGE OF PEPTIDE BONDS

A. Introduction

Studies of peptide bond cleavage using reagents not depending upon high or low pH values have been largely regarded as of academic interest only, and might at first sight seem out of place in this chapter. However, most of the reagents described here can completely solubilize collagen in the form of degraded fragments, indicating a random hydrolysis. It must remain theoretically possible that by selective hydrolysis control a partial treatment would lead to a situation where a subsequent hot water extraction could produce a material similar in characteristics to gelatin.

In two cases this has already proved to be true. Thus hydroxylamine ruptures certain "ester-like" bonds in collagen, and this reagent has been used in gelatin preparation (Zimkin *et al.*, 1965). Also hydrogen peroxide in the presence of cupric ions will solubilize cattle hide (Deasy, 1966), while a less extensive treatment has been used as a pretreatment system (Johns and Courts, 1970). The gelatin prepared by this method had good physical properties, and could be prepared from both ossein and limed splits.

Thus it is possible that a treatment which causes limited but selective peptide bond cleavage could either supplement conventional pretreatment processes, or be used as a pretreatment system in its own right.

While this section deals with peptide bond breakage, it should be pointed out that if the collagen crosslinks occur near the end of the collagen molecule, cleavage of peptide bonds adjacent to the crosslink will lead to removal of the crosslink region. Thus in these circumstances peptide bond breakage would be effective in reducing the degree of crosslinking, which is itself a stage in the pretreatment chain.

B. Use of Hydrogen Peroxide

Hydrogen peroxide, where it is used in gelatin manufacture, is usually restricted to bleaching amber coloured technical gelatins, to aiding sterilization and to destroying nucleic acids. Its use as an agent to assist in pretreatment by breaking covalent bonds is a more recent development.

Bone collagen is not solubilized at 20°C in 1.7 M- hydrogen peroxide and this system does not alter the collagen sufficiently to constitute a pretreatment stage. Extensive conversion to gelatin by this system can occur however when a lyotropic salt is added (Courts, 1961) and the conversion to a free radical mechanism was proposed. Gallop, Seifter and Meilman (1958) found that soluble collagen from carp swim bladder buffered at pH 7 was unaffected by 1.7 M peroxide at 0°C, but at 37° it dissolved completely to

give units of 10,000 molecular weight or lower. A similar reaction was observed using potassium ferricyanide (0.1 M to 1.0 M) and some oxidation of certain amino acids was suggested.

Any oxidation almost certainly relates to the severe conditions used and Gustavson (1958) cites the milder conditions provided by 0.5 M sodium periodate at 20°C, with which Ts can reduce from 65°C to 40°C with the major effect likely to be on associated hexoses.

When rat tail tendon fibres were treated with hydrogen peroxide at concentrations in the range 10^{-3} to 1.0 molar at 21°C, breakage of internal bonding led to elongation of the fibres (Pospisilova, 1966). A higher concentration was used (up to 30% w/w) by Deasy and Michele (1965) in studies of its action on mature steer hide collagen. The collagen was completely solubilized after 5 days' treatment with this reagent. In further work at the same laboratories Deasy (1966) investigated the effect of hydrogen peroxide on steerhide in the presence of various metal ions, and copper was found to be most effective in catalysing this process. It was proposed that the reaction proceeded by free radical attack, on the basis of inhibition studies by free radical scavengers. Surprisingly in this context it was found that ferrous ion was ineffective as a catalyst in this system, although it is widely used as a free radical promoter in Fenton's reagent (Larsen and Smidsrod, 1967).

An alternative mechanism has been proposed by Smejkal and Blazej (1969) who reacted calf-skin with hydrogen peroxide in the presence of copper ions. They proposed that oxidative deamination occurred where copper ions bind to collagen and that the complex reacts with atomic oxygen from the hydrogen peroxide. There was evidence of cleavage of inter- and intramolecular crosslinks and peptide bond breakage, along with a change in the contents of glutamic and aspartic acids, methionine, histidine and tyrosine.

This reaction has been used to pretreat both ossein and limed splits by using copper and peroxide concentrations which were low enough not to cause solubilization of the raw material. Extraction was carried out in the usual way after removal of the pretreating reagents (Johns and Courts, 1970). The yields of gelatin were high, and the properties were typical of good quality gelatins.

C. Use of Hydrazine and Hydroxylamine

Hydroxylamine in alkaline solution has been used as a reagent for cleavage of "ester-like" bonds for some time in both denatured soluble collagen and commercial gelatin (Gallop *et al.*, 1959), and much effort has been directed to characterizing the fragments produced by this reagent. When denatured soluble collagen was used, approximately 6 bonds per 100,000 units of molecular weight were cleaved, each bond being converted to an equivalent

amount of hydroxamic acid. The fragments had molecular weights of 34,000, 18,000 and 8,000 (Blumenfeld *et al.*, 1965), and could be separated by gel filtration into discrete fractions. Thus the reaction occurred at specific sites in the collagen molecule, and did not lead to non-specific degradation. While hydroxylamine has often been the reagent of choice, hydrazine is equally effective (Burde *et al.*, 1963), and both reagents have similar effects (Blumenfeld and Gallop, 1962).

Studies of the fragments have also been made by Heidemann and Heinrich (1970). Of the bonds affected by the hydroxylamine reaction, Butler (1969) finds that an asparagine-glycine bond is specifically cleaved. Bornstein (1969) claims that a cyclic imide is involved, but notably a cyclic imide involving aspartic acid or asparagine. Neither author finds an "ester-bond" to be necessary for hydroxylamine reaction, but rather a specific peptide bond.

In the more difficult realm of insoluble collagen Burde *et al.* (1963) studied the effect of hydrazine on cattle hide. At a concentration of 40–50% (8–10 molar) some peptide bond breakage occurred, but this did not occur in hide which had been limed, indicating that hydrazine sensitive bonds were also alkali labile. This finding agrees with that of Hörmann (1961) who found a reduction of hydroxylamine sensitive bonds during lime pretreatment. At these high concentrations only some 10% of limed or unlimed hide was solubilized, and the authors suggest that this indicates that little crosslink breakage had occurred. However, in view of the current ideas of crosslink breakage during pretreatment referred to later, this conclusion seems to be unjustified.

This reaction appears to be more effective at higher pH values, since in the presence of lime, 0.75 M hydroxylamine caused complete dissolution of insoluble collagen after 10–20 days (Hörmann, 1965). About 20% of the product was classified as tropocollagen, while the rest consisted of dialysable peptides. The tropocollagen had suffered some peptide cleavage which was mild enough not to cause uncoiling of the helix. After denaturation the product had an average molecular weight of only 50,000.

The effect of hydroxylamine on lime pretreatment was studied by Zimkin *et al.* (1965). During liming of ossein a short additional treatment in an acid solution containing 1–2 M hydroxylamine was included. This had the effect of reducing the liming time to 10–12 days. A number of different procedures were suggested, all of which reduced the time necessary to treat with lime, yet still producing high yields of good quality gelatin.

While most of this work with hydrazine or hydroxylamine has required high concentrations of reagents, it is interesting to note that at concentrations as low as 10^{-3} molar at pH values in the range pH 3.0–9.0, both hydrazine and hydroxylamine reduce the tensile strength of rat skin (Brown *et al.*, 1969). The site of reaction is not known since a change in tensile strength can be due

to changes in either the collagen or the non-collagen matrix. This kind of treatment could be commercially attractive where an accelerated pretreatment is required, and at such low concentrations the reagents are not unduly expensive.

D. Use of Cyanogen Bromide

The use of cyanogen bromide has contributed a great deal to our present knowledge of the structure and mechanism of crosslinking of collagen since it was first used (Bornstein and Piez, 1965; Nordwig and Dick, 1965). It has considerable advantages over hydroxylamine since the site of the reaction of cyanogen bromide is specific for methionyl bonds, which are cleaved in high yields with few side reactions. Typical α -chains of collagen produce 8 or 9 peptides according to the number of methionine residues present. In most cases the total number of amino acids present in the starting material can be accounted for in the fragments which are produced (Butler *et al.*, 1967). The fragments show enormous variation in molecular size, for example the $\alpha 1$ chain of rat skin collagen gave rise to fragments ranging in molecular size from 476 to 24, 581. The different size and composition of these fragments suggest that no well defined subunits exist in the chains.

Parallel studies have been applied to many other collagens with similar results, and both hard and soft tissue collagens have proved to behave similarly (Lane and Miller, 1969). The $\alpha 1$ component from chick bone behaved similarly to the $\alpha 1$ component from rat skin with this reagent, as was also true for the $\alpha 2$ components from these tissues. In both tissues the $\alpha 1$ and $\alpha 2$ components showed quite different behaviour.

By comparing fragments from $\alpha 1$ and $\beta 11$ components, it has been possible to isolate the region which is involved in the crosslinking process. Here a peptide present in the digest of $\alpha 1$ components is replaced by a fragment of about twice the size in digests of $\beta 11$ components (Bornstein and Piez, 1966). Piecing together these component studies has been of major significance in proposing a sequence of reactions which could occur during intramolecular crosslinking.

While most studies have been carried out on purified α - or β -components, cyanogen bromide is effective in degrading native soluble collagen (Bornstein *et al.*, 1966). In this instance limited cleavage occurred, principally in the region of the intramolecular crosslink, so that the product consisted of modified α -components, with no trace of β -components. Presumably this was due to the removal of the region containing the crosslink. While this treatment has not as yet been reported as applicable to insoluble collagen, if it could be used to remove the crosslinking components of mammalian collagen, the reagent suggests itself as useful in facilitating pretreatment, especially in view of its specificity. Since the reaction is carried out in acid

solution, cyanogen bromide might be considered as an adjunct to acid process liquors, or even used as a pretreatment method in its own right.

E. Use of Collagenase

While conventional proteolytic enzymes have little effect on native collagen, the family of enzymes prepared from certain clostridia bacteria causes complete destruction of collagen with conversion to small peptides. These enzymes are specific for collagen and related proteins.

During degradation of ichthyocol (a soluble fish collagen) with collagenase it was found that the viscosity falls before the optical rotation, indicating that in the early stage of the reaction, small peptides (hence lower viscosity) are produced which retain their helical structure (hence no reduction in optical rotation) (Steifter *et al.*, 1958). However after prolonged digestion, the optical rotation falls, indicating that the final peptides are too small to support the collagen helix.

The products of collagenase digestion have been examined in detail after digestion of both soluble collagen (e.g. Manahan and Mandl, 1968) and insoluble collagen (e.g. Greenberg *et al.*, 1964). With both starting materials the product consisted largely of tripeptides. End group studies were used to indicate the specificity of the enzyme giving also some ideas of the amino acid sequence in collagen.

There are certain attractions in considering enzymes for pretreatment purposes since these systems present minimum effluent disposal problems, but collagenase carries the reactions too far, apart from practical reasons against its use.

F. The Effect of Radiation

The effect of high energy radiation on both collagen and gelatin has often appeared to be contradictory since some work suggests that it induces cross-linking while other work suggests that it induces degradation. Interpretation of results needs care since various types of radiation such as γ -rays, X-rays, or ultraviolet radiation, each of which has a different energy, have been described.

Soluble rat tail tendon collagen was polymerized after ultraviolet radiation, and the viscosity of the solution increased seven-fold. The altered value for the intrinsic viscosity (51 dl/g) represented an axial ratio of 400, which is just twice that for native soluble collagen. This suggested that end to end polymerization had occurred (Fujimori, 1965). A similar effect also occurred when mouse skin (living mouse) was irradiated with ultraviolet light so that the proportions of neutral salt soluble and acid soluble collagen fell. It was suggested that these components were crosslinked in the skin (Schuster and Bottoms, 1967). These authors had shown previously that mouse skin and

kangaroo tail tendon were further crosslinked by ultraviolet light at -20°C as indicated by a decreased ability to take up water, and by local extension experiments (Bottoms *et al.*, 1966).

When ultraviolet radiation was applied to denatured soluble collagen, some degradation was found to occur and many of the fragments were not able to re-form the collagen helix when renaturation conditions were applied (Davidson and Cooper, 1967). Similar degradative effects were noted when native soluble collagen solutions were subjected to γ -radiation (Cooper and Russell, 1969) or when collagen gels were subjected to X-rays (Caneghem and Lapiere, 1967).

When either rat tail tendon or kangaroo tail tendon fibres were subjected to γ -rays from a ^{60}Co source, degradation was shown by both a fall in breaking strength and a fall in shrinkage temperature (Mohanaradhakrishnan *et al.*, 1968). It is perhaps significant to note here that a fall in shrinkage temperature is a characteristic feature of pretreatment processes.

The dosage of radiation clearly has some influence on the reaction, but the mode of influence is not clearly resolved. Thus when soluble collagen was irradiated, if the dose was less than 30K rad, degradation occurred, as shown by a fall in denaturation temperature. At higher doses, intermolecular crosslinking occurred, and at very high doses a covalently crosslinked gel was produced (Bailey, 1967). The reverse was observed by Kubota *et al.* (1969) where γ -irradiated soluble collagen was precipitated as fibres in sodium chloride solutions.

There is in fact no reason why intermolecular crosslinking and peptide bond hydrolysis should not take place simultaneously by γ -irradiation, as can be demonstrated with a gelatin sol which can show an intermediate fall in molecular weight but finish up as an insoluble crosslinked gel.

The effect of radiation is a highly complex process, with free radicals involved. It has been claimed that radiation facilitates lime pretreatment (Lowry, 1962). Splits were either limed in the usual way, or irradiated from a ^{60}Co source with 1.5×10^6 rad and limed for a shorter time. Gelatin was extracted at an acid pH value, and it was found that the irradiated splits gave a higher yield of gelatin which had a higher gel strength, viscosity and molecular weight than gelatin from the control limed splits. Thus it is evident that radiation had assisted pretreatment in some way. Prolonged exposure to radiation may be a contributory factor in ageing although final assessment must await the results of further studies.

REFERENCES

- Alexandrescu, G. and Súszer, A. (1956). *Rev. Chim. (Bucharest)* **7**, 360-7.
- Ames, W. M. (1944). *J. Soc. Chem. Ind.* **63**, 200-4.
- Ames, W. M. (1944a). *J. Soc. Chem. Ind.* **63**, 231-41.
- Ames, W. M. (1944b). *J. Soc. Chem. Ind.* **63**, 277-80.
- Ames, W. M. (1949). *J. Soc. Leather Trades Chemists* **33**, 407-21.
- Ames, W. M. (1957). *J. Sci. Food Agric.* **8**, 169-73.
- Armour and Co. (1959). *Brit. Pat.* 817,357.
- Armour and Co. (1963). *Brit. Pat.* 940,363.
- Babloyan, O. O. (1968). *Myas. Ind. S.S.S.R.* **39**, 38-40.
- Babloyan, O. O., Steshov, G. I., Istranov, L. P., Kaspar'yants, S. A. and Pereverzev, N. A. (1966). *Izv. Vysshikh Ucheba. Zavedenii Tekhnol Legkoi Prom* **92-96**.
- Bailey, A. J. (1967). *Radiation Res.* **31**, 206-14.
- Bailey, A. J., Peach, C. M. and Fowler, L. J. (1970). *Biochem. J.* **117**, 819-31.
- Bavington, J. H. (1969). *J. Amer. Leather Chemists' Assoc.* **64**, 96-100.
- Beier, G. and Engel, J. (1966). *Biochemistry* **5**, 2744-55.
- Bello, J. and Bello, H. R. (1967). *Biochim. Biophys. Acta* **147**, 272-9.
- Blazej, A. and Galatik, A. (1966). *Kozarstvi* **16**, 359-63.
- Blumenfeld, O. O. and Gallop, P. M. (1962). *Biochemistry* **1**, 947-59.
- Blumenfeld, O. O., Rojkind, M. and Gallop, P. M. (1965). *Biochemistry* **4**, 1780-8.
- Boedtker, H. and Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 4267-80.
- Bogatyrev, A. N., Gaevoi, E. V., Kovalenko, L. V., Morozov, V. M., and Radkevich, D. P. (1969). *U.S.S.R. Pat.* 237,308.
- Bornstein, P. (1969). *Biochem. Biophys. Res. Comm.* **36**, 957-64.
- Bornstein, P. and Piez, K. A. (1965). *Science* **148**, 1353-5.
- Bornstein, P. and Piez, K. A. (1966). *Biochemistry* **5**, 3460-73.
- Bornstein, P., Martin, G. R. and Piez, K. A. (1964). *Science* **144**, 1220-1.
- Bornstein, P., Kang, A. H. and Piez, K. A. (1966). *Biochemistry* **5**, 3803-12.
- Bottoms, E., Cater, C. W. and Schuster, S. (1966). *Nature, Lond.* **211**, 97-8.
- Brown, L., Harkness, M. L. R. and Harkness, R. D. (1969). *Acta Physiol.* **36**, 157-69.
- Burde, R. de La., Peckham, L. and Veis, A. (1963). *J. Biol. Chem.* **238**, 189-97.
- Butler, W. T. (1969). *J. Biol. Chem.* **244**, 3415-7.
- Butler, W. T., Piez, K. A. and Bornstein, P. (1967). *Biochemistry* **6**, 3371-80.
- Caneghem, P. Van and Lapiere, C. M. (1967). *C. R. Soc. Biol.* **161**, 2311-4.
- Cassel, J. M., Mandelkern, L. and Roberts, D. E. (1962). *J. Amer. Leather Chemists' Assoc.* **57**, 556-575.
- Christiensen, R. G. and Cassel, J. M. (1967). *Biopolymers* **5**, 685-9.
- Cooper, D. A. and Russell, A. E. (1969). *Biochem. J.* **113**, 263-9.
- Courts, A. (1954). *Biochem. J.* **58**, 70.
- Courts, A. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 145-8. Pergamon Press, London.
- Courts, A. (1960). *Biochem. J.* **74**, 238-47.
- Courts, A. (1961). *Biochem. J.* **81**, 356-65.
- Courts, A. (1961a). *Nature, Lond.* **191**, 1097.
- Courts, A. (1962). *Biochem. J.* **83**, 124-9.
- Courts, A. (1965). *Brit. Pat.* 1,082,852.
- Courts, A. and Johns, P. (1970). *Brit. Pat.* 1, 184, 502.
- Courts, A. and Little, K. (1963). *Biochem. J.* **87**, 383-6.

- Courts, A. and Stainsby, G. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 100. Pergamon Press, London.
- Crosby, N. T. and Stainsby, G. (1962). *Research* **15**, 427-35.
- Crosby, N. T., Higgs, D. G., Reed, R., Stainsby, G. and Ward, A. G. (1962). *J. Soc. Leather Trades Chemists* **46**, 152-61.
- Davidson, R. J. and Cooper, D. R. (1967). *Biochem. J.* **105**, 965-9.
- Deasy, C. L. (1966). *J. Amer. Leather Chemists' Assoc.* **61**, 258-69.
- Deasy, C. L. and Michele, S. C. (1965). *J. Amer. Leather Chemists' Assoc.* **60**, 665-74.
- Deshmukh, K. and Nimni, M. E. (1968). *Biochim. Biophys. Acta* **154**, 258-60.
- Deshmukh, K. and Nimni, M. E. (1969). *Biochem. J.* **112**, 397-405.
- Deutsch Gelatine-Fabriken (1952). *Brit. Pat.* 682,829.
- Dick, Y. P., Engel, J. and Nordwig, A. (1965). Abs. Fed. Eur. Biochem. Soc. Vienna, 2nd. p. 215.
- Doty, P. and Nishihara, T. (1958) "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 92. Pergamon Press, London.
- Doyle, R. J. and Bello, J. (1968). *Biochem. Biophys. Res. Comm.* **31**, 869-76.
- Dumitru, E. T. and Garrett, R. R. (1957). *Arch. Biochem. Biophys.* **66**, 245-7.
- Eanes, E. D. and Miller, E. J. (1969). *Arch. Biochem. Biophys.* **129**, 769-71.
- Elliott, A. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 267-8. Pergamon Press, London.
- Engel, J. (1962). *Arch. Biochem. Biophys.* **97**, 150-8.
- Fessler, J. H. and Bailey, A. J. (1966). *Biochim. Biophys. Acta* **117**, 368-78.
- Fleischmajer, R. and Krol, S. (1968). *Proc. Soc. Exptl. Biol. Med.* **128**, 1061-5.
- Flory, P. J. and Weaver, E. S. (1960). *J. Amer. Chem. Soc.* **82**, 4518-25.
- Freeman, I. L., Steven, F. S. and Jackson, D. S. (1968). *Biochim. Biophys. Acta* **154**, 252-4.
- Fujii, T. (1966). *Bull. Soc. Sci. Phot. Japan No. 16*, 30-7.
- Fujii, T. (1967). *U.S. Pat.* 3,314,861.
- Fujii, T. (1969). *Hoppe-Seylers' Z. Physiol. Chem.* **350**, 1257-65.
- Fujimori, E. (1965). *Biopolymers* **3**, 115-9.
- Fysh, D. (1958) "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 140-144. Pergamon Press, London.
- Gallop, P. M., Seifter, S. and Meilman, E. (1959). *Nature, Lond.* **183**, 1659-61.
- Gallop, P. M., Seifter, S. and Meilman, E. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 82-6. Pergamon Press, London.
- Glimcher, M. J. and Katz, E. P. (1965). *J. Ultrastruct. Res.* **12**, 705-29.
- Grassmann, W. (1965). *Das Leder* **16**, 32-8.
- Grassmann, W., Riedel, A. and Altenschöpfer, T. (1962). *Kolloid, Z.* **186**, 50-7.
- Grant, M. E., Freeman, I. L., Schofield, J. D. and Jackson, D. S. (1969). *Biochim. Biophys. Acta* **177**, 682-5.
- Greenberg, J., Fishman, L. and Levy, M. (1964). *Biochemistry* **3**, 1826-31.
- Grettie, D. P. (1945). *U.S. Pat.* 2,384,673.
- Grettie, D. P. (1965). *J. Amer. Leather Chemists' Assoc.* **60**, 572-80.
- Gross, J. (1958). *Nature, Lond.* **181**, 556.
- Gross, J., Highberger, J. H. and Schmitt, F. O. (1954). *Proc. Natl. Acad. Sci. (U.S.)* **40**, 679-88.
- Gustavson, K. H. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 259. Pergamon Press, London.
- Haas, H. C., Manning, M. J. and Mach, M. H. (1970). *J. Polym. Sci. Part A-1* **8**, 1725-30.

- Harding, J. J. and Wesley, J. M. (1968). *Biochem. J.* **106**, 749-57.
- Harkness, M. L. R. and Harkness, S. R. D. (1966). *Nature, Lond.* **211**, 496-7.
- Harrap, B. S. (1969). *Int. J. Protein Chem.* **1**, 245-52.
- Heidemann, E. (1964). *Das Leder* **15**, 148.
- Heidemann, E. and Heinrich, W. (1970). *Eur. J. Biochem.* **14**, 61-9.
- Herbage, D., Hanus, A. and Valet, G. (1968). *Biochim. Biophys. Acta* **168**, 544-54.
- Herfeld, H. and Schubert, B. (1966). *Das Leder* **17**, 25-35.
- Herring, G. M. and Kent, P. W. (1963). *Biochem. J.* **89**, 405-14.
- Hey, C. D. and Stainsby, G. (1965). *Biochim. Biophys. Acta* **97**, 364-6.
- Hey, C. D. and Stainsby, G. (1965a). *Biochim. Biophys. Acta* **100**, 596-7.
- Heyns, K. and Legler, G. (1958) "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 186. Pergamon Press, London.
- Higgs, D. G. and Reed, R. (1963). *Biochim. Biophys. Acta* **78**, 265-77.
- Hikaku, N. and Kaisha, K. (1963). *Brit. Pat.* 929,137.
- Hörmann, H. (1965). In "Structure and Function of Connective and Skeletal Tissue" (G. R. Tristram and S. Fitton-Jackson, eds), pp. 48-51. Butterworth, London.
- Hörmann, V. H., Riedel, A., Altenschöpfer, Th. and Klenk, M. (1961). *Das Leder* **12**, 175-80.
- Istranov, L. P., Baramboim, N. K. and Chernov, N. V. (1967). *Nauchn. Tr. Mosk. Tekhnol. Inst. Legkoi Prom* **33**, 20-6.
- Izmailova, V. N., Pchelin, V. A. and Ali, S. A. (1965). *Vysokomolekul* **7**, 1985-8.
- Jackson, D. S. and Neuberger, A. (1957). *Biochim. Biophys. Acta* **26**, 638-9.
- Janus, J. W., Tabor, B. E. and Darlow, R. L. R. (1965). *Kolloid Z.* **205**, 134-9.
- Japan Leather Co. (1967). *Brit. Pat.* 1,062,083.
- Japan Leather Co. (1968). *Brit. Pat.* 1,119,342.
- Japan Leather Co. (1970). *Brit. Pat.* 1,192,314.
- Johns, P. and Courts, A. (1970). Unpublished Results.
- Kang, A. H., Faris, B. and Franzblau, C. (1970). *Biochem. Biophys. Res. Comm.* **39**, 175-82.
- Keil, H. L. and Cavanaugh, E. F. (1963). *U.S. Pat.* 3,073,702.
- Kozlov, P. V., Undzenas, A. I., Marzlov, V. P. and Rozenberg, S. G. (1969). *Doklady Nauk. S.S.S.R.* **185**, 118-21 (Eng. Transl. pages 167-9).
- Kühn, K. and Zimmermann, B. K. (1965). *Arch. Biochem. Biophys.* **109**, 534-41.
- Kühn, K., Hannig, K. and Hörmann, H. (1961). *Das Leder* **12**, 237-41.
- Kühn, K., Tkocz, C., Zimmermann, B. K. and Beier, G. (1965). *Nature, Lond.* **208**, 685.
- Kubota, M., Kimura, S. and Ohashi, T. (1969). *Hikaku Kagaku* **15**, 8-13.
- Küntzel, von A., Cars, N. and Heidemann, E. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 149-55. Pergamon, London.
- Lane, J. M. and Miller, E. J. (1969). *Biochemistry* **8**, 2134-9.
- Larsen, B. and Smidsrod, O. (1967). *Acta Chem. Scand.* **21**, 552-64.
- Loeven, W. A. (1954). *J. Soc. Leather Trades Chemists*, **38**, 117-26.
- Lowry, J. R. (1962). *Can. Pat.* 652,161.
- McClain, P. E., Pearson, A. M., Miller, E. M. and Dugan, L. R. (1968). *Biochim. Biophys. Acta* **168**, 143-9.
- Manahan, J. and Mandl, I. (1968). *Arch. Biochem. Biophys.* **128**, 6-18.
- Manning, A. B. and Schryver, S. B. (1921). *Biochem. J.* **15**, 523-9.
- Miller, E. J., Martin, G. R., Piez, K. A. and Powers, M. J. (1967). *J. Biol. Chem.* **242**, 5481-9.

- Minkin, E. V. and Shestakova, I. S. (1962). *Nauchn. Tr. Mosk. Tekhnol. Inst. Legkoi Prom* 52-7.
- Minkin, E. V., Steshov, G. I., Shestakova, I. S. and Golovteeva, A. A. (1963). *Nauchn. Tr. Mosk. Tekhnol. Inst. Legkoi Prom* 60-66.
- Mitsuda, H., Matsuda, A. and Yasumoto, K. (1967). *Agr. Biol. Chem.* **31**, 1124-9.
- Mobbs, D. R. A., (1966). *Arch. Oral Biol.* **11**, 1071-9.
- Mohanaradhakrishnan, V., Ramanathan, N. and Nayudamma, Y. (1968). *Sci. Cult.* **34**, 76-80.
- Naghski, J., Wisnewski, A., Harris, E. H. and Witnauer, L. P. (1966). *J. Amer. Leather Chemists' Assoc.* **61**, 64-74.
- Nichols, J., and Oneson, I. B. (1969). *Fr. Pat.* 1,577,617.
- Nimni, M. E. (1966). *Biochem. Biophys. Res. Comm.* **25**, 434-40.
- Nimni, M. E., Deshmukh, K. and Bavetta, L. A. (1967). *Arch. Biochem. Biophys.* **122**, 292-8.
- Nishihara, T. (1962). *U.S. Pat.* 3,034,852.
- Nishihara, T. (1963). *Jap. Pat.* 9295/63.
- Nishihara, T. (1963a). *Ger. Pat.* 1,145,904.
- Nishihara, T. (1969). *Jap. Pat.* 69,11,037.
- Nishihara, T. and Miyata, T. (1962). *Collagen Symposium* **3**, 66.
- Nordwig, A. and Dick, Y. P. (1965). *Biochim. Biophys. Acta* **97**, 179-182.
- Okamura, H. (1969). *U.S. Pat.* 3,441,470.
- Okamura, H. and Kawamura, A. (1967). *Nippon Chikusan Gakkai-Ho* **38**, 385-91.
- Piez, K. A. and Carillo, A. L. (1964). *Biochemistry* **3**, 908-14.
- Piez, K. A., Weiss, E. and Lewis, M. S. (1960). *J. Biol. Chem.* **235**, 1987-91.
- Pospisilova, J. (1966). *Nature, Lond.* **211**, 536.
- Pouradier, J. and Venet, M. (1952). *J. Chim. phys.* **49**, 85.
- Privalov, P. L. and Tiktopulo, E. I. (1970). *Biopolymers* **9**, 127-39.
- Puett, D., Rajagh, L. V. and Ciferri, A. (1968). *J. Macromol. Sci. Chem.* **2**, 111-33.
- Purcell, A. W., Jahn, A. S. and Witnauer, L. P. (1966). *J. Amer. Leather Chemists' Assoc.* **61**, 273-4.
- Radkevich, D. (1966). *Myas. Ind. S.S.S.R.* **37**, 48-51.
- Reich, G., Walther, S. and Stather, F. (1961). *Ges. Abhandl. Deut. Lederinst. Freiberg/Sa* **17**, 127-33.
- Reich, G., Walther, S. and Stather, F. (1962). *Ges. Abhandl. Deut. Lederinst. Freiberg/Sa* **18**, 15-23.
- Reich, G., Walther, S. and Stather, F. (1962a). *Ges. Abhandl. Deut. Lederinst. Freiberg/Sa* **18**, 24-30.
- Reiss, W. (1964). *Das Leder* **15**, 233-7.
- Rigby, B. J. (1967). *Biochim. Biophys. Acta* **133**, 272-7.
- Rigby, B. J. (1967a). *Nature, Lond.* **214**, 87-8.
- Rudenko, S. V. and Levi, S. M. (1968). *Vysokomolekul Soedin Ser. A.* **10**, 647-51.
- Russell, A. E. and Cooper, D. R. (1969). *Biochem. J.* **113**, 221-3.
- Russell, A. E. and Cooper, D. R. (1969a). *Biochemistry* **8**, 3980-90.
- Russell, G. (1967). *J. Phot. Sci.* **15**, 236-40.
- Russell, T. J. and Winkelmann, R. K. (1966). *Fed. Proc.* **25**, 424.
- Sanger, F. (1952). *Adv. Prot. Chem.* **7**, 1.
- Saunders, P. R. and Ward, A. G. (1955). *Nature, Lond.* **176**, 26.
- Schiffmann, E. and Martin, G. R. (1970). *Arch. Biochem. Biophys.* **138**, 226-32.
- Schuster, S. and Bottoms, E. (1967). *Nature, Lond.* **214**, 599-600.

- Seifter, S., Gallop, P. M. and Meilman, E. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 164-9. Pergamon, London.
- Seymour-Jones, F. L. (1923). *J. Soc. Leather Trades Chemists* **7**, 93-4.
- Shimizu, M., Golub, L. and Glimcher, M. (1968). *Biochim. Biophys. Acta* **168**, 356-8.
- Smejkal, P. and Blazej, A. (1969). *Kozarstvi* **19**, 180-3.
- Steshov, G. I. and Golovteeva, A. A. (1965). *Izv. Vysshikh Ucheba. Zavedenii Tekhnol. Legkoi Prom* 75-80.
- Steven, F. S. (1964). *Ann. Rheum. Dis.* **23**, 300-1.
- Steven, F. S. (1965). *Biochim. Biophys. Acta* **97**, 465-71.
- Steven, F. S. (1967). *Biochim. Biophys. Acta* **140**, 522-8.
- Steven, F. S. and Jackson, D. S. (1967). *Biochem. J.* **104**, 534-6.
- Steven, F. S. and Tristram, G. R. (1962). *Biochem. J.* **85**, 207-10.
- Steven, F. S., Jackson, D. S. and Broady, K. (1968). *Biochim. Biophys. Acta* **160**, 435-46.
- Sussman, M. V. and Katchalsky, A. (1970). *Science* **167**, 45-7.
- Tkocz, C. and Kühn, K. (1969). *Eur. J. Biochem.* **7**, 454-62.
- Todd, A. (1961). *Nature, Lond.* **191**, 567-9.
- Tristram, G. R., Worrall, J. and Steer, D. C. (1965). *Biochem. J.* **95**, 350-3.
- Tu, S. (1966). *U.S. Pat.* 3,269,851.
- Veis, A. (1964). "Macromolecular Chemistry of Gelatin." Academic Press, New York.
- Veis, A. and Cohen, J. (1960). *Nature, Lond.* **186**, 720-1.
- Veis, A. and Schnell, J. (1967). *Symp. Fibrous Proteins, Int. Conf.* pp. 193-204.
- Veis, A., Anesey, J. and Cohen, J. (1960). *J. Amer. Leather Chemists' Assoc.* **55**, 548-63.
- Veis, A., Anesey, J. and Cohen, J. (1961). *Arch. Biochem. Biophys.* **94**, 20-31.
- Veis, A., Anesey, J. and Cohen, J. (1962). *Arch. Biochem. Biophys.* **98**, 104-10.
- Veis, A., Bhatnagar, R. S., Shuttleworth, C. A. and Mussel, S. (1970). *Biochim. Biophys. Acta* **200**, 97-112.
- Vlidick, A. and Newly, E. J. (1966). *Int. Congr. Gerontol., Proc. 7th* **1**, 173-5.
- von Hippel, P. H. and Harrington, W. F. (1959). *Biochim. Biophys. Acta* **36**, 427-47.
- von Hippel, P. H. and Wong, K. (1963). *Biochemistry* **2**, 1399-413.
- von Hippel, P. H. and Wong, K. (1963a). *Biochemistry* **2**, 1387-98.
- Ward, A. G. (1953), Private communication and B.G.G.R.A. Report CI.
- Ward, A. G. and Stainsby, G. (1965). *Brit. Pat.* 990,276.
- Williams, A. P. (1961). *J. Appl. Chem.* **11**, 100-3.
- Wood, G. C. (1963). *Biochem. Biophys. Res. Comm.* **13**, 95-9.
- Woodlock, A. F. and Harrap, B. S. (1968). *Aus. J. Biol. Sci.* **21**, 821-6.
- Worrall, J. and Steven, F. S. (1966). *Biochim. Biophys. Acta* **130**, 184-9.
- Yates, J. R. (1968). *J. Soc. Leather Trades Chemists* **52**, 425-35.
- Zimkin, E. A., Devyatov, Ya. B. and Maklakov, A. G. (1965). *Zh. Prikl. Khim.* **38**, 2581-5.
- Zimmer, E. and Kühn, K. (1963). *Abstr. Interm. Collagen Symp.* The Hague, The Netherlands. In "Collagen Currents (1963)" **4**, No. 4 p. 30.

Chapter 6

The Gelatin Gel and the Sol-Gel Transformation

G. STAINSBY

*Procter Department of Food and Leather Science, The University of Leeds,
Leeds, England*

I Introduction and Scope	179
II Factors Influencing Gelation	181
A. Thermal History	181
B. Other Factors	184
III Events in Gelation:	186
A. Aggregation	186
B. Setting	188
C. Melting	189
D. Changes in the Set Gel	194
IV Nature of the Links	203
V Isoelectric Gels	205
References	206

I. INTRODUCTION AND SCOPE

When a hot, aqueous solution of gelatin is cooled the viscosity progressively increases and, in time, the liquid changes almost imperceptibly into a gel if the concentration is sufficiently great and if the temperature is low enough. Gelatin is unusual, among the proteins, in this ability to transform liquids into materials which, like solids, retain their shape and have elastic properties. Among the gel-forming agents of natural origin—most of which are carbohydrates—gelatin is exceptionally versatile, giving stable gels over a very wide range of pH values and not requiring the addition of specific ions or other chemicals. The range of existence of gelatin gels, however, is only about 35°C, at most. The upper temperature limit is rather imprecise on account of the gradualness of the change from sol to gel, and vice versa. In contrast, the lower limit is well defined and is the temperature at which ice forms in the system.

Very concentrated gels, after storage, give an X-ray diffraction pattern that indicates the presence of very small locally ordered regions (or crystallites),

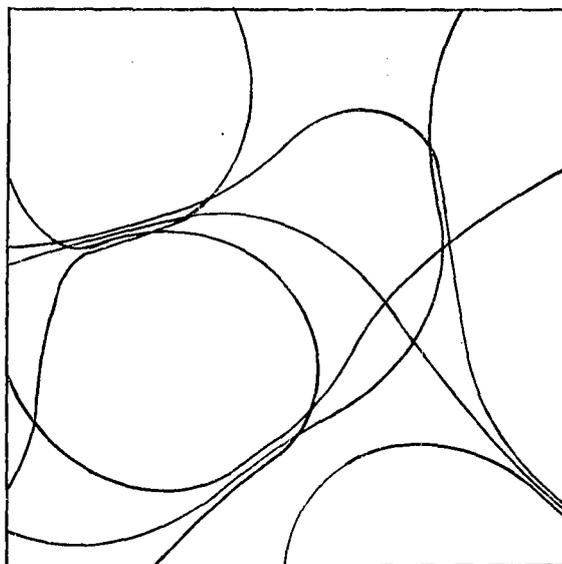


FIG. 1. Schematic diagram of part of network formed by flexible polymer.

although there is no long-range ordering of these crystallites. On this evidence, Herman and Gerngross (1930, 1932) were the first to suggest that a gel could be considered as a three dimensional network of gelatin molecules, with solvent entrapped in the meshes. The "links" of the network, where a number of polypeptide chains meet, were believed to be the crystallites. Between the links the chains, or strands, were thought to be flexible. Any particular strand could be involved in several links (see Fig. 1).

Ferry (1948a), in a comprehensive review of protein gels, pointed out that many of the properties of gelatin gels could be explained at least qualitatively on the basis of such a network structure, with some 5 or 6 sites for linking per molecule. The links themselves were thought to be stabilized by secondary forces, rather than by covalent bonds, since the gel melts readily. Meyer (1942) had earlier suggested that hydrogen bonds play a predominant role in gelation with gelatin.

The phenomenon of gelation has continued to be of major interest and several further reviews have appeared, e.g. Saunders and Ward (1958); Ward (1959); Idson and Braswell (1957, 1960); Veis (1964). These show that the more recent studies generally support the earlier ideas of the network theory, but it is rather frustrating that our understanding of the detailed mechanism is still far from complete. Little has been added to make Ferry's views more quantitative, so that it is still not possible to explain why the rigidity (or strength) of a gelatin gel is approximately proportional to the square of the

protein concentration (except for very dilute gels, near the minimum concentration for gelation). Nor is there a quantitative explanation of the strong dependence of rigidity on temperature.

In qualitative terms, the progressive decrease in rigidity with increasing temperature reflects the decreasing strength of the hydrogen bonded links in the network. The addition of hydrogen bond disrupting agents (such as urea, KSCN, LiBr, etc.), also weakens the network and may be considered as equivalent to an increase in the temperature.

Important progress has been made, however, through the increasing realization that the phenomenon usually termed "gelation" embraces a number of events, and that each event has distinctive characteristics. The first event, on cooling a solution, is partial aggregation of the gelatin molecules. This takes place even if the solution is too dilute to form a gel. Next, the aggregates link together to form a weak network, i.e. the solution sets. On further cooling, or by holding the temperature constant, the set gel develops increasing strength. During this stage, a variety of structures can develop, by varying the thermal history of the set gel, through the continuous breaking and reforming of the links in the network. Finally, when a gel is warmed it eventually melts, and the solution contains aggregates which disperse to separate molecules in time at a high enough temperature. Through the changes that take place in the life time of the set gel, the melting becomes less and less like a reversal of the setting. Moreover, the factors which govern the initial setting are not generally of similar importance in the development of the set gel, so that the properties of the gel need not be reflected by the ability of the solution to set.

This Chapter deals briefly with the factors which govern the gelation of gelatin, and particular emphasis is given to the effects of thermal history. As these effects are most apparent in dilute gels the more concentrated gels receive only a very scant mention, but their properties are considered more extensively in Chapter 8. Each event in gelation is considered in turn, in so far as this is possible at present. Attention is also paid to recent thermodynamic approaches, which have the aim of determining the strengths of the links in the gel network, and to the behaviour of gels in the ultracentrifuge. The chemical nature of the links is then discussed and a theory of the gelation, in molecular terms, concludes the survey.

II. FACTORS INFLUENCING GELATION

A. Thermal History of the Gel

It has long been known that many of the physical properties of a gel change continuously with time, even at a constant temperature. Thus, for useful

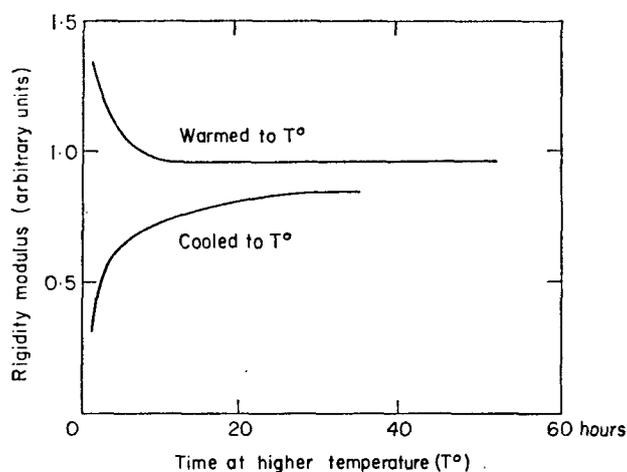


FIG. 2. Change of rigidity modulus with time at 15°C after warming from 0°C. (After Ferry (1948b).)

comparisons to be made, for systems cooled to and held at one fixed temperature, it is essential to specify the age of the gels as well as their temperature. The rigidity modulus, for instance, increases rapidly for several hours after setting and then continues to increase but at a diminishing rate. It has never been observed to reach a constant value. Ferry (1948b), however, observed that an approximately constant value could be attained in a few hours at the measuring temperature if the gel was first pre-cooled and aged at a much lower temperature. Quite probably a further, very slow change would take place at the measuring temperature, but the apparent constancy led to the use of the term "equilibrium gel" (Fig. 2).

Though this procedure is a useful practical device, the description of the gel implies that a unique state has been reached. This is not so. If the gel is cooled again, and then re-warmed to the measuring temperature, the properties will almost certainly re-appear. But if the gel is taken to a higher temperature, and then brought back to the measuring temperature, the properties of the "equilibrium gel" are never regained.

This behaviour is not exceptional but is typical of polymer network structures that are stabilized by secondary forces. The formation of the network involves the delicate balance between polymer-polymer interactions and polymer-solvent interactions, and the balance is strongly dependent on temperature. The structure of the network depends on the mode of formation, which in turn depends on the thermal history of the system. If a gelatin solution is cooled very rapidly, then the molecules are in effect "frozen"

initially in the configuration they had immediately prior to chilling. The system is devoid of order, if the cooling is sufficiently rapid, and through contacts between neighbours, which are there by chance, many weak links form between entangled polymer chains. This is called a "fine" gel network. Since the domain of one gelatin molecule in solution overlaps the domains of neighbouring molecules unless the concentration is less than about 2 mg/cm^3 (Veis, 1964), this type of network will arise whenever a gelatin gel is made by snap-chilling.

In contrast, very slow cooling leads to the formation of a "coarse" network, particularly for the more dilute gels. In a coarse network the links are regions of ordered, co-operative interchain bonding. Chance contact between chains in solution leads to interchain bonding, but only the strongest bonds survive at these higher temperatures. Once a stable bond is present, additional bonds can develop in close proximity whenever the thermal motions of the chains bring the bonding sites sufficiently close. (This can be considered to resemble the closing of a zip-fastener once a start has been made.) In this way aggregates first form and then a complete network slowly develops. Even after the gel network has extended throughout the whole solution, the links are continually strengthened, for the chain-segments between the links are still undergoing ceaseless thermal motions—though the mobility is somewhat hampered as compared with the movements in solution—and new bonding possibilities arise. Naturally the development of the network slows down with age. These continual adjustments to the network structure are particularly favoured for gelatin gels, on account of the very flexible nature of the polypeptide chains due to a high glycine content and the dynamic nature of [NH - - - O] hydrogen bonding.

Similar, but much less extensive, changes in the structure occur with the fine network type of gel at low temperatures, when the chain mobility is greatly reduced and each hydrogen bond is more stable. On heating, however, a fine network can become more and more organized. In the limit, by holding the gel for a long time at a temperature just below the melting point, the fine network is changed to a coarse network. Subsequent cooling, however, does not reverse the change, and the original structure is no longer attainable. The more stable coarse network persists at all lower temperatures and, superimposed on it, a less ordered structure develops utilizing such bonding sites that remain. The structure of any gel, therefore, can be considered as a blend of fine and coarse networks, the proportions depending on the entire thermal history of the gel. At the molecular level the structure is non-uniform, and a spectrum of bond strengths is present, but the whole gel behaves as an isotropic solid. This qualitative model of a gelatin gel will suffice for the present for the interpretation of most of the results given in the next sections of the Chapter.

B. Other Factors

The remaining factors that influence gelation may be divided conveniently into two groups. The first of these is concerned with the gelatin itself, whilst the second group concerns the solvent.

1. *The gelatin*

In order to form a gel at all there must be at least two bonding sites per gelatin molecule, and hence a minimum molecular weight is needed. Ward and Saunders (1958) have suggested that this minimum value is about 15,000, or about one sixth the weight of an α -chain. Above this value the ability to form a gel and the properties of the gel, improve with increase in molecular weight until this is about 90,000. Larger molecules than this, however, give slightly poorer gels as judged by the rigidity modulus of molecular weight fractions at 10°C (Stainsby, Saunders and Ward, 1954).

The most likely explanation for this decrease in jelly strength with increase in molecular weight stems from the fact that each of the larger molecules must consist of several polypeptide chains linked by covalent bonds. These inter-chain bonds aid the formation of interchain hydrogen bonds, when the system is cooled, and so reduce the possibilities for intermolecular hydrogen bonding. In this way the network of intermolecular bonds is weakened.

At present there is only one published investigation of the properties of essentially pure α -chain gelatins (Fujii, 1966). These unusually homogeneous materials were made by pretreating finely ground collagen, from calfskin or pigskin with Pronase in the presence of 0.4 M CaCl_2 and, after inactivating the enzyme and removing the inorganic salts, extracting the gelatin at 80°C and pH 7.0 for 5 minutes. Steerhide, similarly treated, gave a gelatin with about 60% as α -chains. Sedimentation patterns in the ultracentrifuge confirmed the homogeneity of the skin gelatins and provided a guide to the proportion of larger molecules in the hide gelatin and in several commercial preparations. Bloom jelly strength measurements showed that closely similar and high values were obtained for all the gelatins and that the strength decreased as the proportion of β , and higher, components increased in the other gelatins.

Although this investigation gives useful support to the view that the best gels are made from intact α -chains, and therefore that the jelly strengths of the more usual polydisperse commercial gelatins will depend on their α -chain contents, it would be wrong to conclude that a difference in strength between two gelatins could be ascribed entirely to a difference in molecular weight distribution. Saunders and Ward (1955) have shown that another feature of the gelatin, still unidentified, governs the level of gel rigidity modulus or the related Bloom jelly strength that is attained by high molecular

weight fractions. This feature has been termed the rigidity factor. Differences in rigidity factor are best demonstrated using concentrated solutions cooled quickly to 0°C or 10°C, i.e. for fine network gels. The factor is resistant to acid or enzyme degradation, but is reduced by heating gelatin in neutral or alkaline solutions (see Chapter 8). Cold, alkaline pretreatment of collagen, however, causes no loss of this feature—though losses may take place in the subsequent extractions of gelatins. The pH and the temperature during manufacture affect gelling ability both through the rigidity factor and through the molecular weight distribution.

Chemical modification of the reactive side groups of gelatin (e.g. O- and N-acetylation, esterification of carboxyl groups, etc.—see Chapter 7), provided that degradation is avoided, has almost no effect on the Bloom jelly strength of fine network gels. Under these conditions for gelation, changes in the charge pattern of the molecule—as, for example, by replacing $-\text{NH}_3^+$ by $-\text{NHCOCH}_3$ —are expected to be of little consequence. Such changes become important, however, in more dilute gels, especially at higher temperatures, so that setting times and melting points can be altered even though the Bloom strength remains constant (Ledward, 1966). Most chemical modifications result in delays in setting and lower melting points, but the introduction of additional guanidino groups (Janus, 1958) enhances setting rates. The nature of this unexpected and specific effect, involving the bulky guanidinium ion, is still not properly understood.

2. The solvent

The environment of the gelatin molecules is only of secondary importance so far as the strength of fine network gels is concerned. Thus, the rigidity modulus of concentrated gels is almost independent of pH in the range from 4 to 10 pH units, but outside these limits there is a sharp fall in rigidity which cannot be ascribed to degradation (Chapter 8). The more dilute the gel, the more the importance of pH (Bello *et al.*, 1962a) and the greater the change of melting point or setting time with pH.

The small amount of ash in commercial gelatins is insufficient to change the jelly strength, but at much high concentrations of electrolytes the strength decreases quite appreciably (see Chapter 8).

Non-electrolytes such as sugar, increase the jelly strength and melting point, but the increases do not seem to be as great as one would expect by replacing part of the water by an equal volume of an inert substance, e.g. at 10°C the Bloom strength of fine gels rises by about 20% when half the water is replaced by glycerine. The rigidity at 25°C, however, for coarse gels, is enhanced several fold and the melting point rises several degrees. It may be that glycerol, and sugar, can reinforce the bonding in the gel if the temperature and age are suitable, but this has not been properly examined as yet.

Non-electrolytes that precipitate gelatin from aqueous solution, such as alcohols or acetone, have similar effects when present in moderate concentrations. At much higher concentrations, however, the gels lose strength as the solute/solute interactions predominate over the solute/solvent interactions until, eventually, the gelatin is completely precipitated.

Other chemicals, both electrolytes and non-electrolytes (e.g. KSCN, LiBr, CaCl₂, urea, phenols) have the power of completely preventing gelation, when they are present in sufficient concentration. Mandelkern (1962) has suggested that, through binding to the peptide bonds in the main backbone of the protein, there may be induced electronic rearrangements which have the effect of preventing the formation of the hydrogen bonds that are needed to stabilize the gel network. Other writers ascribe the observed effects to a similar interference in the structure of liquid water. At the present time these phenomena are still very poorly understood, and von Hippel (1967), among others, has drawn attention to the need for an explanation that is quite general in scope and not aimed specifically at systems involving gelatin, since similar effects are now being observed increasingly with a variety of other proteins and with other macromolecules, including DNA.

III. THE EVENTS IN GELATION

A. Aggregation

The viscosity of molecularly dispersed solutions of gelatin, at 40°C or higher, is Newtonian in character and falls with time through degradation involving mainly hydrolysis of peptide bonds. At somewhat lower temperatures the viscosity increases with time, even if no setting to a gel eventually occurs, and it may become non-Newtonian, the viscosity decreasing as the shear is increased. This transition in viscous behaviour at about 38°C was noted by Davis and Oakes (1922) and by many later workers, and is due to the linking of gelatin molecules into aggregates. In a more recent study Stainsby (1962) measured the apparent viscosity—taken as directly proportional to the flow-time in a wide bore capillary viscometer—as a function of time at 31°C, and then followed the loss in viscosity on re-warming at 40°C. These gelatin solutions would not set at 31°C. Typical results are given in Fig. 3. The form of presentation used in Fig. 3a is needed in order to isolate the considerable effect of pH on aggregation from the effect of pH on the viscosity of molecular solutions (see Chapter 4). Figure 3b shows that the aggregates, once formed, do not disperse entirely at 40°C, even though no formation of aggregates can be observed at 40°C. Moreover, the longer the system is held at 31°C, the more difficult it becomes to

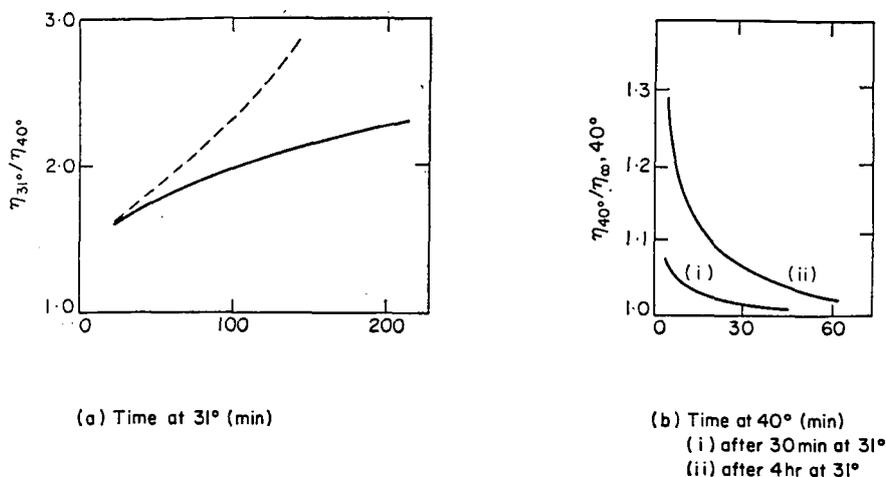


FIG. 3. Aggregation (a), at 31° and dis-aggregation (b), at 40°C. The broken line in Fig. 3a represents the behaviour of a solution which would set. (After Stainsby (1962).)

resolve the aggregates at 40°C. These observations accord with the views expressed earlier. Some intermolecular linking occurs, once gelatin solutions are below the collagen denaturation temperature, and in these concentrated solutions only a very limited regeneration of a collagen-like structure is possible. (Extensive reversion to collagen is undesirable as this could lead to collagen-collagen interactions and eventual insolubility, so that reversion and gelation are competing processes.) In time, through the considerable molecular mobility at this temperature and through localized rebuilding of hydrogen bonds, the aggregates become more stable to thermal disruption.

In a much more limited fashion, aggregation like this takes place before setting, and the slower the rate of cooling the more extensive the aggregation. The progressive linking to form a gel, therefore, has sometimes been considered as a two-stage process—aggregation, and then setting. Bourgoin and Joly (1958) have called the first of these stages pregelation and studied the considerable changes in optical and rheological properties that arise through the continual increase in the number of intermolecular links. The molecular model used by Bourgoin and Joly for the interpretation of their results differs in detail from the view expressed here, principally by distributing the links uniformly throughout the whole system and by keeping the stability of the links constant at a given temperature, regardless of time. In consequence, these authors have claimed that pregelation does not involve the formation of aggregates, i.e. there is no preferential linking together of only some of the

molecules. The fact that some of the gelatin can be washed out of a weak gel favours the alternative view that is given here.

B. Setting

There is a continuous rise in viscosity, and in the rate of the increase, as a gelatin solution cools and sets, and there is no apparent and sharp discontinuity as the liquid thickens to a gel. A sudden onset of weak elastic properties can be demonstrated, however, by the "spring-back" of entrapped air bubbles when gentle stirring is stopped. Prior to this indication of the onset of setting the entrapped bubbles continue to follow the direction of movement, when stirring ceases, until the viscous drag brings them to a halt. Richardson (1933) investigated these properties quantitatively, using the damping of a light disc.

The setting of a gelatin sol depends on its mechanical as well as its thermal history. Thus, mechanical disturbances may delay setting, and the temperature for setting is higher when the sol is cooled slowly than when it is chilled quickly. Sheppard and Sweet (1921) demonstrated these effects using air bubbles, i.e. using an indication of the onset of setting. Olsen (1932), on the other hand, confirmed the effects for what might be termed full setting, since no flow had to take place when a test-tube containing the solution was inverted, i.e. when the set gel was strong enough to support its own weight.

In use, the setting time is generally of more interest than the setting temperature. When a moderate rate of cooling is involved the criterion for setting can be any agreed state between the onset (by air bubbles) or fully set (no flow) condition. Sometimes an intermediate degree of setting (e.g. the ability to support 0.2 cm³ carbon tetrachloride, as in B.S.S. 757 (1959)) is appropriate. In some applications an extremely rapid rate of cooling is used, and Janus *et al.* (1965) have described a useful technique for measuring setting temperatures under these conditions. Uniform drops of solution (at 40–50°C) are placed at suitable time intervals on the polished surface of a level, hollow, metal bar through which water flows at a constant temperature. When some drops have set, the bar is turned through 90° and the setting time deduced from the position of the first drop, after those which have set, to run and the timing interval. Thus, in effect the drop has to become just firm enough to support its own weight. The bar has to be in an enclosure at high humidity to prevent the loss of water from the relatively large surface of the small drop, or a skin may form and give misleading results. This method is particularly suitable for measuring short setting times (less than 10 minutes).

There are no reports of correlations between the various empirical methods, nor are there reports to show whether the ranking order of a given series of gelatins depends on the method of test. In fact, there is a scarcity of published

information on setting behaviour. The most comprehensive study is that of Janus *et al.* (1965) who have shown, using the bar method, that the logarithm of the setting time at 20°C is linearly related to the logarithm of the gelatin concentration, at least up to 10% concentration. The precise relationship varies with temperature and solvent, but the slope of the log-log plot lay between 2.5 and 3.1 for all the systems studied. Thus, when a 10% solution sets in 20 seconds a 3% solution of the same gelatin, in similar conditions, needed 500 seconds to set. Neutral salts, such as KCl, have only a minor effect on setting time. For example at 0.1 molal concentration, KCl increased the setting time of a 6% gelatin solution from 86 to 90 seconds. In contrast, sharp changes in setting time arise when the pH is changed, particularly in the region of the isoionic point, and these effects are shown by quite concentrated gelatin solutions.

Using molecular weight fractions, Janus (1958) has established that setting time (T) and L.V.N.—which is a reflection of molecular weight (see Chapter 4)—are related, over a very wide range of molecular weights, by the expression $T(LVN)^2 = \text{constant} = 1/S$ for a given solvent, temperature and gelatin concentration. S has been termed the setting rate parameter. For four single extract gelatins, of different Bloom jelly strengths, S is related directly, though not quite linearly, to the rigidity for gels held 24 hours at 0°C. (S is calculated from T , for the solution at 20°C, and L.V.N., and is used in place of T to allow for differences in molecular weight.) Thus the rigidity at 0°C and the setting time at 20°C are both reflections of the intrinsic ability of each sample to give a gel. This does not mean that setting and the subsequent development of rigidity are identical processes, though there must be some common features. Some of the factors of importance in setting—for instance, the pH value of the solvent, the molecular weight of the gelatin, the extent and nature of chemical modification of the side groups of the gelatin—have been shown to be of negligible influence on the properties of well-developed gels at low temperatures. It should be recalled that the evidence at present available refers only to fine network gels and to solutions set quickly at 20°C. Care should be taken, therefore, in using these results to predict the behaviour of systems with very different thermal histories, particularly if the solutions are set by slow cooling and if the coarse network type of gel is developed, for extensive structural reorganizations in the sol and in the gel would then be possible.

C. Melting

It used to be thought (e.g. Olsen, 1932) that the sol would set and the gel melt at the same temperature if the system was cooled and then warmed exceptionally slowly. The differences in setting and melting temperatures that

TABLE I. Rigidity modulus as a function of temperature in the region of the setting temperature

Temperature (°C)	Rigidity modulus*	
	Gel 1	Gel 2
29	2.24	1.40
30	1.54	1.13
31	1.00	0.92
32	0.52	0.62
33	0.20	0.33
34	0.03	0.11
35	zero	0.01

* In arbitrary units.
Gel 1 held first at 25°C, gel 2
at 27°C.
Heating schedules as in text.

had been observed were thus thought to arise from the methods of measurement. It is now known, however, that the differences are real and that they may be magnified, not diminished, by holding a weak gel at temperatures in the vicinity of the melting point. The figures in Table I illustrate the effect. These were obtained as follows. A 6% solution of a low-pI gelatin in water was cooled slowly to either 25°C or 27°C and the gel which formed was matured overnight. The next day the rigidity modulus was measured and the temperature of the gel then slowly raised 1°. After a further day the modulus was again measured and the temperature raised another degree. Only the results for temperatures of 29°C or higher, are shown, for convenience. At 29°C the gel matured at 25°C had the higher initial rigidity, and melted above 34°C. The second gel became the stronger at 32°C, and melted above 35°C. The original solution, and those from the melted gels, failed to set after one day at 31°C. After cooling quickly to 25°C and holding at 25°C for only one hour, the gel melted at 31.4°C when the temperature was raised steadily at the rate of 0.25°C per minute. Such effects clearly demonstrate that the gel network, once formed, is continually being reorganized to include links of increasing thermal stability.

Melting point studies prove only very limited information about the network as they give only an arbitrary measure of the strength of the last bonds that maintain the network throughout the system. Weaker links are broken at lower temperatures and probably even stronger links remain in the aggregates which persist after melting.

The existence of aggregates in freshly melted gels has been demonstrated by Boedtger and Doty (1954) using the technique of light-scattering. Weak gels, conditioned first at 0°C, were held at 17.4°C until the scattered light intensity

became constant, i.e. until an apparent equilibrium in the gel state was reached. Then the temperature was raised to 25°C and the gels melted. During melting the scatter intensity changed, but then it remained constant for several hours. The level of intensity, however, corresponded to scattering from aggregates, and not from discrete molecules. Heating to 40°C caused a fall in scatter to that for a true molecular solution. This technique is potentially capable of yielding quantitative information regarding the average size and shape of the stable aggregates remaining after melting gels, but remains unexploited.

The melting points of gelatin gels, unlike the setting times, have formed the subject of many investigations. Much of the earlier work is of very limited use, generally on account of poor characterization of the sample or of the experimental conditions. Most of the more useful contributions from the past are summarized by Bello *et al.* (1962a), who have carefully studied the effects of concentration, pH, time and dilute electrolyte on the melting of gels made from pigskin gelatin (pI 9.2), limed ossein gelatin (pI 5.1) and limed calfskin gelatin (pI 4.8), all substantially free from ash. As with setting, so for melting an empirical definition of the phase change has to be used, and Bello *et al.* chose to take as the melting point the temperature at which a specified Neoprene ball, inserted under the gel surface, reached the bottom of a tube of gel that was heated at about 5°C per hour. The gel was formed at 0°C and matured for 20 hours at 0°C before the melting experiment, i.e. fine gels were used. The melting point of 1 to 5% gels was almost independent of pH from pH 2 to 11, under these conditions, the 5% gel having a melting point approximately 5°C higher than that of the 1% gel, for the pigskin gelatin, regardless of pH in this range. This is taken to mean that neither the carboxyl nor amino groups on side chains participate in the stability of the gels, in keeping with Ferry's proposal that the insensitivity of melting to the presence of sodium chloride is evidence for non-participation by charged groups (Ferry, 1948a). The view is confirmed (Bello *et al.*, 1962b) by determination of the melting points of 5% gels made from modified gelatins having each of the groups (carboxyl, amino, guanidino) blocked by a small non-ionizing substituent. The melting points were close to those of unmodified but partially hydrolysed gelatins of comparable molecular weights, as determined by a sedimentation-equilibrium method using an ultracentrifuge.

Very weak gels, of concentration less than 1%, show a marked dependence of melting point on pH, after one day's storage at 0°C. The unmodified pigskin gelatin, for example, gave no gel for the pH range 3 to 4, nor at pH 9, yet at pH values of 2, 5 and 12 the melting point was just over 15°C. In time, however, homogeneous gels formed at all pH values in the range from pH 2 to 12, and the melting points were all in the region of 15 to 20°C after 4 days at 0°C. On more prolonged storage a small additional rise in melting point

occurred. Modified gelatins behaved similarly. These observations on the time taken to gel at 0°C support the evidence of Janus *et al.* (1965) concerning the rate of setting at 20°C, and show that charged groups are to some extent involved in the earliest stage of gel formation. It is probable that the relative ease of setting at pH 2 and pH 12 arises from charge-screening due to the electrolyte added to change the pH. As with the changes in solution viscosity (Stainsby, 1952), the effects due to intermolecular repulsion are best seen in dilute systems prepared from deionized gelatin, and are readily lost either by raising the gelatin concentration (when more acid is needed to change the pH) or by the direct addition of sodium chloride.

Stainsby (1962, 1965) has made an extensive study of the changes in melting point that take place under a wide variety of storage conditions for unmodified gelatins. The movement of a small drop (0.2 ml) of coloured CCl₄ was used to indicate melting, and the gels were heated at 0.25°C per minute. The concentration range studied was from 1 to 10%, and the results for gels cooled quickly to 0°C and then matured at that temperature support and extend the report of Bello *et al.* (1962a). Thus, the melting point increases with concentration, but for high grade gelatins the increase is very small once the concentration exceeds 5%. Furthermore, it was shown that quite significant increases in melting point are obtained by storing gels at 25°C, even for moderately concentrated systems. For example, the melting point of a 6% gel rose from 31.5 to 34.5°C during one day's storage at 25°C, but in the same time at 10° it changed from 30.3 to only 30.4°C. The gel melting point, therefore, can provide a useful guide to the changes in structure that can arise during storage. Not surprisingly, the structures that develop at room temperature persist on cooling so that the melting point of a gel that is cooled very slowly is higher than that of one which has been cooled rapidly to the same temperature.

Eldridge and Ferry (1954) have also noticed some increase in melting point on holding a gel at 15°C, and have mentioned that the melting point is higher if the gel is heated more slowly than is usual in a melting experiment.

Gel melting point also increases with increase in the average molecular weight of the gelatin. Eldridge and Ferry (1954), for example, showed that the reciprocal of the melting point in degrees absolute was approximately linearly related to the logarithm of the weight average molecular weight, \bar{M}_w , for gels at the same concentration and with the same thermal history. The samples used in this investigation were all prepared from a single gelatin by hydrolysis (conditions not given) and in consequence \bar{M}_w was rather low (3×10^4 to 7×10^4 only). In this range, gel strength also increases with increase in molecular weight, so that gel strength and melting point appear to be directly related. This is not generally true, however, as Pouradier and Venet (1950, 1952) and Stainsby *et al.* (1954) have shown, using molecular weight

TABLE II. Variation of melting point and rigidity modulus with L.V.N.
(Private communication from P. R. Saunders)

<i>Gelatin E Fractions</i>		
L.V.N.	M. Pt. °C	Rigidity modulus 10 ³ dynes/cm ⁻²
104	28.0	45
78	27.9	48
57.5	27.5	49
43.5	27.0	50
24	22.6	30.5

<i>Gelatin A Fractions</i>		
L.V.N.	M. Pt. °C	Rigidity modulus 10 ³ dynes/cm ⁻²
101	32.2	63
81	31.0	64
64	30.2	69
53	30.0	71
43.5	28.8	74
31	26.0	63

L.V.N. obtained from dilute solution viscosities at 40°C by extrapolating η_{sp}/C to $C = 0$.

Melting points and rigidities refer to snap-chilled 5.8% (w/w) systems, held 18 hours at 10°C.

fractions that covered the very much wider range from about 4×10^4 to 1×10^6 . For any one gelatin the melting point rises with increase in reduced viscosity (and therefore \bar{M}_w), though the gel strength is nearly constant for all but the low molecular weight fractions (see Table II). The relationship between melting point and molecular weight varies, however, from one gelatin to another, showing that a further factor is involved. At present this additional factor—most probably the rigidity factor (page 185)—has not been identified with any detailed chemical or structural aspect of gelatin molecules. Thus, in practical terms, the melting point reflects neither the viscosity nor the Bloom strength alone, but is a complex property. It is, therefore, possible to have high melting points yet only moderate gel strengths, in extreme examples, and Selby (1951) has drawn attention to the desirability of using such gelatins in jellied products for the food industry.

Strong gels, made by snap-chilling to 10°C, bring out most clearly the influence of rigidity factor on the melting point. This influence is diminished when dilute gels, stored at room temperature, are studied and in such situations other factors, such as pH, ionic strength and thermal history assume a greater importance, just as with setting. This area is still largely unexplored.

D. Changes in the Set Gel

Every feature which influences the melting point of a gel contributes also to the value of the strength of the gel, which is an indication of the number of links, per unit volume, in the network. The main physical properties of gels are fully described in Chapter 8, so that only a few topics have been selected for discussion here. They are

- (i) the enhancement of gel strength by pre-maturation,
- (ii) the change in optical rotatory power during gelation,
- (iii) the strengths of the links, as deduced from gel strengths and melting points, and
- (iv) ultracentrifugal studies of gels, and the proportion of gelatin involved in gelation.

All these investigations have been concerned particularly with the structural changes that can take place in weak gels, and in this way they have provided useful information for our current explanations at the molecular level of the events in gelation.

1. *Gel strength and thermal history*

It is well known that the strength of a gelatin gel, like the melting point, increases continually with time, at a given temperature, and that the rate of increase falls off as the gel ages. Storage at room temperature (20–25°C) leads to the formation of a coarse network type of gel, containing only a few, highly ordered links, so the gel is rather weak. On cooling to 10°C, however, a fine network develops on the existing framework and the composite gel has a higher rigidity modulus than that for the gel which is formed quickly and aged at 10°C (see Stainsby, 1962, 1969). The difference in strength at 10°C, between these two types of gel, increases with the time that the coarse network is held at the higher temperature, and the enhancement of rigidity is relatively more important the smaller the gelatin concentration, for in both of these ways the structural changes at the higher temperature are optimized. For example, the increase in strength at 10°C was 10% for a 5% gel held only 3 hours at 25°C; but 43% if the time at 25°C was 3 days. The increase was 80% for a 2% gel, made from the same gelatin and held also 3 days at 25°C. With the more dilute gels, the increase in rigidity at 10°C can be greater than the rigidity that develops at the higher temperature for some gelatins, but this is not always so as gelatins vary widely in this respect. On warming the composite gel the fine network melts first, and the associated rigidity is lost. The broad spread of the rigidity-temperature curve (see Fig. 4) for the additional fine network contrasts with the narrow melting-curve for the more highly ordered, but weak coarse network gel. This is as expected as the fine network, whether alone or superimposed on a coarse network, is built from a

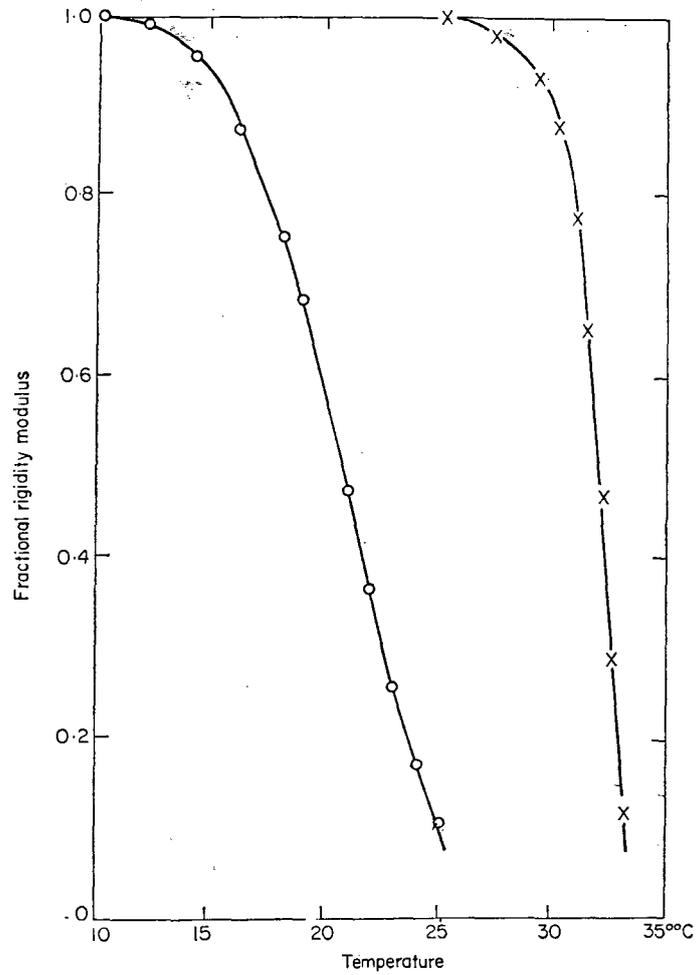


FIG. 4. The loss of rigidity modulus on heating a gel.
x gel held first at 25°C for 4 days
o as above, but then held one day at 10°C.

wide variety of combinations of chain segments as the system is essentially frozen in position and only the nearest neighbouring protein chains are available for linking. (This situation is analogous to that for renaturation of collagen in solution at low temperatures—see, for example, Beier and Engel, 1966.)

Ledward (1966) has shown that the magnitude of the enhancement of

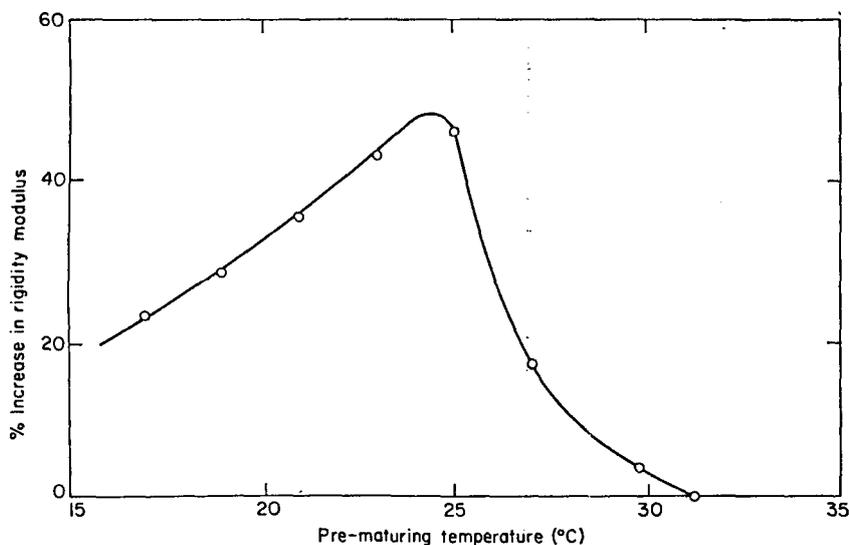


FIG. 5. Increase in rigidity modulus, as a function of pre-maturing temperature.

rigidity varies considerably among commercial gelatins, and depends on pH and, more importantly on the temperature of pre-maturing, as well as on gelatin concentration. Figure 5 shows that there is an optimum temperature for the pre-maturing stage. As a useful working rule, this is several degrees below the setting temperature for the system. Extensive aggregation, without setting, provides no proper network on which to build additional links at the lower temperature, so no enhanced rigidity is produced. Aggregation competes with gel formation so that the quicker the set the smaller the wastage of links in aggregates and the better the development of the coarse network. As the pre-maturing temperature is lowered, however, the selectivity of the linking diminishes (see page 204), and the mobility of the free chains between the links also falls off quite sharply. The network is thus less able to become re-organized in time, i.e. it includes a greater proportion of random links, as in a fine network, and the essential feature of the pre-maturing stage is less marked. A more detailed description in molecular terms is not available at the present time.

2. Optical rotation and gel strength

The specific optical rotation $[\alpha]$, for a gelatin solution, or for a set gel, is negative and is independent of the gelatin concentration. ($[\alpha]$ is calculated from the observed rotation in degrees, α , and the path length in decimeters, l , by the standard relationship $[\alpha] = 100 \alpha / l \cdot c$ where c is the protein concentra-

tion in g per 100 cm³.) A typical value for $[-\alpha]$ for an aqueous solution of gelatin at 40°C is 120 when sodium *D* light is used, the precise value depending on the amount of residual collagen-like structure (see Chapter 4). When the solution sets to a gel the rotation becomes much more negative, e.g. $[-\alpha]_D$ reaches 350. Saunders and Ward (1958) have shown how $[-\alpha]_D$ varies with temperature for a series of gels made from molecular weight fractions of a single gelatin. The procedure was to prepare and age a gel at 0°C, measure $[-\alpha]_D$, warm to say 5°C and hold until $[-\alpha]_D$ became essentially constant, warm again, etc. (i.e. the "equilibrium" method).

Todd (1961), using a more sensitive polarimeter, similarly determined how $[-\alpha]_D$ varied with temperature and in addition measured the variation of the rigidity modulus, G . Three whole gelatins, having similar viscosities at 40°C but different Bloom jelly strengths were used and a single expression $[-\alpha]_D = K.G^{\frac{1}{2}} - I$ fitted the observations. Veis (1964) has commented that the results for the fraction of similar viscosity studied by Saunders and Ward also fit this relationship. The constant K changes for the remaining fractions. Ledward and Stainsby (unpublished) have extended the investigation to weaker gels (at 20 mg/cm³ instead of 58 mg/cm³) and to a variety of thermal histories, and also used a number of chemically modified gelatins. In all instances $[-\alpha]_D$ and $G^{\frac{1}{2}}$ are linearly related, though the constant K varies with concentration since $G^{\frac{1}{2}}$, unlike $[-\alpha]_D$, varies with concentration. Modification of the gelatin, by substitution at ϵ -amino groups, leads to no change in K for a given gelatin at a selected concentration.

The specific rotation $[-\alpha]$ varies sharply with wavelength, λ , becoming progressively larger in value as λ falls in the range 600 to 250 nm. For example, $[-\alpha]_{313}$ for a gelatin solution at 40°C is about 800, as compared with 120 at $\lambda = 586$ nm. As with other proteins, $[-\alpha]_{\lambda}$ can vary quite considerably with the nature of the solvent and with temperature. In order to compare data, therefore, it is essential to correct for effects due to differences in λ , T and solvent.

In the near ultraviolet ($\lambda > 250$ nm) and visible regions of the spectrum the dependence of $[-\alpha]$, suitably corrected for solvent and temperature, on λ is exceptionally straightforward, the rotatory dispersion being represented by just a single-term Drude equation such as $[-\alpha]_{\lambda, T, \text{solvent}} = \alpha_0 \lambda^2 / (\lambda^2 - \lambda_0^2)$ where α_0 and λ_0 are constants obtained by fitting the data to this expression. This type of dispersion is called simple dispersion. Moreover, the constant λ_0 is essentially identical for native collagen, gelatin and for partially denatured or renatured collagen. The collagen \rightleftharpoons gelatin transition is most unusual, among protein denaturations, in this respect. It is on account of this feature that the change in $[\alpha]_{\lambda, T, \text{solvent}}$ can be used as a measure of the helix content of the system. It is customary to take the gelatin solution as having no helix content for this purpose, and to take the appropriate value of the specific

rotation for native collagen as 100% helix. On this basis, matured gelatin gels can have as much as 70% of the protein in helical conformation. This does not mean that there has been a 70% reversion to the structure of native collagen, for this is quite impossible to achieve at the concentrations needed to form a gel (see Chapter 4). 70% of the protein, in this example, is involved in collagen-like helices in the gel network, and the remainder is still in the random-coil conformation, linking helical regions. These regions in the gel, however, are generally of lower thermal stability than the collagen helix (i.e. have a lower melting point), and their wider temperature range for melting suggests that very many different combinations of chain associations, to form helical regions, are present in a gel whilst in collagen there is a very highly specific, even unique, mode of chain-association to give the characteristic and highly stable helical structure.

Since $[-\alpha]$ is related to the total content of collagen-like structure present in a gel whilst the rigidity, G , is a measure of the number of network links per unit volume, the constant K in Todd's relationship may be regarded as a measure of the fraction of non-useful links that form, i.e. chain associations which do not contribute to gel strength. Thus the larger the value of K the smaller the rigidity for a given conversion of gelatin to collagen-like structure. On this view, Saunders and Ward's results indicate that with increasing molecular weight from 70,000 to 270,000 there is an increase in the proportion of non-useful chain associations. The covalent bonds between chains in multi-chain gelatins would thus seem to inhibit the development of useful network links.

Ledward and Stainsby (unpublished) have studied the complementary situation, by decreasing the molecular weight through acid conditions in order to avoid the complications that would arise if the rigidity factor also changed. The constant K increased as \bar{M}_w fell from 200,000 to 40,000, presumably on account of the increased content of highly degraded molecules. Further work is required before the correlation of $[-\alpha]$ and G^* is more fully understood.

The gelatins used by Todd had different rigidity factors and similar, high molecular weights. On the evidence available at present it seems reasonable to conclude that though the rigidity factor may define the total capacity for linkage it is not simply a measure of the ability to form useful links (or K would have varied from one sample to another).

Bello (1965) has followed the change in $[-\alpha]$ when a 5% gel, aged for a day at 4°C, is melted and has shown that a third of the collagen-like structure that had developed during gelation persisted on melting. This contrasts with the very small change in $[-\alpha]$, noted by Ledward (1966), for aggregating solutions which are just too warm to set, and so again emphasises the differing pathways for gelling and melting.

Bello has also used optical rotation changes in extensive studies of the actions of salts on gelation. Copper ions in alkaline solution are exceptionally powerful inhibitors of gelation and at pH 10, when no soluble hydroxy complexes are present, all the Cu^{++} from a very dilute solution binds to the peptide bonds in gelatin, except those involving imino acids. Under these conditions both the gel strength at 4°C and $[-\alpha]$ decrease linearly with increase in copper content. The melting point also falls, but more sharply as the copper content rises. In contrast the transition temperature stays constant. (The transition temperature is the steepest point on the graph of $[-\alpha]$ against time when the gel is heated at 0.1°C per min.) All the available evidence suggests that the copper-peptide bond complexes are thermally stable and retain their conformation without change, so that the entire change in $[-\alpha]$ on heating arises from conformational changes in those regions which are free from copper, i.e. the imino-acid rich segments. Bello (1965) claims that the data from such studies are consistent with the view, expressed by von Hippel and Wong (1963), that the formation of collagen-like structures in regions rich in pyrrolidine residues is an all-or-nothing process. Complexed copper prevents the development of collagen-like structure beyond the imino-acid rich segments, without physically cleaving the polypeptide chain, and so destroys the network.

Other salts that inhibit gelation, such as LiBr , CaCl_2 , behave quite differently from alkaline copper ions, as Bello (1965) has shown. In these instances the transition temperature is also lowered, although the content of collagen structure as judged by the value of $[-\alpha]$ for the system remains unchanged. von Hippel (1967) has urged that no attempt should be made to try to account for these specific effects until a general explanation is available for the outstanding changes produced by these salts with other macromolecules and with simple aqueous solutions.

At wavelengths below 230 nm collagen and gelatin, like other proteins, strongly absorb UV radiation and exhibit a very dramatic change of $[-\alpha]$ with λ . As λ is decreased, $[-\alpha]$ at first increases extremely sharply, then passes through a maximum, decreases even more sharply and changes sign for wavelengths less than about 200 nm. The study of this general phenomenon, the Cotton effect has become possible only very recently, using commercial spectropolarimeters. The effect is thought to be associated mainly with the conformation of the polypeptide backbone, and may prove useful in connection with the sol-gel transition, but to date there is no published work on this aspect.

3. Bond strengths

Eldridge and Ferry (1954) suggested that the melting temperature of an "equilibrium" gelatin gel, prepared in the presence of 0.15 M NaCl to reduce

electrostatic interactions between polypeptide chains to a minimum, could be treated thermodynamically just like the gelling point of a non-ionizable polymer. At the gel point just enough interpolymer bonds have been formed to create an infinite three dimensional network: at the melting point there are just enough links to hold the network together. Stockmayer's method of analysis was adapted [Stockmayer, (1944)], as this applies to any distribution of molecular weights and presumes that the sites for crosslinking are randomly distributed along each macromolecule. If all linking is fruitful (i.e. leads to a network), then $m_{cl} = c/2\tilde{M}_w$ where m_{cl} is the concentration of crosslinks, in moles per cm^3 , at the gel point—and two sites make one link, c is the polymer concentration in g/cm^3 and \tilde{M}_w is the weight average molecular weight. Since in gelation all the linking is not fruitful (some links lead to aggregation and not to a network), Eldridge and Ferry defined a coefficient f ($f < 1$) to represent the fraction of the total number of links that is useful. f is likely to depend on c , \tilde{M}_w , and thermal history. m_{cl} , in the above expression, is thus replaced by $f m_{cl}$. If the total concentration of free crosslinking sites is m_i , and the breaking of the links during melting is represented by the process 1 mole crosslinks \rightarrow 2 moles crosslinking sites then the equilibrium constant, K , for this "reaction" is given by

$$K = m_{cl}/m_i^2 = c/f \cdot 2\tilde{M}_w \cdot m_i^2.$$

Thus

$$d/dT (\log K) = d/dT (\log c) - d/dT (\log f) - d/dT (2 \log m_i)$$

at constant \tilde{M}_w . But

$$d/dT (\log K) = -\Delta H/2 \cdot 303 RT^2,$$

where ΔH may be termed the heat of crosslink breaking, and is a measure of the stability of the links. If it is assumed that $d(\log f)/dT = 0$ and $d(\log m_i)/dT = 0$, then

$$-\Delta H/2 \cdot 303 RT^2 = d (\log c)/dT,$$

and a plot of $\log c$ against the reciprocal of the absolute melting temperature will be a straight line if ΔH is independent of temperature. If the plot is curved, then ΔH at any selected temperature is obtained from the slope at that temperature. In physical terms, the assumptions mean that the coefficient f (i.e. the fraction of useful links) is constant for a series of concentrations that give gels of different melting points. This is doubtful.

The plots of $\log c$ versus $1/T$ are shallow, fairly parallel curves for ossein gelatins degraded to give samples of different average molecular weights. The slopes yield a value of about 130 kcal/mole of crosslinks for gels (20 to 55 mg/cm^3) precooled for a day at 0°C and then "equilibrated" at 15°C . The

same procedure was used for gels made and held at 0°C, and for this purpose they could also be said to be in equilibrium as it is unlikely that the melting point varied with time at 0°C, after one day. A value of 50 to 70 kcal/mole was found for ΔH . Thus the links that just hold the gel together at 0°C have about half the stability of the links just holding the gel together at 15°C.

An alternative, and independent assessment of ΔH was made by Eldridge and Ferry (1954) by taking the concentration as fixed and developing an expression, along the lines given above, for the variation of melting temperature with molecular weight. In this approach ΔH was calculated as about 50 kcal/mole, for gels held at 0°C for a day. This is in quite reasonable agreement with the value deduced from the concentration dependence of the melting temperature.

Pouradier (1967) has pointed out that Eldridge and Ferry's calculations have to utilize melting points, which are determined only by empirical and arbitrary methods, and that no information can thus be obtained on the stabilities of most of the links that hold the gel together. Consequently, Pouradier has extended this approach by calculating ΔH , for the complete array of links, through the variation of the rigidity modulus with temperature. The modulus, G , is first related to the concentration of crosslinks, using the analysis given by Flory (1944). This analysis was developed for lightly vulcanized polymers, but Pouradier considers that it is also applicable to gelatin gels even though the distance between links in a gel may be relatively small. Eldridge and Ferry's coefficient is again used to allow for the formation of non-useful links so that Flory's relationship takes the form $f \cdot m_{cl} = 1000 G/2RT$. Again the equilibrium constant K is used, and again $d(\log f)/dT$ and $d(\log m_i)/dT$ have to be taken as zero. The final expression then becomes

$$-\Delta H/2.303 RT^2 = 1/T - d(\log G)/dT.$$

When calculations are made in this way using the data of Saunders and Ward (1958) for hide gelatin gels at a concentration of 58 g/cm³, ΔH varies regularly from about 5 kcal/mole at 5°C to about 20 kcal/mole at 20°C. Moreover ΔH is independent of the molecular weight of the fractions studied by Saunders and Ward, and similar values of ΔH have been calculated from the gel strengths of the degraded ossein gelatins studied by Ferry (1948c). Although Pouradier states that the value of ΔH may perhaps be underestimated at the higher temperatures, owing to some lack of precision in the rigidities, so that it does not compare too well with Eldridge and Ferry's value, there is no reason why this average value of ΔH should be close to the figure determined from melting temperatures if there is a broad spectrum of stabilities in the links. In fact, the stabilities calculated by Pouradier support the views given by Stainsby (1962)—that, on sudden cooling, a gelatin solution is immobilized in its most random form and many weak bonds develop to form

the gel; and that on raising the temperature the links that remain are strengthened through the limited mobility of the interlink sections of each chain and the rapid local rebuilding of hydrogen bonds.

These views are further supported by the results of Petrie and Becker (1970), who used differential scanning calorimetry. Structures of greater thermal stability formed during annealing at room temperature, or above, through the formation of more highly ordered and/or larger ordered regions in gelatin gels.

4. *Ultracentrifuge studies, and the proportion of gelatin involved in gelation*

Further support for this view comes from the studies of Johnson and his co-workers, who have used the novel approach of subjecting the gel to high gravitational fields in the ultracentrifuge. In these conditions, a weak gel at 18°C (e.g. 2% lime-pretreated ossein gelatin) flows as though only a few stable bonds are present. At 4°C, in contrast, the relatively large flow rate, and its high concentration dependence, suggest that there are many weak links present (Johnson, 1968). Moreover, only part of the sample flows as the gel, the remainder behaving just like a polymer solution, i.e. having a broad, spreading boundary that migrates more slowly than the gel interface. This fraction has been called the solution component and a similar material can be obtained from a finely divided gel by extraction with water. The proportion of solution component, obtained by ultracentrifugation, increases with time during the early stages of gel maturation. Then it remains constant, even though the rigidity continues to increase, so that the change in rigidity cannot then be due to incorporating more gelatin into the gel and is due to structural reorganization. Even in the early stages after setting the change in rigidity with time is far too big to be due entirely to the incorporation of some solution component. The lower the maturing temperature, the lower the proportion of the solution component, e.g. with a 2% gel the soluble fraction fell from 40% (1 hour at 20°C) to about 3% (1 hour at 3°C). Thus the gelling ability of any particular sample of gelatin is not an all-or-nothing property, but is a matter of degree and varies with temperature of maturing, concentration of gelatin and the solvent.

The solution component is more polydisperse and has a lower mean molecular weight than the gel component. When N-terminal groups are determined the solution component has a very much higher content of imino acid end groups than the gel component (Grand and Stainsby, 1975). This is more important than the low molecular weight, and no doubt varies with the method of manufacture and the source of the collagen precursor. Though the overall amino acid compositions of the two components are similar (King, 1968) evidence is accumulating (Gross and Rose, 1975) that the solution component consists mainly of α_2 chains or their fragments.

IV. A THEORY OF GELATION

All the recent studies of gelation gels, outlined above, support the view expressed by Ferry (1948a) that the sol \rightarrow gel transformation involves the creation of a network throughout the system and that the very limited number of intermolecular contacts which are needed to form the network are stabilized by secondary forces rather than by covalent bonds. Intermolecular attractions between the links are negligible. Veis (1964) has drawn attention to the similarities between gelatin gels and the gels that can be formed by many synthetic linear polymers. In both types the balance between solute-solute interactions and solute-solvent interactions is particularly delicate when only secondary forces stabilize the system, and is strongly dependent on the solvent environment and the temperature. Thus the structure of the network is determined by the mode of formation and by the subsequent thermal history of the gel, and one given solution can be made to produce a variety of gels, some of which may be converted into other forms by suitable thermal treatments (see page 183). The structural elements of these gels have been made visible through the use of electron microscopy (Tonka, 1975).

The setting of a gelatin solution, and subsequent changes in the gel network, arise through the *partial* return of disordered gelatin molecules to the collagen structure. The more extensive reversion to collagen that can be induced in very dilute solutions of exceptionally homogeneous gelatins is discussed in Chapter 4. The mechanism of gel formation is thought to be essentially similar to reversion, though modified by the polydispersity in size and in detailed chemical composition of the gelatin molecules present and by their much higher concentration.

Once a molecular network has developed additional gelatin molecules become attached through a less specific form of intermolecular bonding, essentially electrostatic in character (as in micro-fibril formation). This leads to thickening of the framework and a stiffening of the gel, and is more likely to be important in concentrated gels and at low temperatures (when the smallest proportion of a gelatin sample remains in the fluid phase). Maturation thus involves two distinctly different processes, occurring simultaneously—the increasing order and stiffening of the junctions in the molecular network and a general thickening of the whole network. The thickening cannot be very extensive or opacity would result, accompanied by syneresis (“weeping”)—as is prevalent with many polysaccharide gels. This view gives a quite detailed description of the nature and properties of the intermolecular links in the gel network, but it does not lead to a quantitative understanding of the mechanical properties of the gels.

Ledward and Stainsby (1966) have put forward the following description of the network. At temperatures close to the setting temperature only a few

links are present and form the network. Each link is thought to involve an imino-rich section from each of three polypeptide chains, as in collagen. Each section is particularly rich in gly-pyrrol-pyrrol triplets so that, following the sequence of events in collagen renaturation as described by Josse and Harrington (1964), it has developed on each chain from a primary nucleus of locked, contiguous pairs of pyrrolidine residues by incorporating adjacent triplets of similar high stability and, possibly, some triplets containing only one pyrrolidine residue (though these are less stable). The remainder of each chain—and the major part of it—is disordered, and the average distance between links is great.

On cooling this very weak gel, additional parts of each polypeptide chain become ordered as triplets of decreasing stability are able to survive. The additional ordering is by growth on existing links and by the formation of new, but much less stable, links. In these ways the average distance between links decreases. As the temperature falls the remaining disordered sections of the chain become less mobile and the hydrogen bonding between chains, in the linked regions, becomes less readily disrupted. Thus the ability to reorganize to a more collagen-like structure is lost, although it is never lost entirely. This type of gel is, at low temperature, essentially an open network of fairly long stiffened, stable links on which a fine network of many shorter and weaker links is superposed. A snap-chilled gel made from the same solution would, at the same low temperature, be almost devoid of the long, stiffened, ordered, collagen-like links and could not acquire these characteristics by ageing. Conversely, the slower the cooling rate the more the stiffened regions develop.

On warming a gel, the least stable, short links in the fine network—the links with the lowest content of gly-pyr-pyr triplets and the loosely held additional molecules—are the first to become disordered, or melt. The strongest links remain intact to the melting point of the whole gel, and some persist in the viscous solution for a few degrees above the melting temperature.

This view requires that there is a spectrum of bond-stabilities and that the stability is related to the sequence of the residues in the triples in the linked region. It distinguishes a gelatin gel from a lightly vulcanized polymer gel, for which the network link involves only one residue from each component chain. (At present, there is unfortunately no quantitative formulation, analogous to the rubber-like elasticity theory, for systems of molecules linked over relatively long regions.) This molecular picture clearly emphasizes the differences between setting, ageing and melting. Setting involves a series of temperature and concentration dependent reactions—ordering of chain segments, random contact of similar segments and stabilization of a compound helix of the collagen-type. Maturing involves further gradual stabilization of the network and thickening. Melting, on the other hand, requires

the co-operative disruption of sets of weak bonds between chain segments.

The structural feature called the rigidity factor is still not revealed, however. It is possible that the number and distribution of covalent crossbonds, in the multistrand molecules of many high grade gelatins of commerce, may determine this factor. For instance, suitable crossbonding may favour intramolecular aggregation, and so effectively remove many imino-rich chain segments. (This would be renaturation competing with gelation.) Alternatively, the rigidity factor may reflect the proportion of chain cleavage—inevitable in manufacture—that has occurred in imino-rich sections. Quite a small change in the number of intact imino-rich sequences could prevent the formation of the interchain links required to make a gel. At present, however, techniques for the quantitative determination of imino end groups (i.e. for the proportion of cleaved imino-rich triplets) are still little used.

Veis (1964) has developed a similar molecular description of gelation, but the differences from the views expressed here are not insignificant. Veis favours the association of only two ordered chain segments, which he has called collagen folds, to create a stable unit or junction point in the network. This is thought to be insufficiently stable, particularly at the higher temperatures. Veis considers the nucleation stage to be the rate determining step and proposes that electro-static forces are involved in the alignment of segments in the formation of the collagen fold. These forces prohibit certain segments from linking, rather than acting to aid fold formation. The underlying idea is that each folded apolar region behaves as a dipole on account of the localization of predominantly charged residues in the adjacent regions. The negative "end" of one fold then prevents association with another fold that is similarly oriented in space. This is a highly questionable proposition as ionic strength does not play a major part in the stabilization of collagen, nor do pH and ionic strength have much effect on the melting point and strength of fine network gels. Veis considers that the folded units differ in stability as they are formed by random contact and that the degree of order can be improved by ageing the gel. In these respects there is no difference of opinion, but Veis states that "this reorganization can apparently take place without any major change in the net content of collagen-folded units". The views given here, on the contrary, suggest that reorganization leads to an increase in helix content through the incorporation of previously disordered sections of polypeptide chains, with a consequent shortening of the distance between the stiff links.

V. ISOELECTRIC GELS

Thus far the transparent, quivery gels that are usually encountered have been considered. These hold all the solvent when kept. Gels made from the

protein in its isoelectric condition are quite different. Such gels exude a small proportion of the solvent (or show syneresis) and are often opaque. Eagland (1974) has suggested that at concentrations only just above the threshold for gelation, and in a salt-free environment, the isoelectric gel arises mainly from the predominance of van der Waals-London attractive forces between hydrated gelatin particles. The molecules in each particle have assumed a collagen-fold configuration, prior to setting, and the inter-particle attractions are transmitted through the structured hydration regions, once the particles are sufficiently close together. At higher concentrations a more complex situation prevails, partly through the increase in counter-ion concentration, and it is probable that the mechanism for gelation then bears a close resemblance to that for the more usual gel, away from the isoelectric point. Evidence for this view is not yet wholly satisfactory, because the α -gelatin used—from rat tail tendon—was claimed to be isoelectric at a rather low pH, (Stainsby, 1974). Further progress towards a full understanding of the sol-gel transformation for gelatin will occur only through the use of well-characterized and homogeneous gelatins, and by the study of a very wide range of experimental properties, including those (such as the change in volume, or the change in heat capacity) that require extremely sensitive techniques. Unfortunately, at present, attention has concentrated almost exclusively on inhomogeneous gelatins and on commercially useful properties.

REFERENCES

- Beier, G. and Engel, J. (1966). *Biochemistry* **5**, 2744.
 Bello, J., Bello, H. R. and Vinograd, J. R. (1962a). *Biochim. Biophys. Acta.* **57**, 214.
 Bello, J., Bello, H. R. and Vinograd, J. R. (1962b). *Biochim. Biophys. Acta.* **57**, 222.
 Bello, J. (1965). *Biochim. Biophys. Acta.* **109**, 250.
 Boedtke, H. and Doty, P. (1954). *J. Phys. Chem.* **58**, 968.
 Boedtke, H. and Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 4267.
 Bourgoin, D. and Joly, M. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 204. Pergamon Press, London.
 Davis, C. E. and Oakes, E. T. (1922). *J. Amer. Chem. Soc.* **44**, 464.
 Eagland, D., Pilling, G. and Wheeler, R. G. (1974). *Faraday Disc.* **57**, 181 and 208.
 Eldridge, J. E. and Ferry, J. D. (1954). *J. Phys. Chem.* **58**, 992.
 Ferry, J. D. (1948a). *Advances in Protein Chem.* **4**, 44.
 Ferry, J. D. (1948b). *J. Amer. Chem. Soc.* **70**, 2244.
 Ferry, J. D. (1948c). *J. Chem. Phys.* **64**, 1616.
 Flory, P. J. (1944). *Chem. Rev.* **35**, 51.
 Fujii, T. (1966). *Bull. Soc. Sci. Plot. Japan.* **16**, 30-37.
 Grand, R. J. A. and Stainsby, G. (1975). In "Photographic Gelatin II" (R. G. Cox, Ed.) p. 11, Academic Press, London.
 Gross, S. and Rose, P. I. (1975). *J. Phot. Sci.* **23**, 33.
 Herman, K., Gerngross, O. and Abitz, W. (1930). *Z. physik. Chem.* **B10**, 371.
 Herman, K. and Gerngross, O. (1932). *Kautschuk*, **8**, 181.
 Idson, B and Braswell, E. (1957). *Advances in Food Research*, **7**, 236.

- Idson, B. and Braswell, E. (1960). In "Physical Functions of Hydrocolloids", pp. 25-36. American Chemical Society, Washington, D.C.
- Janus, J. W. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 214. Pergamon Press, London.
- Janus, J. W., Tabor, B. E. and Darlow, B. L. R. (1965). *Koll. Zeit*, **205**, 134.
- Johnson, P. (1968). In "Solution Properties of Natural Polymers", p. 243. Special Publication No. 23. Chemical Society, London.
- Johnson, P. and King, R. W. (1968). *J. Phot. Sci.* **16**, 82.
- Josse, J. and Harrington, W. F. (1964). *J. molec. Biol.* **9**, 269.
- King, R. W. (1968). Ph.D. Thesis, University of Cambridge.
- Ledward, D. A. (1966). Ph.D. Thesis, University of Leeds.
- Ledward, D. A. and Stainsby, G. (1966). G.G.R.A. Research Panel Paper, No. 66/1.
- Mandelkern, L., Halpin, J. C., Diorio, A. F. and Posner, A. S. (1962). *J. Amer. Chem. Soc.* **84**, 1383.
- Meyer, K. H. (1942). "Natural and Synthetic High Polymers", Interscience. New York.
- Olsen, A. G. (1932). *J. Phys. Chem.* **36**, 529-533.
- Petrie, S. E. B., and Becker, R. (1970). *Analytical Calorimetry* **2**, 225.
- Pouradier, J. (1967). *J. Chim. Phys.* **64**, 1616.
- Pouradier, J. and Venet, A. M. (1950). *J. Chim. Phys.* **47**, 391.
- Pouradier, J. and Venet, A. M. (1952). *J. Chim. Phys.* **49**, 85 and 239.
- Richardson, E. G. (1933). *Trans. Faraday Soc.* **29**, 494.
- Saunders, P. R. and Ward, A. G. (1955). *Nature, Lond.* **176**, 26.
- Saunders, P. R. and Ward, A. G. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 197. Pergamon Press, London.
- Selby, J. W. (1951). *Food*, p. 284.
- Sheppard, S. E. and Sweet, S. (1921). *Ind. Eng. Chem.* **13**, 423.
- Stainsby, G. (1952). *Nature, Lond.* **169**, 662.
- Stainsby, G. (1962). In "Scientific Photography" (H. Sauvenier, ed.), p. 253. Pergamon Press, London.
- Stainsby, G. (1969). In "Proceedings of 1st Internat. Congress of Food Science and Technology, London, 1962" (J. M. Leitch, ed.), Vol. 1, pp. 743-52. Gordon & Breach, London.
- Stainsby, G. (1974). *Faraday Disc.* **57**, 207.
- Stainsby, G., Saunders, P. R. and Ward, A. G. (1954). *J. Polymer Sci.* **12**, 325.
- Stockmayer, W. H. (1943). *J. Chem. Phys.* **11**, 45.
- Stockmayer, W. H. (1944). *J. Chem. Phys.* **12**, 125.
- Todd, A. (1961). *Nature, Lond.* **191**, 567.
- Tomka, I., Bohonek, J., Shühler, A. and Ribeaud, M. (1975). *J. Phot. Sci.* **23**, 97.
- Veis, A. (1964). "The Macromolecular Chemistry of Gelatin". Academic Press, London.
- von Hippel, P. H. (1967). In "Treatise on Collagen" (G. N. Ramachandran, ed.), Vol. I, p. 253. Academic Press, London.
- von Hippel, P. H. and Wong, K.-Y. (1963a). *Biochemistry* **2**, 1387.
- von Hippel, P. H. and Wong, K.-Y. (1963b). *Biochemistry* **2**, 1399.
- Ward, A. G. (1959). *Rev. pure appl. chem. (Australia)* **9**, 87.
- Ward, A. G. and Saunders, P. R. (1958). In "Rheology, Theory and Applications" (F. R. Eirich, ed.), Vol. 2, p. 313. Academic Press, N.Y.

Chapter 7

The Chemical Reactivity of Gelatin

R. C. CLARK AND A. COURTS*

Gelatine and Glue Research Association, Birmingham, England

I Introduction	209
II The Constitution and Occurrence of the Side-chain Groups in Gelatin	210
III Gelatin Modification Reactions	212
A. Experimental Conditions	212
B. Chemical Modification of Side-chain Groups	213
C. Crosslinking of Gelatin	223
References	241

I. INTRODUCTION

The need for chemical techniques whereby the constitution of proteins may be studied has stimulated an interest in reagents which will react with the protein molecule. A number of reviews on this field of activity have already been published (Putnam, 1953; Olcott and Fraenkel-Conrat, 1947; Harriott, 1947; Whitfield and Wasley, 1964; Gustavson, 1956; Hirs, 1967). The consequent effects that chemical modification have on the behaviour of proteins has also interested the technologist, who has contrived all manner of purposes to which this new generation of materials could be put. Gelatin has been recognized as a protein which can be particularly amenable to chemical activity. The number and the variety of reactive groups along the chain opens up the field to a very long list of potential reagents. Its ready solubility in water is both a hazard and an advantage. The advantage lies in the subsequent speed and controllability of chemical reactions; the disadvantage is that because gelatin reactions proceed most favourably in water the choice of reagent must be limited to those with suitable aqueous stability. Thus a chemical reaction in aqueous solution often depends upon selecting conditions for which the speed of reaction is faster than the rate of hydrolysis of the reagent.

* Present address: Imperial College of Science and Technology, London, England.

Technically the rewards are important.

Activity in some fields, photography for example, is prolific. Much of this type of work appears in the form of patent publications which are written to support claims rather than to explain the chemistry involved. This makes the lot of the reviewer not a particularly happy one. Furthermore, like the proverbial iceberg, a considerable amount of know-how is submerged in the confidential files of industry.

Gelatin is employed commercially for one or more of the following properties, gel formation, emulsion stabilizing, foam stabilizing, flocculant, film forming, adhesive, and as a protective colloid.

It is likely therefore that any structural alteration to the reactive group could modify the physical behaviour. This is often what is sought in the study of chemical modification. Reactions can take the form of substitution at one or more types of reactive sites which will be termed "chemical modification", or a reaction may be polyfunctional in which case it can bridge adjacent protein chains. This will be included under "crosslinking".

II. THE CONSTITUTION AND OCCURRENCE OF THE SIDE-CHAIN GROUPS IN GELATIN

Modification of the chemical constitution of gelatin can involve reactions with either the polypeptide backbone of the molecule or more commonly with the side-chain groups which are attached to the backbone. Figure 1 shows the distribution of these side-chain groups along the polypeptide backbone. Table I gives details of the structure and proportions in which the various side-chain groups occur in gelatins from three origins. Proline (2-pyrrolidine-carboxylic acid) and hydroxyproline (4-hydroxy-2-pyrrolidinecarboxylic acid) are exceptions to the general amino acid structure in that they are imino acids and due to their cyclic structure introduce a "corner" into the polypeptide chain via a keto-imide link. The chemical behaviour of the hydroxyl group of hydroxyproline is similar to that of serine or threonine. The data in Table I show that the differences between the three gelatins is only minor, and thus the chemical behaviour would be expected to be very much the same.

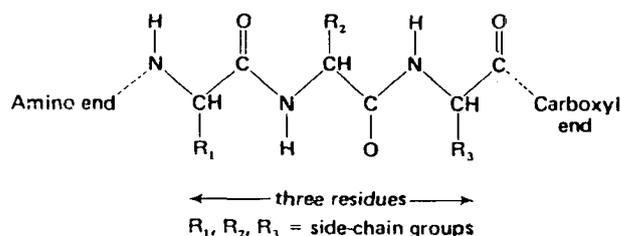


TABLE I. The side-chain groups in gelatin (Eastoe, 1955)

Amino Acid	Side-chain structure	Number occurring per 1000 residues		
		Lime process ox skin	Lime process ox bone	Acid process pork skin
amide NH ₂	see text	7.5	15.7	40.8
alanine	-CH ₃	112.0	116.6	110.8
glycine	-H	333	335	326
valine	-CH(CH ₃) ₂	20.1	21.9	21.9
leucine	-CH ₂ CH(CH ₃) ₂	23.1	24.3	23.7
isoleucine	-CH(CH ₃)CH ₂ CH ₃	12.0	10.8	9.6
proline	see text	129.0	124.2	130.4
phenylalanine	-CH ₂ C ₆ H ₅	12.3	14.0	14.4
tyrosine	-CH ₂ C ₆ H ₄ OH	1.5	1.2	3.2
serine	-CH ₂ OH	36.5	32.8	36.5
threonone	-CH(OH)CH ₃	16.9	18.3	17.1
arginine	-(CH ₂) ₃ NHC(NH)NH ₂	46.2	48.0	48.2
histidine	-imidazolylmethyl	4.5	4.2	6.0
lysine	-(CH ₂) ₄ NH ₂	27.8	27.6	26.2
aspartic acid	-CH ₂ COOH	46.0	46.7	46.8
glutamic acid	-(CH ₂) ₂ COOH	70.7	72.6	72.0
hydroxyproline	see text	97.6	93.3	95.5
hydroxylysine	-(CH ₂) ₃ CH(OH)NH ₂	5.5	4.3	5.9
methionine	-CH ₂ CH ₂ SCH ₃	5.5	3.9	5.4

One significant difference that can occur is the amount of amide ammonia that may be present. A proportion of the acidic amino acids occur as the side-chain amides, glutamine and asparagine in collagen. Pretreatment procedures, which can be alkaline or acid, bring about some degree of hydrolysis of these amide groups. On one extreme, prolonged alkaline treatment can bring about almost total loss of amide ammonia, whereas short acid treatment can give rise to a gelatin in which practically all the amide groups have remained intact. Amide hydrolysis unmasks acidic groups which will lower the iso-ionic point (pI) of the gelatin. Acid processed pigskin gelatin is usually isoionic in the pH range of 8.5 to 9.4 whereas alkali processed materials are isoionic at about pH 5, with a minimum at pH 4.8.

Molecular weight of gelatins are heterodisperse; weight averages can vary from values like 50,000 to 200,000 or more (Courts and Stainsby, 1958). There is evidence that more than one polypeptide chain is present per molecule, these chains ranging in weight about a value in the region of 50,000 to 80,000 (Courts, 1954; Steven and Tristram, 1963). This means that there is about one α -amino group per 750 to 800 residues, i.e. one α -amino group per approximately 25 ϵ -amino groups. The involvement of the α -amino group in the overall amino group chemistry of gelatin is thus only a minor one. Materials of molecular weights significantly below the limits described tend to be regarded as glues. In this category they are often associated with sub-

stances of non-collagenous origin. High molecular weight can be advantageous in that the chemical functionality is increased. This is an important factor in matters such as crosslinking.

III. GELATIN MODIFICATION REACTIONS

A. Experimental Conditions

Water is by cost and convenience the most attractive solvent in which to dissolve gelatin. Other alternatives are anhydrous liquefied ammonia, dimethylsulphoxide, formamide and dimethylformamide. Damschroeder and Gates (1950) have laid claims with respect to the use of non-aqueous solvent systems for the chemical modification of gelatin. Where no solvent is suitable it becomes necessary instead to react the gelatin in the form of a finely ground suspension, and in the majority of cases it has been found that the reaction is both slow and inefficient. Where water is used there is the problem of hydrolytic destruction of the reagent. Careful manipulation of the reaction conditions is necessary in these cases to secure optimum results; in some instances the excessive rate of decomposition of the reagent, for example, acetyl chloride, will preclude its use in water. In order to limit the destruction of the gelatin molecule in aqueous solution it is necessary to maintain temperatures below about 80°C. This temperature can only be exceeded if reaction times are short. Gelation or "setting" of gelatin places a lower temperature limit at about 35°C. pH limits for most purposes lie at about 3 and 10; here again these can be exceeded provided the time factor is kept short. Cast gelatin films can generally withstand greater extremes of reaction temperature and pH, depending on the amount of water present in the film. Control of pH in stirred aqueous media is best effected by use of an automatic titrator/pH-stat, which detects the pH by means of the usual glass electrode and controls the addition of acid or alkali to bring about the necessary correction of the pH. Many reagents are water insoluble, and particularly if solid, have to be added in suitable solvents such as dioxane, methyl ethyl ketone, or acetone. The need for very efficient stirring during these reactions is often critical, especially if the reagent being added is a crosslinker. This is because local gelation can occur around droplets of a non-dispersed reagent. After the chemical modification reaction is complete, the necessary acid or alkali is usually added to bring the final pH to between 6 and 7. Cooling will bring about setting if the dilution is not too great. The gel can then be shredded, noodled or diced and then washed in chilled water until small molecules, mainly salts arising from the chemical modification reaction have all been removed. The product can then be dried at ambient tempera-

ture in an air stream, which at the latter stages of the drying process can be heated to between 30 and 40°C. Grinding in a hammer mill then gives a powder.

B. Chemical Modification of Side-Chain Groups

1. Removal of side-chain groups

(a) Deguanidation of the arginine residue

Kennington (1958) and Davis (1958) have carried out alkaline hypobromite oxidation of gelatin. The reaction does not proceed quantitatively and is accompanied by side reactions which include degradation and cross-linking. Removal of the highly basic guanidino group gives rise to the new basic amino acid ornithine, reducing the isoelectric point of the protein. Davis notes that the hypobromite oxidation of gelatin destroys the protective colloid properties. Berger *et al.* (1958) have deguanidated gelatin by use of sodium in liquid ammonia.

(b) Deamination of amino groups

This reaction, carried out with the use of nitrous acid, has been the basis of the traditional van Slyke technique for the determination of the amino group content in proteins through the measurement of the nitrogen gas that is evolved. The amino groups, which are on the whole the most common participants in side-chain modifications of gelatin, become replaced by a less reactive hydroxyl group. The chemical properties of the deaminated product is thus significantly altered. Kennington (1961), Lichtenstein (1940) and Hitchcock (1923) have reported on the deamination of gelatin, Kennington has found that nitrous acid also gives rise to side reactions including cross-linking to an insoluble gel. The reaction probably involves the formation of a carbonium ion and hence its use as a specific side-group modifier is not regarded with favour.

2. Substitution at side-chain groups

(a) The amino groups

These residues, as already mentioned, are the most amenable sites for modification in aqueous solution. There is the α -amino group of the N-terminal amino acid, the proportion of which is small, and the ϵ -amino side-groups of lysine and hydroxylysine. The imidazolylimino group of histidine participates in a number of reactions as well. The guanidino group of arginine, being protonated at the usual mild alkaline reaction conditions, is involved to a negligible degree.

(i) Organic acid halides and anhydrides

Optimum reaction conditions in aqueous gelatin solution for these reagents

TABLE II. Properties of N-acetyl gelatins

Gelatin and derivative	Substitution (%) at		pI	Rigidity modulus* (dynes/cm ²)	Viscosity† (cP)
	(amino)	(amino + imino)			
parent—277	—	—	5.20	77,300	6.5
acetyl—277	35	35	4.75	77,000	7.0
acetyl—277	63	69	4.51	79,000	7.4
acetyl—277	90	97	4.31	77,300	7.6
parent—281	—	—	9.07	89,000	7.8
acetyl—281	31	32	5.73	87,700	7.5
acetyl—281	59	68	5.15	87,700	7.2
acetyl—281	92	96	4.75	89,000	7.7

* Rigidity moduli were measured on 6½% gels matured for 18 hours. at 10°C.

† Viscosity measurements were on 6½% solutions at 40°C.

are secured with pH values in the region of 8 to 9. Typical temperatures used are 40 to 50°C. Since the reagent, liquid or solid, is usually poorly soluble in water and the reaction products cause a fall in the pH value, it is necessary to mix vigorously while adding the reagent, in a suitable solvent carrier if needed, slowly through a fine jet in a thin stream. Substitution of the α and ϵ -amino groups takes place, while hydroxyl carboxyl and guanidino groups are not usually attacked. The imidazolylimino group of histidine is a difficult case on which to generalize as the stability of the substitution products are rather variable according to the reagent involved. Thus, Clark (1963) has shown that arylsulphonylation at pH 8 to 9 with benzenesulphonyl chloride, followed by adjustment of the product to neutrality, gave a gelatin with a substituent at the imidazolylimino group. Aroyl substitution with benzoic anhydride, on the other hand, gave a gelatin with no substituted imidazolylimino groups. Measurement of imidazolyl group substitution by means of titration curves gives rise to a certain amount of difficulty owing to a small degree of overlap between the carboxyl and histidyl regions of the curve. Carboxyl groups are in vast excess compared with histidine, as can be seen from Table I.

Kennington (1958) has acetylated gelatin using acetic anhydride in aqueous and non-aqueous media. In the latter instance he found that, besides acetylating the amino groups, hydroxyl groups were also converted to the O-acetate. Physical properties of the N-acetyl derivative were found to be similar to that of the unmodified gelatin. Water solubility, viscosity and gel strength are the usual criteria for comparison. Due to masking of the amino groups it was found that the pI of the protein was reduced. This effect has been extensively followed by Gibbs (1964) and the results are summarized in Table II.

Sanders and Nassau (1960) and Riso (1964) have described the structure of

the Maypon Trade Mark protein detergents (Maywood Chemical Works, U.S.A.). Casein or gelatin is lauroylated at the amino groups to give a detergent-like product; the protein serves as the ionic region and the fatty acid chains function as the non-ionic "tails". The products are claimed to have excellent scouring properties. They are non-toxic and non-irritant (making them suitable for hair shampoos). These detergents are also biodegradable. Reaction products of fatty acids and gelatin have also been used by Monier (1944) as additives to phenol-formaldehyde resins.

In the dicarboxylic acid series succinic anhydride has been investigated by a number of workers (Yutsy and Frame, 1950; Klotz and Heiney, 1962; Klotz *et al.*, 1965; Bogomolova and Znamenskaya, 1967). Unlike the monocarboxylic series which give rise to a neutral amide, succinylation attaches an additional carboxyl residue at each site of reaction, and thus it would be expected that these proteins would be isoelectric at pH values even lower than encountered with the monocarboxylic acid series. The advantages in these reactions of the acid anhydrides over acetyl chlorides are twofold. Firstly no salt impurities are introduced into the reaction mixture and secondly the rate of reagent hydrolysis is generally slower. Tourtellotte and Williams (1958) have succinylated gelatin for the purpose of using it as a blood plasma expander.

Derivatives of maleic anhydride and *o*-phthalic anhydride have also proved successful (Gibbs, 1964) in high level substitution. Gibbs' results are summarized in Table III.

TABLE III. Properties of N-diacyl gelatins

Gelatin and derivative	Substitution (%) at		pI	Rigidity modulus (dynes/cm ²)	Viscosity (cP)
	(amino)	(amino + imino)			
parent—277	—	—	5.20	77,300	6.5
maleyl—277	81	96	4.03	71,300	8.9
succinyl—277	81	99	4.19	72,000	9.4
phthalyl—277	86	99	—	57,700	8.5

Phthaloyl—fatty acid substituted gelatins have been utilized by Gates and Miller (1960) for coagulation of photographic emulsions.

With a special reference to the succinylation of porkskin gelatin, Tourtellotte and Markes (1963) consider this derivative to have significantly improved drying rate characteristics from the gel state and improved viscosity stability as a sol. Both properties are important in its utilization for pharmaceutical capsules.

Benzoylation of gelatin may be carried out in aqueous solution with the use of benzoyl chloride. Gibbs (1964) discusses the properties of this reagent and

demonstrates the advantages of benzylation using benzoic anhydride, which hydrolyses relatively slowly and does not lead to a high ash content of the gelatin derivative. Considerably lower quantities of benzoic anhydride are required for high substitution levels, for example 120% of theoretical give nearly 100% substitution compared with a requirement of over 400% of benzoyl chloride. The product resembles the original gelatin in respect to gel strength and viscosity, but differs in that its solubility in water when iso-electric is considerably reduced. This is attributed to the presence of the now numerous bulky hydrophobic aromatic residues covalently bound to the protein. When the reaction is performed on an alkali process gelatin of pI in the region of 5, a product of pI about 4 to 4.5 will be produced. This modified gelatin will remain in aqueous solution in similar concentrations to the original gelatin provided the pH is in excess of about 6. Slow reduction of the pH will give rise to a turbidity about pH 5.5. At about pH 4.3 a dense protein-rich gummy plastic phase, termed a protein coacervate, separates from the solution. If the pH is lowered even further, at 3.5 it is found that a slow resolution of the coacervate will commence, the process being accelerated by further reduction in the pH. This phenomenon is reversible by readjustment of the pH to higher values again, provided that highly acidic or alkaline conditions, which hydrolyse the protein, have not been sustained for prolonged periods. Modified gelatin coacervates are generally found to be more soluble in non-aqueous solvents than the unmodified gelatins. Practical uses to which this pH coacervating property may be put are discussed in Chapter 13, III.

Alkylsulphonylation of gelatin presents a number of problems. Treatment of gelatin with methanesulphonyl chloride has been described by Clark *et al.* (1965). The reagent is rapidly hydrolysed by water, making it necessary to add it in amounts which are in considerable excess of the theoretical requirement. Where a large excess of reagent is employed to overcome hydrolysis, this can lead to crosslinking and insolubility. Where a particular type of gelatin is amenable to this condition, for example 400% theoretical of methane sulphonyl chloride has been used to effect full substitution, then degradation prevents the isolation of a satisfactory material. The pI of the product is again lower than that of the original gelatin, thus the 17% substitution of a gelatin of pI 5.20 gave a derivative at pI 4.78 while following 27% substitution it was at pI 4.29. Since the substituent introduced is, like acetyl, of such a small size it is found not to give rise to a region of coacervation from aqueous systems.

Arylsulphonylation using halosulphonyl reagents such as benzenesulphonyl chloride and toluenesulphonyl chloride is reported by several investigators (Yutzy and Russell, 1952; Lowe and Griffin, 1952; Lowe and Frame, 1952; Yutzy and Frame, 1952; Yutzy and Frame, 1955; International Polaroid Corp., 1966; Gevaert Photo-Producten, 1961a; Gurin and Clarke, 1934;

Wood, 1952; Clark *et al.*, 1965). When the reaction is carried out in aqueous solution at pH values in the region of 9 the α and ϵ -amino groups become converted to the sulphonamides. Under these reaction conditions it is found that the guanidino group is not involved to any detectable degree. Gurin and Clarke (1934), on the basis of a positive Pauly test on benzenesulphonylated gelatin, assumed that the imidazole of histidine had remained unsubstituted. In view of the instability of m-benzenesulphonylimidazole to moderately strong acid or alkaline conditions (Staab and Wendel, 1960) it is possible that the test conditions brought about hydrolysis of the benzenesulphonylimidazole group. Staab and Wendel's work shows that benzenesulphonylimidazole is moderately stable in neutral aqueous solution. The alkaline conditions which they chose for their hydrolysis studies are unfortunately excessively drastic for useful comparison with the conditions utilized for the benzenesulphonylation of gelatin. Drawing from the experiences with the fluorescent end-group reagent 1-dimethylaminonaphthalene-5-sulphonyl chloride (Gray, 1967) it would seem that the imidazole substitution products are formed. Hydrolytic breakdown of these groups is more rapid than the derivatives of the α - and ϵ -amino groups, for under the conditions used for cleavage of the protein to amino acids (typically 6N HCl at 110°C for 18 to 24 hours) the imidazole-sulphonamide substituent is removed. Leach (1966) does not support the opinion that benzenesulphonylation of the imidazole groups in gelatin takes place. Conclusions are based upon the results of a modified formol titration procedure.

Physical properties of the arylsulphonylated gelatins resemble the description already given for the benzoylated gelatins. The pH dependant coacervating property of these modified proteins is widely used for the coagulation of photographic emulsions. This step is useful for the elimination of unwanted salts. Presumably these proteins could be used in a similar way for the clarification of water. A further important use is in the preparation of microscopic capsules (Clark *et al.*, 1967; Gevaert Photo-Producten, 1963b). Solids or water-immiscible liquids, when present in aqueous suspension or emulsion (the droplets or particles being typically 10 microns in diameter) can be coated with arylsulphonylated or aroylated gelatin by adding the gelatin derivative to the aqueous phase at the pH in which it is readily soluble, and then slowly adjusting the pH to a value which will bring about coacervation of the protein. Separation from the aqueous phase onto the particle-water interface takes place, thereby encapsulating the discontinuous phase of the emulsion. Microscopic capsules are used in large amounts for the manufacture of carbonless pressure-sensitive copying paper. Dalton (1965) describes other uses.

Alternative reactions leading to acylation or aroylation can be performed with the use of activated esters (Ellins *et al.*, 1956). Examples of such reagents

are *p*-nitrophenyl acetate, *isopropenyl* acetate, methyl *o*-chlorobenzoate, *p*-nitrophenyl benzoate and phenyl salicylate. The ester breaks down to yield the acyl or aroyl residue which then combines with the amino groups of the protein.

(ii) Miscellaneous aryl-substituting reagents

Aryl isocyanates and isothiocyanates, for example, the Edman reagent, phenyl isothiocyanate, will combine with the amino groups of gelatin to give the corresponding substituted urea or thiourea derivatives (Clark and Courts, 1965). These derivatives show the same coacervating properties of the arylsulphonylated and aroylated gelatins already described. Indanetrione (ninhydrin) can also be used to produce a gelatin derivative which will coacervate in acid aqueous media.

(iii) Reaction with activated haloorganic reagents

Aryl halides, such as benzyl bromide, biphenyl-halomethanes, benzoyl halomethanes and phenyl benzoylhalo-methanes can be reacted with gelatin amino groups (Gevaert Photo-Producten, 1962a, 1962b, 1965). 1-Fluoro-2:4-dinitro-benzene, first described by Sanger (1945) as an end-group reagent for insulin will generate 2,4-dinitrophenyl substituted amino groups on the gelatin molecule, giving a derivative which coacervates from aqueous solution in mildly acid media. It was shown by Courts (1954) that the 2:4 dinitrophenyl derivatives of alkali-processed gelatins were more hydrophobic than those derived from acid-processed materials of similar number average chain weight so that the effect of acidification might be regarded as precipitation rather than coacervation. β -ketoalides (Gevaert Photo-Producten, 1965, 1965a), α -haloaliphatic acids (Miller *et al.*, 1966) and β -halonitriles (Butskus *et al.*, 1960) have been used to modify gelatin. Carboxymethylation of amino groups readily takes place if the gelatin in aqueous alkaline solution is treated with chloracetic or bromacetic acid (Gates *et al.*, 1965). There are numerous other interesting reactive haloorganic structures utilized by the covalent-binding dye industry, for example, α -haloacylaminomethyl groups (Blass and Wehrli, 1965) and α -halogeno-acrylic acid amides (Schoenauer and Siegrist, 1965). Yet more active halogen groupings are listed by the Cassella Farbwerke Mainkur A.-G. (1965) which includes chloroderivatives of the *s*-triazines, pyrimidines, pyridazines, pyrazines, pyridazones, quinoxalines, quinazolines, phthalazines, benzoxazoles, benzothiozoles and benzoimidazoles. Some of these covalent-binding dyes, together with other types of covalent-binding dyes, are used for producing coloured gelatins for photographic emulsions (Ciba, 1962, 1966a; Gevaert-Agfa, 1966b).

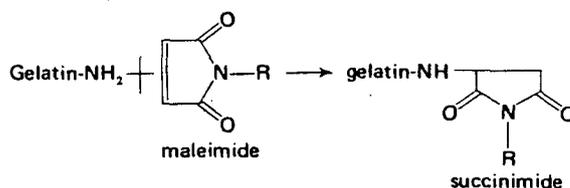
(iv) Carbamoylation

Gelatin, if treated at about neutrality with cyanate or nitrourea in aqueous

solution, gives a product with the amino groups converted to urea radicals (Stark, 1967; Schwander, 1955; Young and Christopher, 1957). The pH solubility range of the protein, along with the other physical properties is not noticeably changed by the modification. The pI is found to be very slightly lowered. Leach (1966) has examined carbamoyl substituted gelatin for the purpose of evaluating a modified formol titration procedure for the measurement of combined amino and imidazolyl group substitution. From the chemical standpoint the reaction amino group of the original gelatin is now replaced by the more inert urea function. Young and Christopher (1960) have combined the field of urea-formaldehyde plastics with protein chemical modification by treating carbamoylated gelatin with formaldehyde to produce a protein-urea-formaldehyde condensate.

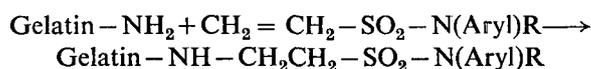
(v) Reaction with double bonds

Kovacic and Hein (1959) have investigated the reaction of the maleimide group with gelatin and have concluded that combination with the amino groups does take place:



The grafting of acrylic type polymers to gelatin has been carried out by numerous workers (Krajewski, 1966; Pieper *et al.*, 1964; Dann *et al.*, 1958, 1959; Societá Generale per l'Industria Mineraria E. Chimica, 1961; Khismatullina *et al.* 1964, 1966; Kodak Ltd., 1960; Butskus *et al.* 1960; Denisova and Sinyakova, 1968; Illingsworth *et al.*, 1958; Caldwell, 1960). Reagents that have been used include acrylamines, acrylamides, acrylonitriles, methylmethacrylates, vinyl sulphones, vinylsulphonate esters and sulphonamides, styrenes and vinylpyridines, allylamines, butadienes, isoprene and chloroprene. Unlike the previous side-chain substitutions already discussed in this chapter, these reactions do not necessarily end when one molecule of the modifying reagent is attached per available site on the protein. The addition can continue, as in vinyl polymerization, to give an extended carbon atom chain attached to the side-group of the gelatin molecule. The length of the side-chain polymer graft formed is dependent upon the reaction conditions prevailing. Peracids or peroxides are used as initiators of the reaction. Surfactants may or may not be necessary to maintain the monomer dispersed in the aqueous reaction medium. The properties of the products can be tailored according to the choice of monomer, these varying in hydrophobic-hydro-

philic character. Water solubility and good compatibility of these products with gelatin in aqueous solution are useful properties for use in photographic emulsions. Sites for side-chain grafting can be expected to be at the amino, imidazole or hydroxyl groups. However, Khismatullina *et al.* (1964) report that modifying with unionizing acrylics (esters, amides and nitriles) brings no great change in the titration curve, implying that the amino group is at least only a minor participant. Krajewski (1966) claims the use of unsaturated polymerizable acidic monomers for the purpose of lowering the isoelectric point of gelatin. Reynolds and Seddon (1966) have grafted vinyls to gelatin and nylon by first chlorinating the peptide nitrogen, to which the graft subsequently becomes attached. Agfa A-G (1961) have reacted N-arylvinylylsulphonamides with gelatin in aqueous alkaline medium, describing the addition as taking place on the amino groups—

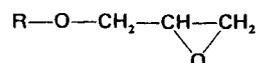


where R may be a hydrogen, alkyl or acyl.

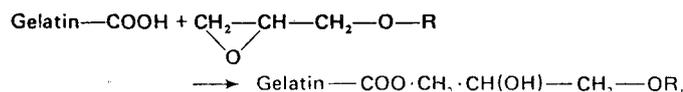
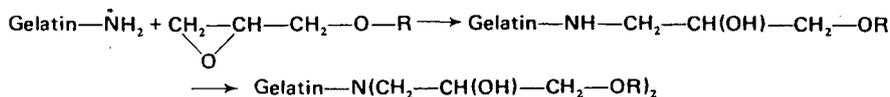
These products, like the arylsulphonylated and aroylated gelatins, coacervate from aqueous solution in mildly acid medium (pH range 2.5 to 4.5) and can therefore be used for the flocculation of photographic emulsions.

(iv) Reaction with epoxides

The chemistry of epoxides is reviewed by Rosowsky (1964). Merrill (1965) has prepared a hot-melt adhesive from gelatin by reaction with glycidyl ethers.



R may be alkyl or aryl, the residue being selected according the requirements of the modification. Reaction can involve either the acidic or amino groups.



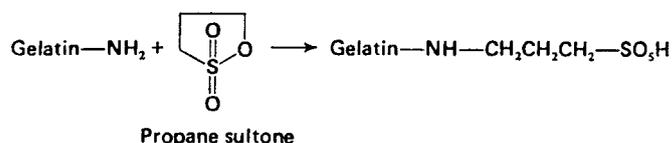
In general the rate of reactivity of gelatin with epoxides is governed by pH. Thus Whiteside *et al.* (1964) give the following pot lives in minutes for a

gelatin reacted with a polyepoxide at the indicated pH values, all measured at 60°C.

360 (pH 5) 210 (pH 6) 180 (pH 8) 56 (pH 9) 9 (pH 10)

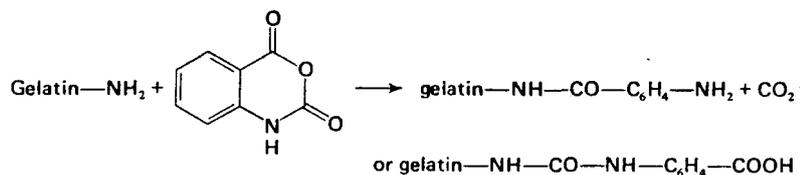
The General Aniline and Film Corporation (1967) describe the reaction of alkylene oxides, for example, propylene oxide with gelatin, catalysed by alkali metal hydroxides or alkoxides. The products are claimed as suitable for the coagulation of photographic emulsions.

(vii) **Reaction with the cyclic molecules, butane sultone, propane sultone and isatoic anhydride**
Sulphoalkylation of the amino residue of gelatin can be easily performed (Kodak, 1966; Fuji Photo, 1968) by the use of propane sultone or butane sultone.



Disubstitution of the nitrogen can take place in an excess of the reagent. Modification is carried out in aqueous alkaline solution. The strongly acidic sulphate group attached to the protein will bring about a fall in the pI. The nitrogen atom retains its basic character; it is possible to quaternize it by reaction with three molecules of the sultone. Physical properties of gelatin are only very slightly altered by sulphoalkylation. Advantageous photographic properties are claimed. The secondary amino group is still amenable to involvement in reaction with hardening agents.

Isatoic anhydride has been used for the amino-benzoylation of gelatin (Kodak, 1951).



Aqueous alkaline medium is suitable in which to carry out this reaction. The covalently bound aromatic amino groups are useful in that they can be diazotized and coupled to give gelatiny azo dye derivatives.

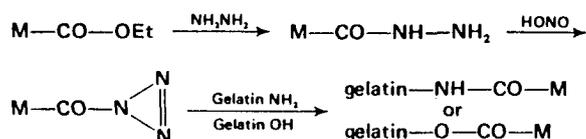
(viii) **Guanidylation**

Kenchington (1958) has carried out this procedure by use of O-methylisourea, which converts the amino groups to guanidino groups in a quantitative yield. Two new amino acids are formed; homoarginine from lysine and

hydroxyhomoarginine from hydroxylysine. Ornithine, formed by breakdown of arginine during collagen pretreatment for gelatin manufacture, is reconverted back to arginine. Viscosity and gel rigidity of the gelatin is altered only slightly, in accordance with the expected amount of degradation of the polypeptide chain that would arise from the high pH conditions that are necessary for the modification. The reaction of cyanamide with gelatin is also reported as increasing cationic properties (American Cyanamid, 1968).

(ix) Carboxylazide modification reaction

Humphrey and Yuill (1939) have used this reaction for the preparation of N-carbobenzoxytyrosyl gelatin. Agfa, A.-G. (1965) has since made use of this reaction for the covalent bonding of chromogen couplers to the gelatin molecule.



where M = modifying substituent

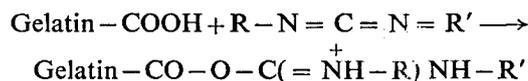
(b) Carboxyl groups

(i) Methylation of carboxyl groups

Kennington (1958) performed this reaction on gelatin by treatment with dry methanolic sulphuric acid. Taking the extreme reaction conditions into account, direct effects on viscosity and gel strength were considered not to have taken place. The methyl ester groupings were found to be labile to acid and alkali. Since the pI, as a result of the carboxyl group masking, was expected to be well into the alkaline range, it was not expected that accurate measurements of the pI could be made by the mixed bed resin ion exchange technique of Janus, *et al.* (1951). Saponification of ester groups would take place during the course of measurement. Combret (1961) has partially esterified gelatin in the course of his studies on the dissociation of carboxyl groups of proteins.

(ii) Treatment with carbodiimide

Activation of the carboxyl group on any protein molecule can be achieved in the following way (Hoare and Koshland, 1966).



Treatment with a compound having an amino group, for example, glycine methyl ester, will give rise to side-chain glycine groups thus,



The amino group involved can equally be that of another protein molecule in which crosslinking will take place. This will be discussed in the appropriate section dealing with crosslinking reactions.

(c) Hydroxyl Groups

(i) Carboxyl azide modification reaction

The conversion of a hydroxyl to an ester by this method has already been described in Section III. B. 2. a. ix.

(ii) Sulphate esterification

Reitz *et al.* (1946) found that if gelatin was carefully treated in the cold with concentrated sulphuric acid, sulphates and sulphonates of the available hydroxyl groups were formed without excessive destruction of the protein taking place. The product would be very acidic in properties.

(iii) Acylation

Kenchington's report (1958) on the acetylation of hydroxyl groups in gelatin using acetic anhydride in waterless media is referred to in Section III. B. 2. a. i. The O-acetyl groups were found to be unstable in mildly alkaline conditions.

C. Crosslinking of Gelatin

1. Covalent crosslinking

(a) General remarks

The literature on this subject is mainly in patent form. It is very extensive and only a limited coverage of the topic will be attempted in a review of this size. The hardening of photographic emulsion coatings has been reviewed by Fuchs (1961) and Hornsby (1956). Bjorksten (1951) has dealt with the crosslinking of proteins in general as well as discussing its significance in relation to human age and mortality (Bjorksten and Andrews, 1960).

Crosslinking of gelatin is most commonly carried out in aqueous solution or on cast layers of the protein as with photographic films and papers or abrasive coated sheets. Concentration is an important factor, as distance between molecules will determine whether linking will take place between each other (intermolecularly) or within a single molecule itself (intramolecularly). Intermolecular crosslinking multiplies the molecular weight, which in the case of a high grade gelatin is already large. In aqueous solution it is manifested by a rise in viscosity. If allowed to continue, the solution becomes viscoelastic; it will ascend up the driving shaft of a rotating immersion type stirrer (known as the Weissenberg effect) and if conditions are right, can be made to siphon itself over the edge of a beaker by tilting the vessel sufficiently

to allow some of the contents to flow over the lip. Further crosslinking causes the flow properties to disappear altogether and the material forms a rigid gel which will either have an elevated melting point or fail to revert to a sol at all when heated. The progress of these effects depends upon the degree of crosslinkage present. Crosslinked gelatin solutions generally give rise to weak gels on cooling, presumably because the covalent links introduced in the sol phase interfere with the freedom of the polypeptide chains to adopt the conformation necessary for the gel structure. On the other hand, if gelatin is crosslinked while in the gel state, dramatic increases in the rigidity occur. The melting point of the gel rises with increased degree of crosslinking until it reaches the boiling point of water, whereupon it is termed infusible. Dried gelatin films or particles on treatment give even "tighter" crosslinked structures than aqueous gels, due again to the concentration factor, but on the other hand it is difficult in these cases to achieve efficient penetration of the crosslinking reagent. Small molecules such as formaldehyde usually are the only effective agents under these conditions. An alternative procedure incorporates the crosslinker into the mix prior to casting of the film and drying. In this case crosslinking should not take place in any substantial amount until casting of the film is complete. In the photographic industry it is frequently the alkalinity of the processing solutions that is used to promote the hardening reaction in the emulsion coat. The crosslinking of gelatin films gives rise to a reduced tendency to swell in the presence of water or humidified air. Dimensional stability and resistance to mechanical damage is much improved. This is an important factor in the case of photographic coatings and abrasive coated media such as sandpaper. A raised melting point or complete resistance to melting is advantageous in tropical environments as well as in various accelerated photographic development procedures where warmed and concentrated processing solutions are used. Jopling (1965) has studied some of the mechanical properties of crosslinked gelatin films. Coopes (1968) has investigated the effect of crosslinking gelatin films on their optical rotation. Crosslinking is synonymous with tanning, a term more familiarly associated with the leather industry. From the chemical standpoint leather tannage and gelatin crosslinking are the same thing. Useful reviews have been written by Gustavson (1949) and Bjorksten (1951). The two fields of leather tannage and gelatin crosslinking have been combined in the following process: unwanted leather off-cuts are comminuted and mixed with gelatin, crosslinkers and a number of other agents which will improve the mechanical properties of the final product. The mixture is pressed into sheets which serve as an artificial leather substitute (United Shoe Machinery Corp, 1962).

As regards hardening in the photographic industry the objectives have to be two-pronged: an improvement in the mechanical properties of the emulsion

coat is sought, but at the same time no deterioration in the photochemical processes must arise. It is in attempts to achieve these aims that the types of reagents that have been examined are so numerous.

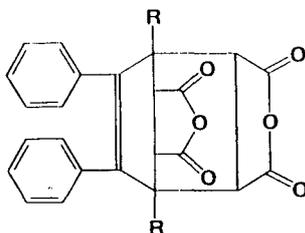
As a prelude to dealing with the formal categories of chemical crosslinking agents there are a few special cases which deserve mention. (i) Gelatin may be rendered insoluble, presumably as a result of the formation of covalent crosslinks, as a result of exposure to radioactivity, X-rays or to an electric corona discharge. The effect of high energy radiation on proteins is discussed by Whitfield and Wasley (1964). A photo-catalytic dye system is mentioned in Chapter 12. (ii) Prolonged conditions of extreme desiccation, such as storage over phosphorus pentoxide or heating in an oven at about 100°C also induces insolubility in water. Loss of water between carboxyl side-groups and hydroxy or amino groups of other gelatin molecules could give rise to ester or amide bonds (Yannas and Tobolsky, 1967). For the behaviour of gelatin after heating at higher temperatures, see Yannas and Tobolsky (1968). See also Chapter 8, VII. A. (iii) The normally monofunctional substituting reagents, acetic anhydride, benzenesulphonyl chloride or methanesulphonyl chloride can under suitable conditions behave in an anomalous manner. The crosslinking action that acetic anhydride can have on collagen and gelatin has been investigated by Bello and Bello (1963) and also observed by Kenchington (1958). Benzenesulphonyl chloride or methanesulphonyl chloride, if in contact with an aqueous solution of gelatin at mildly acid pH (e.g. between 4 and 6) will cause it to form an infusible gel almost instantaneously. In the case of benzenesulphonyl chloride it is found that extremely small amounts of the reagent appear to have been consumed in the reaction (Laird, 1964). Possible mechanism by which the crosslinking could take place is the formation of a mixed anhydride between the reagent and the carboxyl groups of the gelatin, followed by breakdown of this anhydride by involvement of a neighbouring amino group to give a covalent amide crosslink and the release of the sulphonic acid.

(b) Covalent crosslinking reagents

Most of the reagent types described in Section III. B. 2. are represented again here. Instead of there being only one functional group, crosslinkers have two or more and so can be involved in reaction with at least two different gelatin molecules. A few of the reagents, for example the mono-aldehydes, are exceptions to this generalization.

(i) Acid halides and anhydrides

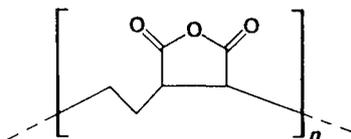
Allen and Carroll (1955) describe the use of anhydride hardeners. As would be expected from the discussions in the earlier part of this review, these reagents work best in the alkaline range (pH 7.5 to 9.0). Examples of



R = CH₃ or H

reagents are 7,8-diphenylbicyclo (2,2,2)-7-octene-2,3,5,6-tetracarboxylic dianhydride

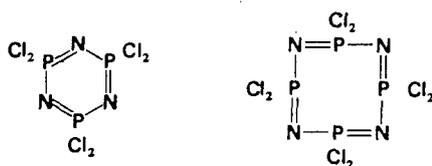
and 3-methoxy-7-methyl-1,4a,5,6,7-hexahydro-1,4-ethanonaphthalene-5,6,9,10-tetracarboxylic acid dianhydride, as well as 1,5-diketopentane-1,2,4,5-tetracarboxylic acid dianhydride and 1,5-diketo-3-methylpentane-1,2,4,5-tetracarboxylic acid dianhydride. Gevaert (1961) make use of the pyromellitic dianhydrides. Polyanhydrides of interest are those formed on copolymerization of maleic anhydride with other vinyl monomers, for example, poly(ethylene/maleic anhydride) of a molecular weight in the region of a quarter- to half-a-million.



These polymers are incorporated into microcapsule mixes (National Cash Register Co., 1967). Use of maleic anhydride copolymers as hardeners is mentioned by Minsk and Cohen (1967) as well as Henkel and Cie G.m.b.H. (1963). Instead of using the anhydrides it is possible to crosslink the protein by heat in the presence of dibasic organic acids (Drew and Dutton, 1946).

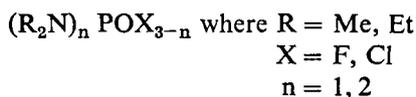
Concerning the acid chloride hardeners, Allen and Carroll (1955a) claim the use of such compounds as terephthaloyl chloride and 4,4'-diphenylmethanedisulphonyl chloride. Reagents of a similar nature are described by Gevaert Photo-Producten (1959, 1960b). *m*-Benzenedisulphonyl chloride crosslinking of gelatin has been studied by Kenchington and Lauder (1958). Fluorosulphonyl crosslinkers are named in a patent by Gevaert Photo-Producten (1960). The tribasic reagent, cyanuric acid, has been put to use in various forms. As cyanuric chloride it will crosslink gelatin most effectively (Leach, 1965). 2,4-dichloro-6-hydroxy-*s*-triazine, in the form of a water-soluble salt, has been claimed as a photographic hardener by the Fuji Photo Film Co. (1966). The reactive cold dyeing Procion dyes (I.C.I., Dyestuffs Division, Manchester, England) generally contain a 2,4-dichloro-*s*-triazin-6-ylamino grouping and hence are capable of crosslinking gelatin (Clark, 1963).

Kinetics of the reaction of these dyes with gelatin has been examined by Mullen (1962). The work of Wirnik and Tschekalin (1962) has shown that the amino groups, including the imidazolylimino nitrogen, are involved in the reaction with these dyes. Ciba Ltd. (1962) have claimed the use of 1-(2,4-dichloro-*s*-triazin-6-ylamino)-benzene-4-sulphonic acid and other similar reagents as hardeners for gelatin. Chloropyrimidines such as 2,4,6-trichloropyrimidine are also effective crosslinkers (Clark, 1963). Also falling into the acid chloride class are a range of phosphorus-containing compounds. Two cyclic phosphonitrilic chlorides are named by Gevaert Photo-Producten (1965a).

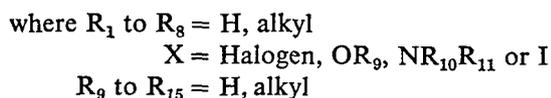
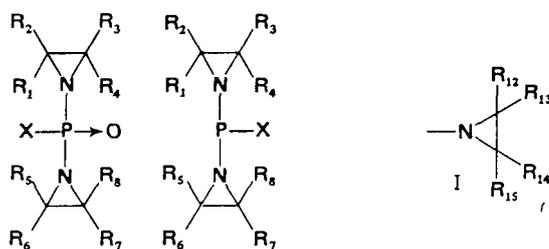


Their chemical behaviour will be similar to that of the chlorotriazines already mentioned. Substituted amino-halophosphine oxides and related compounds are also described as hardeners (Gevaert Photo-Producten, 1963; Harrow, 1966). In addition to the halogen group there are aziridinyl radicals as well. These are discussed in Section III. C. 1. b. iv.

General formulae



and



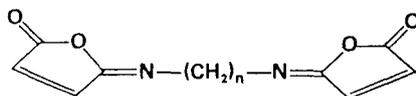
(ii) Activated halogen hardeners

Gevaert Photo-Producten (1965) have hardened photographic emulsions

with compounds such as N,N'-dibromoacetyl- and N,N'-dibromopropionyl-derivatives of hydrazine, dimethylhydrazine, ethylenediamine, propylenediamine and urea. Bis(chloroacetyl) amine is also included. Bishalomethyl esters of the general structure, $(XCH_2OOC)_2M$, where X is halogen and M is either a connecting bond or a divalent radical are claimed as hardeners (Wilson, 1966). Eastman Kodak (1966) describe hardeners with the structure $X-CH_2-CH_2-CO(ACO)_mCH_2COOX$, where X is a C₂ to C₅ alkoxyhalogenated group, m = 0 or 1 and A is $(CR_2)_n$, n being less than 11 and R being H or a C₁ to C₄ alkyl group. Double-ended bisbromo compounds, for example, $Br-CH_2CH_2-CO-NH(CH_2)_6-NH-CO-CH_2CH_2Br$, are used by Burness *et al.* (1967). There are also bis- α -haloacyl esters of polyethyleneglycol (Morgan and Starkey, 1956), taking the general form, $R-CO-(CH_2CH_2O)_n-CO-R$, where R could be CH_2Cl , $-CH_2Br-$, or Cl_3C- . By carrying out polymerization of a monomer having an active halogen group, for example, γ -chloro- β -hydroxypropylacrylate (Gevaert Photo-Producten, 1962), a polyfunctional hardener of a novel type is obtained. Similarly, polymeric chloroacrylamides, such as poly(p-(chloroacetyl-amino)methacrylanilide) have also been prepared (E. I. du Pont de Nemours and Co. 1967). Ciba (1967) claim hardeners with reactive groups comprised from α -haloacrylamide radicals. An example of this compound is 1,4-bis(α -bromoacryloylamino) benzene-2,5-disulphonic acid. Bischloroformyl esters of glycols and polyglycols are described by Putman (1950), and β -chloroethylesters by Perutz Photowerke (1963).

(iii) Active double bond type hardeners

The behaviour of gelatin with the crosslinking reagent, divinylsulphone is reported by Kenchington and Lauder (1958) and is made use of as a hardener by Farbenfabriken Bayer, A.-G. (1944). 1,3,5-triacryloyl-hexahydro-s-triazine and copolymers having active double bond side groups are described by Gates (1966). Birr and Walther (1958) also name similar compounds. Bis(*isomaleimide*) hardeners which have the following structure,



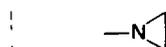
where n = 4 to 10

have been claimed by Burness and Wilson (1966). By conversion of polyvinyl alcohol to a maleic half ester Gevaert Photo-Producten (1963a) have produced a material capable of crosslinking gelatin. Alkylene or arylene bis-maleimides, such as 1,4-di(N-maleimido) benzene are also made use of by Gevaert Photo-Producten (1959a). Minsk and Cohen (1966, 1967) describe acrylic copolymers in which the reactive crosslinking side-chain group is

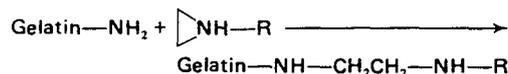
comprized of the maleimido radical attached via the nitrogen atom. Side-chain acrylic groups can also be included. Vinylsulphonyl compounds and vinylcarbonyl compounds are named amongst other reagents as hardeners by V.E.B.-Filmfabrik Agfa Wolfen (1961). Bis[(dialkylamino) methyl] substituted ureas are listed in du Pontder Nemours (1968) and β -chloro- β -benzo acrylic derivatives in Zanker and Reicheneder (1972). The Fuji Photo Film Co (1967) have grafted polymerized vinyl monomers to gelatin. Some of the monomers chosen have active chlorine atoms at the end opposite to the vinyl group. An example is $\text{CH}_2 = \text{CH} - \text{CO} - \text{NH} - \text{C}_6\text{H}_4\text{SO}_2\text{NH} - \text{CH}_2\text{CH}_2 - \text{Cl}$, which will react with the amino groups of neighbouring gelatin molecules and so introduce covalent crosslinkages via the acrylic graft. Hardeners have been prepared by Himmelman and Riebel (1968) by reacting acrolein with a hydroxylated polymer, such as polyvinyl alcohol. This product brings about hardening in gelatin at alkaline pH values.

(iv) Aziridines

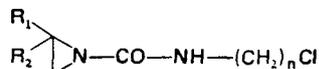
The function group in this class of compounds is



The chemistry is reviewed by Fanta (1964). Reaction with proteins can proceed through ring opening, thus—



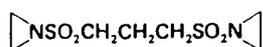
The active hydrogens of other side-chain groups such as the carboxyl and hydroxyl could also participate in a similar manner. The reaction will proceed more readily if R is electronwithdrawing in nature, such as with sulphonylaziridines, ketoaziridines and 2,4,6-triaziridinyl-*s*-triazine. Use, for example, is made of the following reagent by Burness (1967)—



where R_1 and $\text{R}_2 = \text{H}$ or low alkyl
 $n = 2$ or 3

In this case one functional group is the aziridinyl residue, the other being the halogen. Because of the similarity, mention, again, of the aziridinylphosphine oxide type hardeners can be made (Gevaert Photo-Producten, 1963; Harrow, 1966). Structural details of these substances has been given in Section III, C. 1. b. i. The aziridinyl homologue of urea, N,N'-diethyleneurea, has been found

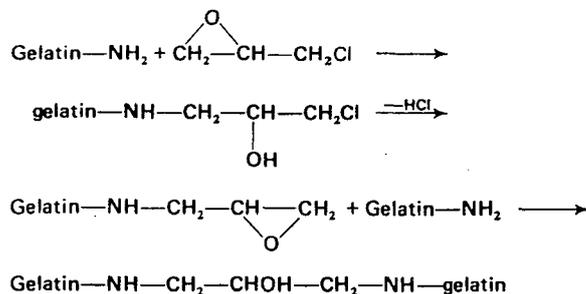
by Allen and Webster (1960) to harden gelatin. Bis- and polyaziridinylsulphonyl agents such as



have also been used (Burness, 1960). This same inventor (1965) has prepared ethyleneimine derivatives of phosphonitrilic polymers and used them as effective gelatin hardeners. Birr and Walther (1958a) also claim the use of ethyleneimino crosslinking reagents. Polyfunctional amino-azinolines are described as hardeners (Eastman Kodak, 1967a).

(v) Epoxides

Epichlorhydrin is the traditional hardener of this series and is believed to perform its function through a two-stage process:



Bifunctional epoxides that have been used include diepoxide substituted aromatic dicarboxylic acids and dihydroxybenzenes (V.E.B.-Filmfabrik Agfa Wolfen, 1960). Quaternary ammonium salts with two or more epoxy groups are described by Burness (1965). Epoxides as photographic hardeners are also listed by V.E.B.-Filmfabrik Agfa Wolfen (1958) and include examples such as triglycidylbenzenesiliconate, bis(β -glycidylloxyethyl) sulphone and diglycidyl ethyl phosphate. Triglycidyl-*iso*-cyanurate features as a hardener in claims by V.E.B.-Fotochemische Werke (1965, 1966). Diglycidyl *iso*-cyanurates are mentioned by Venner (1968). Glycerol derivatives containing epoxide residues are described by Levi *et al.* (1962). Polyepoxides feature in a patent by Baker (1959). Birr and Walther (1958b) claim the use of epoxides, e.g. diglycidyl succinate, together with compounds having di- or polychlorohydrin groupings.

(vi) Di- and polyisocyanates

Kenyon *et al.* (1965) have utilized the bisulphite adducts of diisocyanates for the up-grading of gelatin viscosity. The reagents have the following structure:



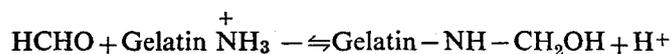
where M represents an alkali metal ion and $n = 2$ to 10

The centre portion of the molecule can also comprise an arylene grouping. The reagent, in the form of this adduct, has the advantage that homogenous and controllable crosslinking takes place.

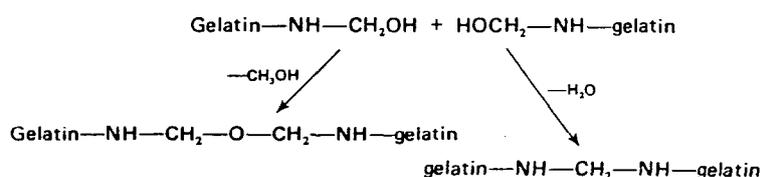
(vii) Aldehydes and ketones

Aldehydes

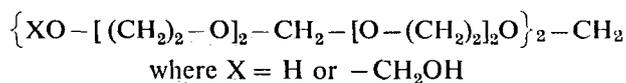
Of this series, formaldehyde, the smallest is probably one of the most widely used hardening agents for proteins. A comprehensive review of its chemistry with proteins has been published by French and Edsall (1945) and its use with gelatin has been described by Kragh (1958) and Fuschs (1961). Its use with gelatin or glue to produce a flocculant is reported by Sutton (1962). Davis and Tabor (1963) have examined the kinetics of the crosslinking reaction. Deamination, acrylation or aroylation or guanidylation of gelatin has been found to interfere with the action of formaldehyde on gelatin. The proposed mechanism of reaction is firstly the formation of a methylol substituent at the amino groups:



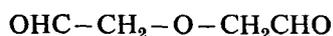
In very dilute solution this reaction does not proceed further as the distance between molecules does not favour mutual involvement. This is especially taken advantage of in the formol titration which measures the amount of hydrogen ion released by N-methylol formation. Details are given in the chapter devoted to analysis. The involvement of a second formaldehyde molecule to give rise to a N-dimethylol group can take place. Two proposals for the crosslinking stage of the formaldehyde reaction are made:



The methylene bridge is considered to be rather short in comparison with the dimethylene ether bridge, which Davis and Tabor support. Reformation on storage to the methylene type bridge is possible. The reaction of formaldehyde with carbamoylated gelatin to give a gelatin-urea-formaldehyde product has been described by Young and Christopher (1960). Formaldehyde in use has the disadvantages of difficult control on reaction rate and a repulsive odour. The use of polyformals in its place is described by Kress (1959). Paraformaldehyde and hexamethylenetetramine are also more easily handled. Products formed from paraformaldehyde and diethylene-glycol of the following structure,



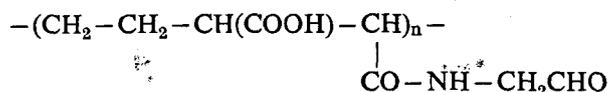
and other variants are also named in the patent by Kress. Another means of moderating the action of formaldehyde and improving its handling characteristics is to use it in the form of an N-methylol derivative of substances such as urea, hydantoin, melamine or benzimidazolinone (Zwicky, 1961; Knott and Pollak, 1956) to name a few examples. These agents are sometimes referred to as formaldehyde donors (Sutton, 1962a). An unusual case is tetrakis (hydroxymethyl) phosphonium chloride reported by Cater (1965). The higher monoaldehydes and dialdehydes are also used as hardeners, although in the case of the monoaldehydes their crosslinking efficiency decreases rapidly with increase in length of the alkyl group. Accelerated hardening by aldehydes in the presence of organic carboxylic acids is reported by Sieg and Killick (1967). Of the dialdehydes, glyoxal and glutaraldehyde give good results. In the homologous series which includes the latter compound, Courts and Homan (1973) have shown the dialdehyde $\text{CHO} - (\text{CH}_2)_n - \text{CHO}$ effectiveness to follow $n = 1 < n = 2 < n = 3 < n = 4$. Patents describing the use of these aldehydes have been published (Allen and Burness, 1966, 1966a; Clark and Miller, 1953, Mueller and Harriman, 1951, Orinik, 1952; Rose, 1968; Zambito and Macek, 1962). Robinson (1964) and Milch (1965) have studied the chemistry of various aldehydes with gelatin. Bisulphite adducts of aldehydes have the advantage and convenience of moderating the reaction with gelatin. Other derivatives of aldehydes in common use are the acylated aldehydes and acetals (Staud and Keyes, 1940; Jeffreys and Burness, 1966). In similar vein are the diacyloxy and acyloxy compounds named in patents by Eastman Kodak (1966 and 1967). Conversion of glycoaldehyde to an ether derivative,



has been claimed (May and Baker Ltd., 1965) to give superior results when crosslinking gelatin. Condensation products of glyoxal with diols or amino alcohols are also described (Kodak Ltd., 1963a). Ilford (1966) name 2,3-dihydroxy-1,4-dioxane, which could be regarded as a cyclic hemiacetal from two glycollic aldehyde molecules.

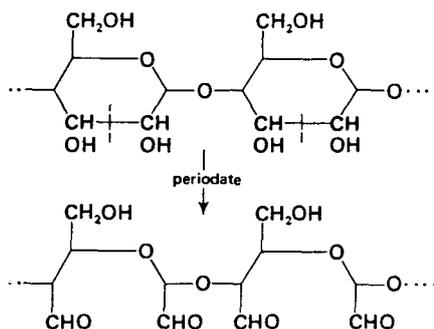
Aldehyde carboxylic acids, such as glyoxylic acid will also harden gelatin (I. G. Farbenindustrie, 1939) as well as ketoaldehydes (Lerner, 1961). Nitroaldehydes have also been claimed as being highly suited for photographic emulsion hardening (Zappert, 1945).

Polymeric aldehydes (Minsk and Cohen, 1966, 1967; V.E.B.-Filmfabrik Agfa Wolfen, 1961) include a substance of the following structure:



where $n \geq 20$

Alkylene glycol esters of polymannuronic or polygalacturonic acids are also claimed (Adox Fotowerke, 1962). In more common use are the oxidation products of oligo- and polysaccharides (Jeffreys *et al.* 1962; Kodak Ltd., 1963; Lemmerling and Vranken, 1962; Graham, 1962; Gevaert Photo-Producten, 1960) which can be prepared by treatment with periodate. In polysaccharides cleavage of the pyranose ring takes place in the following way:

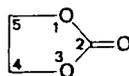


giving a product most suited to polyfunctional combination with protein.

Ketones

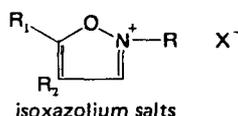
The mechanism of reaction of ketonic structures with proteins has received limited cover in the literature. Possible reaction products with amino groups could be Schiff bases or α -carbinolamines. Acetylacetone and biacetyl have been claimed by Ilford Ltd. (1963, 1966). Hydroxyketones are described, for example, dihydroxyacetone, together with halo-ketones as hardeners by Brunken (1939). Bis(β -acyloxyethyl) ketones of various kinds are used by Burness and Wilson (1967), an example being 1,10-diacetoxy-3,8-decanedione. β -oxyethyl onium salts (Burness, 1967a; Wilson, 1966) are also examples of this type of compound. The enol type acid, dihydroxymaleic acid, with mucochloric acid, have been claimed by Gevaert Photo-Producten (1960a). Robinson (1964) has proposed a mechanism by which mucobromic acid crosslinks gelatin.

(viii) Dioxolanes and isoxazolium salts



dioxolane-2-one

4-chloro-1,3-dioxolane-2-one and its 4,5-dichloro derivative renders gelatin films insoluble in boiling water (Gevaert-Agfa, 1966a).



R = C₁ to C₄ alkyl, with or without sulphonate

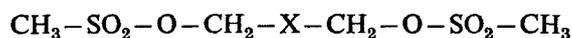
R₁, R₂ = H, alkyl, aryl, with or without halogen hydroxyl or sulphonate, heterocycle such as furyl or R₁ + R₂ = alicyclic ring.

X = solubilizing anion.

Isoxazolium salts are described by Burness and Wilson (1967a) as well as Van Campen and Graham (1967).

(ix) Methanesulphonate biesters

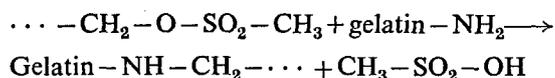
These have the following general formula:



where X may be $-\text{CH}_2-\text{O}-\text{CH}_2-$ or $-(\text{CH}_2)_n$

n = 1 to 4

These hardeners have been claimed in a patent by Kodak Ltd., (1955). Similar agents are described by Ferrania (1958). Polymeric homologues have been prepared from the monomer, methanesulphonoxyethylacrylate (V.E.B.-Filmfabrik Agfa Wolfen, 1961). Mechanisms of reaction are not proposed, but a possibility could be the displacement of the methanesulphonoxy residue involving a protein amino group in the following way.

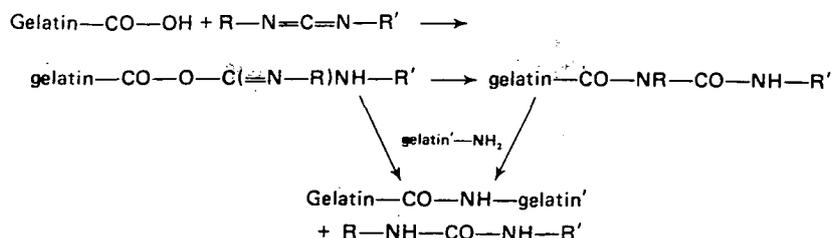


(x) Alkoxymethylurea and alkoxymethylamine type crosslinkers

By the reaction of two moles of an alkoxymethylisocyanate with a base such as ethylenediamine a product is obtained, which when added to gelatin, will elevate its melting point (Gevaert-Agfa, 1966). Alkoxymethylamines are also effective hardeners

(xi) Use of Carbodiimides

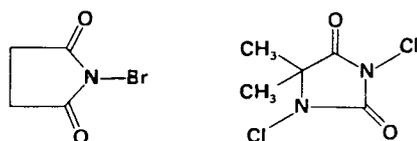
Carbodiimide has already been discussed as a reagent for introducing new substituents at the carboxyl group. Besides attaching small molecules having amino groups it can also be used to introduce amide crosslinks in the following way.



International Polaroid Corporation (1963) claim the inclusion of carbodiimides into photographic reagents. Wilson (1966a) and Robinson (1968) make reference to the use of this reagent as well.

(xii) Miscellaneous oxidants

The use of chlorine gas to upgrade the viscosity of gelatin is described by Farbenfabriken Bayer A.-G. (1965). The gas is bubbled through the solution, causing the viscosity to rise. After reaching a maximum, further chlorination causes a decline in the viscosity. Needles (1967) has found a similar trend when treating gelatin with peroxydisulphate. His findings were that deaminated gelatin crosslinked less readily than ordinary gelatin when treated with this reagent. His proposals as to the nature of the crosslink formed is that the aromatic amino acids are capable of oxidation to quinone-type intermediates which in turn condense with reactive sites such as amino, imino, guanidino or hydroxyl on other gelatin molecules to give rise to a covalent crosslink. Clark (1963) has observed that the following reagents:

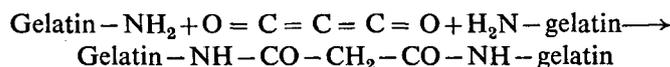


N-bromosuccinimide N,N'-dichloro-5,5-dimethylhydantoin

when added to mildly alkaline aqueous gelatin solution (pH values ranging between 8 and 9.5) gave sharp increments in viscosity up to a point, beyond which if more reagent was added, a decline in viscosity took place. Viscosities which were less than that of the original gelatin could be attained if sufficient reagent was added. N-bromosuccinimide is commonly used in protein research as a specific site cleavage agent at peptide bonds involving unsaturated amino acid. Its action on amino acids and peptides is reviewed by Filler (1963) and Witkop (1968). Deliberate breakdown of gelatin is sought in a patent by Bayer A.-G. (1966) where alkaline hypochlorite treatment is performed to give a degraded material which is suitable for use as a plasma substitute.

(c) Crosslinking combined with peptide backbone hydrolysis

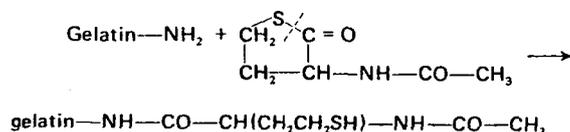
For certain specific uses, blood plasma substitutes in particular, it is found that the dimensions of the gelatin molecule do not suit the necessary requirements. If, however, there is a reduction in the length of the polypeptide chains while still maintaining the molecular weight through increasing the number of side-chain crosslinks, it is possible to produce a material of substantial molecular weight, but with a reduced gelling power and viscosity. Campbell *et al.* (1952) have prepared a plasma substitute which they name oxypolygelatin by first crosslinking gelatin with aldehydes, quinone or carbon suboxide. The crosslink introduced by carbon suboxide is as follows.



The product is then treated with an oxidant such as potassium permanganate or hydrogen peroxide at pH 5.5-7.5. Pyrogenic contaminants in the gelatin are conveniently destroyed by this step. See also Chapter 13, II. H. Similar procedures are described by Schmidt-Thome *et al.* (1962). Biotest-Serum-Institut (1965) have carried out the crosslinking stage with the use of carbodiimides, which introduce a more "natural" amide crosslink. The product of the carbodiimide reagent itself is not included in the structure of the final product, as has already been shown in earlier discussions. Peptide backbone cleavage is carried out by tryptic hydrolysis, this breaking the chain only at the carboxyl sides of arginine and lysine. An alternative method of heating in acid medium is also described. Behringswerke A.-G. (1964, 1965) describe similar processes.

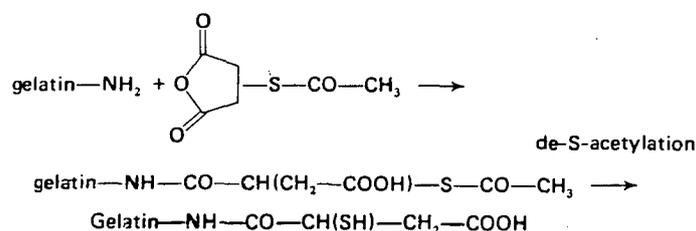
(d) Reactive gelatins with built-in crosslinking action

The existence of the disulphide bridge as a crosslink in nature finds examples in such proteins as keratin and insulin. It is, however, virtually absent in gelatin. Benesch and Benesch (1958) have successfully grafted the thiol residue onto gelatin by means of N-acetylhomocysteine thiolactone.



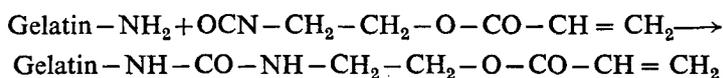
Mild oxidative conditions, such as exposure to the air, will cause the product to crosslink through the formation of intermolecular disulphide bridges (provided the material is not excessively dilute, in which case intramolecular disulphide bridges will be preferentially formed). Reduction will reverse the process. More drastic oxidation conditions will also fission the disulphide bond, in this case, irreversibly. The thiolation of proteins is also described by Benesch and Benesch (1963). Other thiolactones are described by Klotz

and Elfbaum (1964). Klotz and Heiney (1962) and Klotz *et al.* (1965) have used a number of anhydrides for the introduction of sulphur into proteins. S-mercaptosuccinic anhydride is one example.



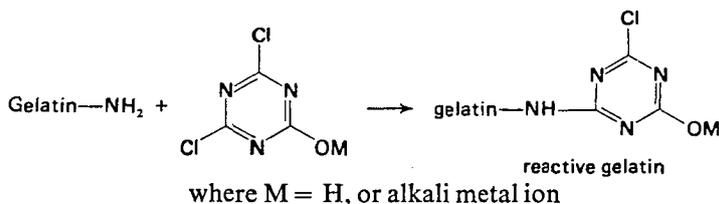
Controlled diffusion and release of therapeutic agents using gelatins of this type has been described by Barron and Tsuk (1967).

By treating gelatin with acryloxyethylisocyanate it is possible to introduce the following "reactive" side-group (Gates and Allentoff, 1966).

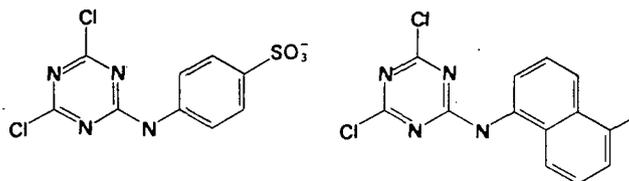


This acryloxyethylcarbamoyl-substituted gelatin, with the numerous vinyl groups attached, will harden unmodified gelatin if allowed to react with it.

Gelatins with "reactive" side-groups have also been prepared by the Fuji Photo Film Co. (1966a), who have made use of dichlorotriazines:



Treatment of this "reactive" gelatin with further amounts of any polymeric amine, including gelatin, will cause displacement of the remaining chlorine atom on the triazine ring to form covalent cross-bridges of the 2-hydroxy- or 2-metaloxy-*s*-triazin-4,6-diylamino structure. Similar "reactive" gelatins were prepared at an earlier date by Clark and Sutton. (1968), making use of the following two reagents which behave in a similar way to 2,4-dichloro-6-hydroxy-*s*-triazine.



Presumably because of the bulky aromatic residue now bound to the gelatin molecule, it is found that these products coacervate from mildly acidic aqueous solution after the same manner as the arylsulphonylated and aroylated gelatins which have been discussed in the early parts of this review. A useful purpose to which this combined "reactive" and coacervating property can be put is in the field of microencapsulation, where these gelatin derivatives can be induced to coacervate onto the interface of an emulsion or suspension. Once in this state, the coating can be stabilized through hardening by the addition of a polymeric amine such as polyethyleneimine or gelatin (Clark *et al.*, 1967). In the reactive gelatin is only partially substituted at the amino groups it will be unstable, but will only self crosslink at a low rate prior to coacervation because of the high dilution. At the high concentration of protein present in the coacervated coating of the microdroplets or microparticles it is possible for the "auto-crosslinking" to take place rapidly to stabilize the capsule layer. Supplements of polymeric amine are in this case not necessary. Sulphoarylaminochlorotriazinyl type reactive gelatins of reduced functionality can also be prepared by carrying out the triazinyl substitution in combination with a reagent such as benzenesulphonyl chloride. This will give a mixed substitution. In this way, any degree of reactive group substitution may be chosen without leaving any unsubstituted amino groups to jeopardize the stability of the product with respect to self-hardening (Clark *et al.*, 1957; Clark and Sutton, 1963).

2. *Non-covalent crosslinking agents:* Electrovalent and co-ordinate interactions (including hydrogen bonding)

The subject of polyelectrolyte complexes has been discussed by Michaels (1965) with special emphasis on the practical uses of these materials. A series of publications (Veis and Aranyi, 1960; Veis, 1961; Veis, 1963) treat the matter from a more theoretical standpoint. The interactions of synthetic detergents with proteins has been specifically dealt with by Putnam (1948).

It is perhaps worth noting in general terms that while covalent crosslinking of gelatin *per se* does not usually cause the product to separate from aqueous solution, numerous cases involving non-covalent type compounds can bring about this phenomenon, particularly if the gelatin solution is dilute.

The treatment of gelatin with metaphosphates for food purposes is described by Grettie (1940), Keil *et al.* (1961), and Kalafatas *et al.* (1968). Gelatin metaphosphate complexes containing a range of prosthetic residues such as calcium, silver, aluminium, *tert*-butylamine, methyl, ammonium, and calcium phytate have been prepared by the General Foods Corporation (1962). Metaphosphate as a precipitant in dilute aqueous solution for ana-

lytical purposes is described by Horvath (1946). The mode of interaction of the metaphosphate and the gelatin molecule is suggested to be through electrovalent links between the negative charges on the metaphosphate ion and the positive charges of the basic groups on the protein. This is augmented by co-ordinate bonds between protein nitrogens and metaphosphate P atoms, as well as between electron-saturated phosphate O atoms and the carbonyl C of the peptide bond (Wilson, 1937; Briggs, 1940). The interaction of calcium phosphates and gelatin have been examined by Visser (1962). Ciba (1964) have utilized the alkyl esters of phosphoric acid as precipitants for gelatin based photographic emulsions.

Polymers containing sulphonate, sulphate or carboxyl residues will also interact with gelatin. Poly-(acrylic acid/vinyl-pyridinesulphonic acid) has been usefully employed to coagulate photographic emulsions (Koeppf and Soehne G.m.b.H, 1962). Polymers of ethylenesulphonic acid, the sulphonamide and styrenesulphonate are utilized by Ohba *et al.* (1962). Sulphate esters for use as photographic hardeners are claimed by Ferrania (1965). These particular hardeners may well operate through ionic bonds with the protein. A possible alternative is the displacement of the sulphate ester group from the hardener by an amino group to give rise to a covalent crosslink. Monofunctional sulphate ester dyes of a similar nature are used by Ciba (1966a) to prepare coloured gelatin films for optical filters. Additions like carboxymethylcellulose, polyacrylates and other types of polycarboxylic substances to gelatin are discussed by Ellenberger (1966).

The coacervation of gelatin by natural polyacidic and polyphenolic substances such as gum arabic, pectins, and tannins is well-known and extensively studied (Zitko *et al.*, 1962; Zitko and Rosik, 1963; Keshishyan *et al.*, 1962; Yoshida and Thies, 1966; Schweikert, 1963; Birr and Walther, 1958c). Their interaction with the collagen as a tanning agent is dealt with by Gustavson (1956a), Shuttleworth (1952, 1967) and Shuttleworth and Cunningham (1948). Their use to flocculate photographic emulsions is reviewed by Fuchs (1963). The coacervation of gelatin with alginate, gum acacia and a range of other materials is described by the Upjohn Company (1963) in their process for preparing microscopic capsules.

Polysaccharide complexes with proteins have been reviewed by Bettelheim-Jones (1958) who confines his interest to those of biological occurrence. Chung and MacMasters (1966), Doyle *et al.* (1967) and Woodside *et al.* (1968) report on polysaccharide complexes with gelatin. These products are found to have an increased solubility in systems of organic solvents. Sedimentation studies of the complexes of gelatin with pectin, heparin and chondroitin sulphate have been carried out by Courts and Giles (1965). These complexes vary in their ability to hydrate, a property related to the efficiency of the gelatin molecule to take up the collagen fold.

3. *Crosslinking with metals*

Along with formaldehyde, chromium must be one of the earliest tanning agents to be put to use. The combined effect of these two substances has been used by Polson and Katz (1968) for the crosslinking of micropearls of gelatin. These, after packing in chromatographic columns, can be used as a combined ion exchange and molecular sieve medium for the resolution of biological polymer mixtures. A monograph on the mechanism of chrome tanning of leather has been written by Shuttleworth (1958). Co-ordination between carboxyl groups of the protein and the chrome ion is proposed as the main bonding force, along with some degree of hydrogen bonding or residual valency (Van der Waal's) forces acting to a less significant extent. Working with soluble collagen, Davjes (1972) has hydrolysed the chrome complex and isolated the crosslink, demonstrating a bonding mechanism to involve glutamic and aspartic acids. The effects of basic chromium sulphates on gelatin in aqueous solution has been studied by Pouradier *et al.* (1952), who have shown that the molecular weight, as measured by osmotic pressure, is substantially raised by the addition of chrome alum. Langston and Wootton (1962) found that additions of chrome alum or chromic chloride to 6.6% aqueous gelatin solutions in the pH range 3.1 to 6.1 gave rise to an increase in the viscosity; if sufficient chromium salt was added at the higher pH values viscoelastic or rigid gels were formed. Tamura *et al.* (1959) have carried out investigations of a similar kind and have concluded that crosslinking in the sol state interferes with the setting and rigidity of the gel, whereas crosslinking while in the gel state promotes increased rigidity. The hardening of gelatin with chrome as well as other types of crosslinking reagents is reported by Pouradier and Venet (1958) as well as Tachibana *et al.* (1958). The use of chrome tanning combined with the incorporation of long-chain fatty acids has been made by Iler (1942, 1945) to prepare water repellent and insoluble coatings from gelatin. Piolat (1959) treats gelatin with dichromate for a similar purpose. Potassium chrome alum and potassium aluminium alum are commonplace additives to fixing solutions used for the processing of photographic films, plates and papers. Of the other metals, iron, titanium and zirconium, the last-named has been used by Corben (1965) as a complexing agent with gelatin for the preparation of microscopic capsules. The text of this patent contains a suggested structure for the zirconium-gelatin complex in which the lone electron pairs of two peptide nitrogens are co-ordinated with the zirconium atom.

The biuret test for protein, which takes advantage of the binding of copper to the peptide backbone to give a purple/blue complex, has been studied by Strickland *et al.* (1961). This phenomenon, is however, not strictly a case of crosslinkage.

(a) Hydrolysis of the polypeptide backbone

For certain uses it has been found that a reduction in the molecular weight of gelatin earns certain advantages. One is that the solubility in non-aqueous solutions is improved. Karjala *et al.* (1966), Bouthilet and Karler (1965), Bouthilet *et al.* (1966) and Burnett (1963) have described how these low molecular weight products can be sorbed onto human hair with favourable cosmetic results. Hydrolysis products of gelatin and glue have also been used for the stabilization of tar acid emulsions for disinfectant use.

REFERENCES

- Adox Fotowerke, Dr. C. Schleussner, G.m.b.H (1962). *Belg. Pat.* 617,929..
Agfa A.G. (1961), *Brit. Pat.* 861,414.
Agfa A.G. (1965), *Belg. Pat.* 662,606.
Allen, C. F. H. and Webster, E. R. (1960). *U.S. Pat.* 2,950,197.
Allen, C. F. H. and Carroll, B. H. (1955). *U.S. Pat.* 2,725,294.
Allen, C. F. H. and Carroll, B. H. (1955a), *U.S. Pat.* 2,725,295.
Allen, C. F. H. and Burness, D. M. (1966). *U.S. Pat.* 3,232,764.
Allen, C. F. H. and Burness, D. M. (1966a). *U.S. Pat.* 3,232,761.
American Cyanamid Co. (1968). *Brit. Pat.* 1,100,842.
Baker, E. B. (1959). *U.S. Pat.* 2,882,250.
Barron, E., Tsuk, A. G. (1967). *U.S. Pat.* 3,329,574.
Behringswerke A.-G. (1964). *Belg. Pat.* 641,705.
Behringswerke A.-G. (1965). *Belg. Pat.* 665,076.
Bello, J. and Bello, H. R. (1963). *Biochim. Biophys. Acta.* **69**, 562.
Benesch, R. and Benesch, R. E. (1958). *Proc. Natl. Acad. Sci. U.S.* **44**, 848.
Benesch, R. and Benesch, R. E. (1963). *U.S. Pat.* 3,111,512, *Brit. Pat.* 906,851.
Berger, A., Kurtz, J. and Noguchi, J. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 271. Pergamon Press, London.
Bettelheim-Jones, F. R. (1958). *Adv. in Prot. Chem.* **13**, 36.
Biotest-Serum-Institut G.m.b.H (1965). *Brit. Pat.* 1,013,577.
Birr, E. J. and Walther, W. (1958). *East Ger. Pat.* 19,892.
Birr, E. J. and Walther, W. (1958a), *East Ger. Pat.* 20,378.
Birr, E. J. and Walther, W. (1958b). *East Ger. Pat.* 20,377 and 20,141.
Birr, E. J. and Walther, W. (1958c). *East Ger. Pat.* 20,032.
Bjorksten, J. (1951). *Adv. in Prot. Chem.* **6**, 343.
Bjorksten, J. and Andrews, F. A. (1960). *J. Amer. Geriat. Soc.* **8**, 632.
Blass, U. and Wehrli, W. (1965). *Brit. Pat.* 1,010,184.
Bogomolova, L. G. and Znamenskaya, T. V. (1967). *U.S.S.R. Pat.* 192,357.
Bouthilet, R. J. and Karler, A. (1965). *Proc. of the Sci. Sect. of the Toilet Goods Assoc.* **44**, p. 27.
Bouthilet, R. J., Karler, A. and Johnson, M. S. (1966). *Proc. of the Sci. Sect. of the Toilet Goods Assoc.* **45**, 27.
Briggs, D. R. (1940). *J. Biol. Chem.* **134**, 261.
Brunken, J. (1939). *Can. Pat.* 383,373, and 383,374.
Burness, D. M. (1960). *U.S. Pat.* 2,964,404.
Burness, D. M. (1962). *Belg. Pat.* 620,602.

- Burness, D. M. (1965). *U.S. Pat.* 3,189,459.
- Burness, D. M. (1967). *U.S. Pat.* 3,338,715.
- Burness, D. M. (1967a). *U.S. Pat.* 3,345,177.
- Burness, D. M., Sterman, M. D. and Van Campden, J. H. (1965). *Belg. Pat.* 656,174.
- Burness, D. M. and Wilson, B. D. (1967). *U.S. Pat.* 3,360,372.
- Burness, D. M. and Wilson, B. D. (1966). *U.S. Pat.* 3,232,763.
- Burness, D. M., Cowan, S. W. and Ford, J. A. (1967). *U.S. Pat.* 2,305,376.
- Burness, D. M. and Wilson, B. D. (1967b). *U.S. Pat.* 3,360,372.
- Burness, D. M. and Wilson, B. D. (1967a). *U.S. Pat.* 3,321,313.
- Burnett, R. S. (1963). Reprint from the "American Perfumer and Cosmetics," published by Wilson and Co. Inc., Research Products Dept., 4200 South Marshfield, Chicago 6, Ill., U.S.A.
- Butskus, P. F., Denis, G. I. and Butskene, A. I. (1960). *Izvest. Vysshikh. Ucheb. Zavedenii, Khim. i Khim. Tekhnol.* 3,469.
- Caldwell, J. R. (1960). *U.S. Pat.* 2,956, 884.
- Campbell, D. H., Koepfli, J. B. and Pauling, L. (1952). *U.S. Pat.* 2,591,133.
- Cassella Farbwerke Mainkur A.-G. (1965). *Brit. Pat.* 1,009,794.
- Cater, C. W. (1965). *J. Soc. Leath. Trad. Chem.* 49, (12), 455.
- Chung, H. Y. and MacMasters, M. M. (1966). *Staerke* 18, (12), 377.
- Ciba Ltd. (1962). *Belg. Pat.* 616,138.
- Ciba Ltd. (1962a). *Belg. Pat.* 615,313.
- Ciba Ltd. (1964). *Belg. Pat.* 642,112.
- Ciba Ltd. (1966). *Swiss Pat.* 411,919.
- Ciba Ltd. (1967). *Neth. Appl.* 6,609,327.
- Clark, R. C. (1963). Unpublished observations.
- Clark, R. C. and Courts, A. (1965). *Chemical Processing, Product Development Supplement*, June, S.6.
- Clark, R. C., Cobbett, W. G., Gibbs, J. A., Jones, R. T., Leach, A. A., Pratt, A. N. and Sutton, D. A. (1965). *J. Appl. Chem.* 15, 479.
- Clark, R. C., Gibbs, J. A., Pratt, A. N., Sutton, D. A., Wootton, J. and Leach, A. A. (1967). *Brit. Pat.* 1,075,952.
- Clark, R. C. and Sutton, D. A. (1968). *Brit. Pat.* 1,101,291.
- Clark, G. R. and Miller, L. F. (1953). *U.S. Pat.* 2,642,362.
- Combret, S. (1961). *Compt. Rend. Congr. Natl. Soc. Sovantes. Sect. Sci.* 86, 277.
- Coopes, I. H. (1968). *J. Poly. Sci., Part A. I.*, 6, (7), 1991.
- Corben, L. D. (1965). *U.S. Pat.* 3,201,353.
- Courts, A. (1954). *Biochem. J.* 58, 70.
- Courts, A. and Giles, B. (1965). "Structure and Function of Connective and Skeletal Tissue", p. 235. Butterworths, London.
- Courts, A. and Stainsby, G. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 100. Pergamon Press, London.
- Dalton, A. S. (1965). *Official Digest*, U.S.A., December, p. 1593.
- Damschroeder, R. E. and Gates, J. W. (1950). *U.S. Pat.* 2,518,666.
- Dann, J. R., Illingsworth, B. D. and Gates, J. W. (1958). *U.S. Pat.* 2,831,767.
- Dann, J. R., Illingsworth, B. D. and Gates, J. W. (1959). *Brit. Pat.* 807,894.
- Davis, P. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 225. Pergamon Press, London.
- Davis, P. and Tabor, B. E. (1963). *J. Poly. Sci. Part A.* 1, 799.
- Denisova, A. A. and Sinyakova, L. I. (1968). *Vysokomol. Soedin., Ser. A.* 10, (2), 357.

- Doyle, R. J., Woodside, E. E. and Fishel, C. W. (1967). *Carbohydrate Res.* 5, (3), 274.
- Drew, R. B. and Dutton, K. R. (1946). *Brit. Pat.* 576,228.
- Eastman Kodak Co. (1966). *French Pat.* 1,446,163.
- Eastman Kodak Co. (1967). *French Pat.* 1,496,008.
- Eastoe, J. E. (1955). *Biochem. J.* 61, 589.
- E. I. du Pont de Nemours and Co. (1967). *Brit. Pat.* 1,091,487.
- Elns, H. J., Bellis, D. C. and Gates, J. W. (1956). *U.S. Pat.* 2,763,639.
- Ellenberger, W. (1966). *Adhäsion* 10, (2), 58.
- Fanta, P. A. (1964). "The Chemistry of Heterocyclic Compounds: Heterocyclic Compounds with Three- and Four-Membered Rings" (A. Weissberger, ed.), Part 1, p. 524. Interscience Publishers, London.
- Farbenfabriken Bayer A.-G. (1944). *Ger. Pat.* 872,153.
- Farbenfabriken Bayer A.-G. (1965). *Ger. Pat.* 1,207,078.
- Farbenfabriken Bayer A.-G. (1966). *French Pat.* M.4072.
- Ferrania Società per Azioni. (1965). *Ital. Pat.* 690,926.
- Ferrania Società per Azioni. (1958). *Ital. Pat.* 574,739.
- Filler, R. (1963). *Chem. Revs.* 63, 21.
- French, D. and Edsall, J. T. (1945). *Adv. in Prot. Chem.* 2, 278.
- Fuchs, E. (1961). *Adhäsion* 5, (5), 225.
- Fuchs, E. (1963). *Adhäsion* 7, (4), 160.
- Fuji Photo Film Co. Ltd. (1966). *Brit. Pat.* 1,022,656.
- Fuji Photo Film Co. Ltd. (1966a). *Brit. Pat.* 1,049,083., *Belg. Pat.* 664,641.
- Fuji Photo Film Co. Ltd. (1967). *Jap. Pat.* 12,550 ('67).
- Fuji Photo Film Co. Ltd. (1968). *Brit. Pat.* 1,107,299.
- Gates, J. W. and Miller, P. E. (1960). *U.S. Pat.* 2,956,880.
- Gates, J. W., Lovett, W. G. and Miller, P. E. (1965). *U.S. Pat.* 3,184,312.
- Gates, J. W. and Allentoff, N. (1966). *U.S. Pat.* 3,255,000.
- General Aniline and Film Corp. (1966). *Belg. Pat.* 672,906.
- General Foods Corp. (1962). *Brit. Pat.* 888,643.
- Gevaert Photo-Producten N.V. (1959). *Belg. Pat.* 571,229.
- Gevaert Photo-Producten N.V. (1959a). *Belg. Pat.* 579,914.
- Gevaert Photo-Producten N.V. (1960). *Ger. Pat.* 1,073,305.
- Gevaert Photo-Producten N.V. (1963). *Brit. Pat.* 918,950.
- Gevaert Photo-Producten N.V. (1960a). *Belg. Pat.* 587,524.
- Gevaert Photo-Producten N.V. (1960b). *Belg. Pat.* 586,694.
- Gevaert Photo-Producten N.V. (1960c). *Belg. Pat.* 592,051.
- Gevaert Photo-Producten N.V. (1961). *Belg. Pat.* 593,710.
- Gevaert Photo-Producten N.V. (1961a). *Belg. Pat.* 606,122.
- Gevaert Photo-Producten N.V. (1962). *Belg. Pat.* 615,548.
- Gevaert Photo-Producten N.V. (1962a). *Belg. Pat.* 611,865.
- Gevaert Photo-Producten N.V. (1962b). *Can. Pat.* 614,426.
- Gevaert Photo-Producten N.V. (1965). *Can. Pat.* 715,561.
- Gevaert Photo-Producten N.V. (1963a), *Ger. Pat.* 1,148,742. and *Belg. Pat.* 597,207 (1961).
- Gevaert Photo-Producten N.V. (1963b). *Belg. Pat.* 628,650.
- Gevaert Photo-Producten N.V. (1965). *Brit. Pat.* 990,275. also *Belg. Pat.* 606,234 (1961), and *Ger. Pat.* 1,130,283. (1962).
- Gevaert Photo-Producten N.V. (1965a). *Brit. Pat.* 997,635.
- Gevaert-Agfa N.V. (1966). *Neth. Appl.* 6,608,174.

- Gevaert-Agfa N.V. (1966a), *Neth. Appl.* 6,609,853.
Gevaert-Agfa N.V. (1966b). *Neth. Appl.* 6,509,591.
Gibbs, J. A. (1964). Unpublished work: private communication.
Graham, J. L. (1962). *U.S. Pat.* 3,058,827.
Gray, W. R. (1967). "Methods in Enzymology" (C. H. W. Hirs, ed.), Vol. 2, p. 139. Academic Press, London.
Grettie, D. P. (1940). *U.S. Pat.* 2,196,300.
Gurin, S. and Clarke, H. T. (1934). *J. Biol. Chem.* **107**, 395.
Gustavson, K. H. (1949). *Adv. in Prot. Chem.* **5**, 354.
Gustavson, K. H. (1956). "The Chemistry and Reactivity of Collagen", pp. 227-259. Academic Press, New York.
Gustavson, K. H. (1956a). "The Chemistry and Reactivity of Collagen", Chapter 13, Academic Press, New York.
Harrow, R. A. J. (1966). *Ger. Pat.* 1,211,070.
Henkel and Cie. G.m.b.H. (1963). *Ger. Pat.* 1,149,517.
Harriott, R. M. (1947). *Adv. in Prot. Chem.* **3**, 169.
Hitchcock, D. I. (1923). *J. Gen. Physiol.* **6**, 95.
Himmelman, W. and Riebel, A. (1968). *U.S. Pat.* 3,382,077.
Hirs, C. H. W. and numerous other contributors (1967). "Methods in Enzymology" (C. H. W. Hirs, ed.), Vol. 2, Sect. 8, pp. 481-711. Academic Press, London.
Hoare, D. G. and Koshland, D. E. (1966). *J. Am. Chem. Soc.* **88**, 2507.
Homan, P. J. and Courts, A. (1967). Private communication.
Hornsby, K. M. (1956). *Brit. J. Photog.* **103**, 17-18, and 28-30.
Horvath, A. A. (1946). *Ind. Eng. Chem., Anal. Ed.* **18**, 229.
Humphrey, J. H. and Yuill, M. E. (1939). *Biochem. J.* **33**, 1826.
I. G. Farbenindustrie (1939). *Brit. Pat.* 499,648.
Iler, R. K. (1942). *U.S. Pat.* 2,273,040.
Iler, R. K., (1945) *U.S. Pat.* 2,381,752.
Ilford Ltd. (1963). *Brit. Pat.* 999,145.
Ilford Ltd. (1966). *Brit. Pat.* 1,040,081.
Illingsworth, B. D., Dann, J. R. and Gates, J. W. (1958). *U.S. Pat.* 2,852,382.
International Polaroid Corporation (1963). *Ger. Pat.* 1,148,466.
International Polaroid Corporation (1966). *Brit. Pat.* 1,018,244.
Janus, J. W., Kenchington, A. W. and Ward, A. G. (1951). *Research, London* **4**, 247.
Jeffreys, R. A., Tabor, B. E. and Burness, D. M. (1962). *U.S. Patents* 3,062,652 and 3,034,894.
Jeffreys, R. A. and Burness, D. M. (1966). *U.S. Pat.* 3,291,624.
Jopling, D. W. (1965). *J. Poly. Sci. Part A, General Papers* **3**, 513.
Kalafatas, N. J., Ehrlich, R. M. and Huntoon, R. B. (1968). *U.S. Pat.* 3,362,832.
Karjala, S. A., Williamson, B. S. and Karler, A. (1966). *J. Soc. Cosmetic Chemist* **17**, 513.
Keil, H. L., Hagen, R. F. and Flawe, R. W. (1961). *Ger. Pat.* 111,917.
Kenchington, A. W. (1958). *Biochem. J.* **68**, (3), 458.
Kenchington, A. W. (1961). Private communication.
Kenchington, A. W. and Lauder, W. E. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 191. Pergamon Press, London.
Kenyon, W. O., Smith, D. A. and Taylor, E. W. (1965). *U.S. Pat.* 3,220,864.
Keshishyan, G. O., Andrew, P. F. and Danilov, L. T. (1962). *Zh. Prikl. Khim.* **35**, 2051.

- Khismatullina, L. A., Levi, S. M. and Kukhtin, V. A. (1964). *Vysokomolekul. Soedin.* **6**, (3), 473.
- Khismatullina, L. A., Levi, S. M., Bogdanov, L. M. and Kukhtin, V. A. (1966). *Zh. Nauchn. i Prikl. Fotogr. i Kinematogr.* **11**, (2), 81.
- Klotz, I. M., Heiney, R. E. (1962). *Arch. Biochem. Biophys.* **96**, 605.
- Klotz, I. M., Martin, Y. C. and McConaughty, B. L. (1965). *Biochim. Biophys. Acta.* **100**, 104.
- Klotz, I. M. and Elfbaum, S. G. (1964). *Biochim. Biophys. Acta.* **86**, (1), 100.
- Knott, E. B. and Pollak, F. (1956). *U.S. Pat.* 2,732,316.
- Kodak Ltd. (1951). *Brit. Pat.* 649,549, *U.S. Pat.* 2,481,540.
- Kodak Ltd. (1955). *Brit. Pat.* 729,345.
- Kodak Ltd. (1960). *Brit. Pat.* 841,136.
- Kodak Ltd. (1963). *Brit. Pat.* 921,120 and 928,591.
- Kodak Ltd. (1963a). *Brit. Pat.* 926,313.
- Kodak Ltd. (1966). *Brit. Pat.* 1,033,189.
- Koepff and Soehne G.m.b.H. (1962). *Brit. Pat.* 911,886, *Ger. Pat.* 1,140,813.
- Kragh, A. M. (1958). *Manuf. Chemist* **29**, 103.
- Kragh, A. M. and Langston, W. B. (1962). *J. Colloid Sci.* **17**, 101.
- Kovacic, P. and Hein, R. W. (1959). *J. Am. Chem. Soc.* **81**, 1187.
- Krajewski, J. J. (1966). *U.S. Pat.* 3,291,611.
- Kress, B. H. (1959). *U.S. Pat.* 2,868,773.
- Laird, R. M. (1964). Unpublished observations, Private Communication.
- Langston, W. B. and Wootton, J. (1962). Private Communication.
- Leach, A. A. (1965). *J. Appl. Chem.* **15**, 78.
- Leach, A. A. (1966). *Biochem. J.* **78**, 506.
- Lemmerling, J. T. and Vranken, M. N. (1962). *Brit. Pat.* 907,675.
- Lerner, H. W. (1961). *U.S. Pat.* 3,012,886.
- Levi, S. M., Smirnov, O. K., Vilenskii, Yu. B., Korneva, E. D., Schvadchenko, L. P., Budurina, N. N. and Dusheiko, D. A. (1962). *U.S.S.R. Pat.* 151,933.
- Lichtenstein, I. (1940). *Biochem. Z.* **303**, 13.
- Lowe, W. G. and Griffin, K. P. (1952). *U.S. Pat.* 2,614,930.
- Lowe, W. G. and Frame, G. F. (1952). *U.S. Pat.* 2,614,931.
- May and Baker Ltd. (1965). *Belg. Pat.* 655,436.
- Merrill, R. E. (1965). *U.S. Pat.* 3,207,613.
- Michaels, A. S. (1965). *Ind. and Eng. Chem.* **57**, (10), 32.
- Minsk, L. M. and Cohen, H. L. (1966). *U.S. Pat.* 3,277,030.
- Minsk, L. M. Cohen, H. L. (1967). *U.S. Pat.* 3,330,664.
- Milch, R. A. (1965). *Gerontologia*, **10**, (2-3), 117.
- Miller, P. E., Haefele, C. R. and Gates, J. W. (1966). *U.S. Pat.* 3,227,571.
- Monier, J. B. (1944). *U.S. Pat.* 2,363,892 and 2,363,893.
- Morgan, J. and Starkey, A. J. (1956). *U.S. Pat.* 2,732,303.
- Mueller, F. W. H. and Harriman, B. R. (1951). *U.S. Pat.* 2,553,506.
- Mullen, T. C. (1962). *J. Soc. Leath. Tr. Chem.* **46**, 162.
- National Cash Register Co. (1967). *Ger. Pat.* 1,256,195.
- Needles, H. L. (1967). *J. Poly. Sci., Part A.* **1**, **5**, (1), 1.
- Ohba, S., Yonezawa, T. and Kumai, A. (1962). *U.S. Pat.* 3,022,172.
- Olcott, H. S. and Fraenkel-Conrat, H. (1947). *Chem. Revs.* **41**, 151.
- Orinik, M. T. (1952). *U.S. Pat.* 2,593,912.
- Perutz Photowerke G.m.b.H. (1963). *Ger. Pat.* 1,155,233.
- Pieper, G., Bayer, O. and Gloxhuber, C. (1964). *Ger. Pat.* 1,165,606.

- Piolat, G. (1959). *French Pat.* 1,199,803.
- Polson, A. and Katz. (1968). *W. Biochem. J.* **108**, 641.
- Pouradier, J., Roman, J., Venet, A., Chateau, H. and Accary, A. (1952). *Bull. Soc. Chim. France* **19**, 928.
- Pouradier, J. and Venet, A. M. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 236. Pergamon Press, London.
- Putnam, R. C. (1950). *U.S. Pat.* 2,523,324, 2,523,525 and 2,523,326.
- Putnam, F. W. (1948). *Adv. in Prot. Chem.* **4**, 80.
- Putman, F. W. (1953). "The Proteins" (H. Neurath and K. Bailey, eds), Vol. 1B, Chapter 10, Academic Press, New York.
- Reitz, H. C., Ferrel, R. E., Fraenkel-Conrat H. and Olcott, H. S. (1946). *J. Am. Chem. Soc.* **68**, 1024.
- Riso, R. R. (1964). *Proc. Sci. Section of the Toilet Goods Assoc.* No. 42, 36.
- Reynolds, R. J. W. and Seddon, J. D. (1966). 30th meeting of the Research Panel of the Gelatine and Glue Research Association.
- Robinson, I. D. (1964). *J. Appl. Poly. Sci.* **8**, 1903.
- Robinson, I. D. (1968). *J. Photogr. Sci.* **16**, (1), 41.
- Rose, H. J. (1968). *U.S. Pat.* 3,383,223.
- Rosowsky, A. (1964). "The Chemistry of Heterocyclic Compounds: Heterocyclic Compounds with three- and four-membered Rings" (A. Weissberger, ed.), part 1, page 1. Interscience Publishers, London.
- Sanders, H. L. and Nassau, M. (1960). *Soap and Chemical Specialities*, January, page 57.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Schmidt-Thome, J., Mager, A. and Schoene, H. H. (1962). *Arzneimittel-Forsch*, **12**, 378.
- Schoenauer, W. and Siegrist, H. (1965). *Brit. Pat.* 1,001,063.
- Schwander, H. R. (1955). *U.S. Pat.* 2,719,146.
- Schweikert, E. (1963). *Rev. Tech. Ind. Cuir.* **55**, (9), 250.
- Shuttleworth, S. G. and Cunningham, G. E. (1948). *J. Soc. Leath. Tr. Chem.* **32**, 183.
- Shuttleworth, S. G. (1952). *J. Am. Leath. Chem. Assoc.* **47**, (9), 603.
- Shuttleworth, S. G. (1958). "The Chemistry and Technology of Leather" **2**, Chapter 23, American Chemical Society Monograph No. 134.
- Shuttleworth, S. G. (1967). *J. Soc. Leath. Tr. Chem.* **51**, (4), 134.
- Sieg, A. L. and Killick, M. W. (1967). *French Pat.* 1,478,140.
- Società Generale per l'Industria Mineraria E Chimica, (1961) *Ital. Pat.* 617,347.
- Staab, H. A. and Wendel, K. (1960). *Chem. Ber.* **93**, 2902.
- Stark, G. R. (1967). "Methods in Enzymology" (C. H. W. Hirs, ed.), Vol. 2, pp. 590-4. Academic Press, London.
- Staud, C. J. and Keyes, G. H. (1940). *U.S. Pat.* 2,172,300.
- Steven, F. S. and Tristram, G. R. (1963). *Biochim. Biophys. Acta.* **71**, 392.
- Strickland, R. D., Freeman, M. C. and Gurule, F. T. (1961). *Anal. Chem.* **33**, 545.
- Sutton, D. A. (1962). Reporting on the work of Wootton, J. and Langston, W. B., *Nature* **196**, 223.
- Sutton, D. A. (1962a). Reporting on the work of Whiteside, I. R., Gibbs, J. A. and Wootton, J., *Nature* **196**, 422.
- Tachibana, T., Inokuchi, K. and Kakiyama, H. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 243. Pergamon Press, London.
- Tamura, M., Kurata, M. and Fujita, K. (1959). *Phot. Sci. Eng.* **3**, 277.

- Tourtellotte, D. and Williams, H. E. (1958). "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), p. 246. Pergamon Press, London.
- Tourtellotte, D. and Markes, E. M. (1963). *U.S. Pat.* 3,108,995.
- United Shoe Machinery Corp. (1962). *Belg. Pat.* 614,984.
- Upjohn Company. (1963). *Brit. Pat.* 930,421.
- Van Campen, J. H. and Graham, J. L. (1967). *U.S. Pat.* 3,316,095.
- V.E.B.-Filmfabrik Agfa Wolfen. (1961). *Ger. Pat.* 1,109,875.
- V.E.B.-Filmfabrik Agfa Wolfen. (1960). *Ger. Pat.* 1,091,322.
- V.E.B.-Filmfabrik Agfa Wolfen. (1958). *Ger. Pat.* 1,085,663.
- V.E.B.-Fotochemische Werke, Berlin. (1965). *Fr. Pat.* 1,410,834.
- V.E.B.-Fotochemische Werke, Berlin. (1966). *East Ger. Pat.* 45,657.
- Veis, A. and Aranyi, C. (1960). *J. Phys. Chem.* **64**, 1203.
- Veis, A. (1961). *J. Phys. Chem.* **65**, 1798.
- Veis, A. (1963). *J. Phys. Chem.* **67**, 1960.
- Veis, A., Cohen, J. and Aranyi, C. (1967). *U.S. Pat.* 3,317,434.
- Venner, H. A. L. (1968). *Brit. Pat.* 1,109,546.
- Visser, S. A. (1962). *J. Dairy Sci.* **45**, 710.
- Whitfield, R. E. and Wasley, W. L. (1964). "The Chemical Reactions of Polymers" (E. M. Fettes, ed.), vol. 19, pp. 367-500. Interscience Publishers, London.
- Whiteside *et al.* (1964). Private communication.
- Wilson, J. A. (1937). *J. Am. Leath. Chem. Assoc.* **32**, 113.
- Wilson, B. D. (1966). *U.S. Pat.* 3,241,972.
- Wilson, B. D. (1966a). *U.S. Pat.* 3,236,882.
- Wirnik, A. D. and Tschekalin, M. A. (1962). *Textil Praxis* **17**, 577.
- Witkop, B. (1968). *Science*, **162**, 318.
- Wood, H. W. (1952). *Sci. et Ind. Phot.* **23A**, 110.
- Woodside, E. E., Trott, G. F., Royle, R. J. and Fishel, C. W. (1968). *Carb. Res.* **6**, 449.
- Yannas, I. V., and Tobolsky, A. V. (1968). *European Poly. J.* **4**, (2), 257.
- Yannas, I. V. and Tobolsky, A. V. (1967). *Nature* **215**, (5100), 509.
- Yoshida, N. and Thies, C. (1966). *Amer. Chem. Soc. Div. Polymer Chem., Preprints* **7**, (1), 245.
- Young, H. H. and Christopher, E. F. (1957). *U.S. Pat.* 2,816,009.
- Young, H. H. and Christopher, E. F. (1960). *U.S. Pat.* 2,923,691.
- Yuttsy, H. C. and Frame, G. F. (1950). *U.S. Pat.* 2,525,753.
- Yuttsy, H. C. and Frame, G. F. (1952). *U.S. Pat.* 2,614,928.
- Yuttsy, H. C. and Russell, F. J. (1952). *U.S. Pat.* 2,614,929.
- Yuttsy, H. C. and Frame, G. F. (1955). *U.S. Pat.* 2,728,662.
- Zambito, A. J. and Macek, T. J. (1962). *U.S. Pat.* 3,028,308.
- Zappert, R. H. (1945). *U.S. Pat.* 2,372,873.
- Zitko, V., Rosik, J. and Vasalko, J. (1962). *Chem. Zvesti.* **16**, 175; *Chem. Zvesti*, **16**, 474.
- Zitko, V. and Rosik, J. (1963). *Chem. Zvesti*, **17**, 109.
- Zwicky, H. (1961). *Phot. Korr.* **4 Sonderheft** Page 24.

Chapter 8

The Physical Properties of Gelatin

C. A. FINCH* AND A. JOBLING†

(*Croda Polymers Ltd., Harefield, Middlesex, England)

(†Lensfield Research and Development Ltd., Maulden Rd, Flitwick, Bedford, England)

I	Introduction	250
II	Swelling and Solubility	251
	A. Swelling and Solubility in Water	251
	B. Swelling and Solubility in Solutions Containing Ions	257
	C. Solubility of Gelatin in Non-Aqueous Solvents	258
III	Mechanical Properties	260
	A. Mechanical Properties of "Solid" Gelatin	260
	1. <i>Effect of conditions of formation and drying</i>	261
	2. <i>Mechanical properties of drawn fibres</i>	262
	3. <i>Internal stresses in films</i>	262
	4. <i>Mechanical properties of plasticized gelatin films</i>	263
	5. <i>Creep</i>	263
	B. Mechanical Properties of Gelatin Gels	263
	1. <i>Introduction</i>	263
	2. <i>Elastic behaviour at small strains</i>	264
	(a) methods of measurement	264
	(b) maturing conditions	264
	(c) effects of magnitude and duration of applied stress or strain	265
	(d) concentration dependence	266
	(e) temperature dependence	267
	(f) effect of pH and added substances	268
	3. <i>Fractionated and degraded gelatins: gel rigidity and molecular weight</i>	268
	4. <i>Elastic behaviour at high strains and rupture</i>	270
	5. <i>Crosslinked gels</i>	270
	C. Mechanical Properties of Gelatin Solutions	271
	1. <i>Introduction</i>	271
	2. <i>Temperature- and concentration-dependence of viscosity</i>	271
	3. <i>Ultrasonic irradiation of gelatin solutions</i>	273
	4. <i>Mechanical degradation in gelatin solutions</i>	273
IV	Diffusion Through Gelatin Films and Gels	273

V	Optical Properties of Gelatin	275
	A. Colour and Clarity of Solutions	275
	B. Optical Rotation	275
	C. Refractive Index	276
	D. Streaming Birefringence	277
	E. Light Scattering	279
	F. Miscellaneous Optical Properties	279
VI	Electrical Properties	279
	A. Irradiation of Gelatin	279
	B. Electrical Conductivity	281
	C. Dielectric Properties	282
	D. Miscellaneous Electrical Properties	283
VII	Thermal Properties	283
	A. Irreversible Changes on Heating	283
	B. Effect of Plasticizing Additives	285
	C. Thermal Conductivity of Gelatin Gels	286
	D. Density of Aqueous Gelatin Solutions	286
VIII	Surface Active Properties	286
	A. Introduction	286
	B. Surface Tension	287
	C. Interfacial Tension	287
	D. Surface Films	288
	References	288

I. INTRODUCTION

In 1861, Graham named a new family of materials, the colloids, after the Greek word ($\kappa\omicron\lambda\lambda\alpha$) for a typical member, animal glue. He could not have known that, in the light of current knowledge over a century later, his exemplar would still embody, probably uniquely in a single material, nearly the whole gamut of physical phenomena characteristic of macromolecular colloids, both natural and synthetic.

Gelatin solutions exhibit viscoelastic flow and streaming birefringence. They can be spun into filaments which can then be drawn into mechanically and optically anisotropic threads like the synthetic polymers which have revolutionized modern textiles.

The ability of aqueous gelatin solutions to modify crystal growth (e.g. of silver halides) and to set to gels through which small ions can pass freely is the main basis of the photographic process. The stress-optical properties of gelatin gels (Bayley, 1959) enabled the engineering technique of photoelastic stress analysis to be developed. The strength and toughness of the gelatin (animal glue) film, probably aided by the low surface tension of aqueous gelatin solutions made animal glue the main structural adhesive for wood and many other materials from the time of the ancient Egyptians until well

into the twentieth century. Gelatin is also a polyampholyte and exhibits in solution the typical behaviour of this class.

The term "gelatin" is used conveniently to describe a group of proteins derived by various means from collagen. Their physical properties depend on the origin of the collagen and the method by which it is treated.

Most of the earlier papers published on the properties of gelatin must be regarded with caution since the origin, method of manufacture, and degree of purity are often not specified. The limitations of this earlier work have been well reviewed by Ward (1954a,b; 1955; 1959; 1961), who has pointed out the difficulties of interpretation. Impurities in gelatin can be of great significance in photographic properties: differences in behaviour involving surface active properties appear to result from trace impurities rather than differences in the main gelatin component.

With these reservations, the following sections critically describe the diverse physical properties of gelatin indicating where possible the *nature of the substances studied*, and the validity of the observations discussed.

II. SWELLING AND SOLUBILITY

The solubility properties of gelatin are of prime importance in both its study and its applications. As with other properties, no clear distinctions can be made of particular phenomena, especially as observed behaviour depends on the origin of the gelatin being investigated (frequently not given), the time-dependence of the solubility process (of which the swelling of "dry" gelatin may be considered as an intermediate state), and the nature of the solvent system. In addition, the pattern of published information suggests both considerable commercial restrictions in certain areas, and that many significant aspects remain uninvestigated. The arrangement of this section reflects these limitations.

A. Swelling and Solubility in Water

If gelatin is put into contact with cold distilled water some of the material dissolves. Pouradier and Abribat (1950) found that, as might be expected, with gelatin with a wide range of molecular weights, material with $\bar{M} = 40,000$ passed more easily into solution than that of $\bar{M} = 94,000$. When a gelatin of nearly uniform \bar{M} was used, both water-soluble and insoluble fractions were of the same \bar{M} (measured by specific viscosity). This suggests that the method of manufacture determines the solubility of the gelatin since viscosity and osmotic pressure measurements of dilute solutions of gelatin in cold water do

not indicate a change in \bar{M} for the cold-water-soluble portion (Pouradier, 1950). Similar conclusions were reached in a review of Russian work on the properties of gelatin and agar-agar solutions (Lapin, 1955) (see also Tya-zhelova, 1937; Lipatov, 1939; and Lapin and Lipatov, 1939) in which gelatin was separated into three fractions (I, soluble at 3°C; II, soluble at 22°C and III, not soluble at 22°C). The molecules of the more soluble fractions appear to be only slightly associated whereas the particles present in the less soluble fractions are micellar aggregates. The viscosity of mixed gelatin solutions is not additive. As the fractions each give similar amounts of nitrogen in the Van Slyke determination, the basic molecules are presumed to be identical. The total number of polar groups is the same in each type of gelatin, which each produce no further heat of solution after absorption of 0.27 g water/g of gelatin. This is considered to be a measure of the "true" hydration by the polar groups. The molecules are associated through their non-polar portions, since the dielectric components of each type I, II and III, are the same at different concentrations whilst that of III varies with temperature parallel to that of water, and unlike that typical of dipolar association. The micellar aggregates swell by osmotic absorption of water. This osmotic hydration is shown by differences in osmotic pressure and viscosity amongst the fractions, which, with gelatin, disappear at 60°C with the breakdown of the micelles. The presence of I and II types in the fluid surrounding the micelles of type III cuts down the osmotic hydration and the swelling. The presence of the dispersed fractions I and II is also considered to lead to breakdown (by peptization) of the micelle, with decreased viscosity and \bar{M} (as measured by osmotic pressure). Heating in the dry state, exposure to ultra-violet light or to high frequency electrical fields increases the micellar pattern, with decreased solubility. The micellar solutions are not in equilibrium, with the micellar pattern increasing on ageing.

Other Russian workers (Zubov, Zhurkina, and Kargin, 1949) have also indicated that the method of preparation of the gelatin is significant in relation to the solubility properties of the product, suggesting that the formation of bonds between polar groups of any gel-forming substance should proceed increasingly by intramolecular processes as the concentration is reduced. Vacuum evaporation of a 0.2% aqueous solution of gelatin at 5°C gave a product which dissolved gradually in water at 10–15°C (instead of the 25–30°C normally required). On heating 2–3% solutions of this product, gelling occurred at 20–22°C. This "globular" gelatin has a negative temperature coefficient of viscosity, indicating that the globules must be particles or aggregates of molecules destroyed at high temperatures. Each molecule of globular gelatin may be regarded as a miniature of the gel structure. Treatment of the gelatin solution before evaporation with 5% of quinone yields a non-gelling product with a low temperature coefficient of viscosity.

In a study of the properties of gelatin films dried under different relative humidities Bradbury and Martin (1952) examined two types of gelatin, first identified by Katz *et al.* (1931) by X-ray diffraction, and showed differences in the diffraction pattern of films dried at 20°C and at 60°C at RH 70%. The cold-dried sample was markedly crystalline, although the hot-dried films still showed some signs of crystallinity. It was suggested that in the "cold-dried" films the molecular forces become associated into large crystallites by gelling forces acting in the initial 5% gel and subsequently during drying as the concentration increases. These preserve the molecules in a partially extended state.

In the "hot-dried" films, where gelling forces cannot operate until an advanced state of drying (or until cooling after drying), the molecules are preserved in the film in the contracted, disordered, and closely packed condition of the concentrated solution. Crystallites formed in the "hot-dried" film are fewer than those in the "cold-dried" film, and are randomly distributed in three dimensions. Contraction of the "cold-dried" films in boiling methanol may be due to contraction of the partially extended molecules when the temperature is high enough for gel bonds to be broken.

These structures allow an explanation of the solubility properties of films, which relate to the mechanical properties. Both "cold-dried" and "hot-dried" films consist of an adequately bound network of molecules, which account for low extensions at low humidities. At higher relative humidities, when the intermolecular bonds have been weakened by absorbed water, the load/extension curves show an apparent yield-point before rupture. Extension of the molecules in the "cold-dried" film is limited by intermolecular gel forces. In the "hot-dried" films, the intermolecular forces are mainly Van der Waals forces, which, weakened by absorption of water, permit free sliding of the molecules.

The first steps in the swelling of gelatin with water were studied by Winther (1954) by measurement of the extinction spectrum of a gelatin interference plate, in relation to time in the presence of water and water vapour. The rate of swelling with water is:

$$v - 84.1 = (V - 84.1) \{1 - \text{antilog}(-0.028 t^{\frac{1}{2}})\}$$

and for swelling with water vapour is:

$$v = V\{1 - \text{antilog}(-kt^{\frac{1}{2}})\}$$

where v is the amount of water absorbed per 100 vols. of gelatin in t secs. and V is the maximum amount of water absorbed per 100 vols. of gelatin.

According to these measurements 84.1% by volume of the water is thus absorbed instantaneously, at the surface of the gelatin, whilst the remaining

water absorbed diffuses through the gelatin at the same rate at which water vapour is absorbed.

Pinoir and Pouradier (1948) found that a "hot-dried" film behaved normally, in that it swelled in water at 18°C and then did not dissolve until the temperature was raised to about 35°C. When placed in water at 25°C, however, part of the film began to dissolve immediately, but the film did not dissolve completely. Conmar Robinson (1953) investigated these effects optically and noted that at 18°C in water, gelatin started to dissolve spontaneously at the surface, but, as water penetrated into the mass, the remainder of the soluble "hot-dried" gelatin was presumably converted into the soluble "cold-dried" form before it could dissolve. Conversion to the "cold-dried" form should be less rapid at 25°C, so more gelatin dissolves at this temperature. This initial cold water solubility of "hot-dried" gelatin was believed to be due to the lack of strong intermolecular (gel) linkages, and the later insolubility to the rearrangement of the molecules to form a continuous gel network.

The swelling of gelatin which had been heated at 45°C for 5 days was studied by Pankhurst (1947) under various conditions of relative humidity. At RH 69%, the gelatin absorbed 640% of water when soaked, but at RH near 100% under the same conditions it absorbed only 430% of water, the melting point of the film was raised, and it exhibited hydrothermal shrinkage similar to that of collagen. Thus gelatin can become insoluble under comparatively mild conditions.

These observations have been confirmed by Jopling (1952, 1953, 1956) who investigated the swelling in water at 20°C of gelatin films about 10 μ thick, dried and conditioned at different temperatures and humidities, using an instrument designed by Lewis and Soper (1950), based on the theory of the parallel plate viscometer. He determined the swell ratio $R_s = l_o/l_s$ where l_o = original thickness and l_s = thickness of swollen film. A film dried at 70° swells more rapidly than a cold dried film during the first few minutes, then contracts again to a lower final R_s , probably due to an initial osmotic effect caused by salts present in the gelatin, since deionized gelatins swell straight to this final R_s . When conditioned at high relative humidity, all layers showed reduced R_s . Typical results (Jopling, 1956) are shown in Figs. 1-3.

The results were explained by assuming that bonds formed during setting and drying are under no strain at the time of their formation and so tend to resist any compressive or tensile strains caused by drying or by increased swelling, respectively. The number and strength of these bonds is controlled by the drying conditions. Differences in the folding chains (Robinson, 1953) and intramolecular structure may also be significant. There is also some evidence to suggest that a slow insolubilizing crosslinking reaction occurs in gelatin upon heating under suitable conditions (Saunders, 1956).

The inter-relation of gelatin and water has been studied as a sorption-desorption phenomenon, with a split hysteresis loop (Subba Rao and Das, 1967). Only limited work has been carried out on the swelling of gelatin prepared by different methods: "alkali-extracted" bone, "acid-treated" bone; "alkali-extracted" hide (Jopling, 1956); and acid-pigskin, limed hide (high and low pH), limed ossein (Simms and Blake, 1960): the latter also considered gelatins of a range of Bloom strengths and viscosities.

The kinetics of swelling in water of hardened and unhardened gelatin films (limed-ossein or limed-hide types, hardened with 2,4-dichloro-6-*p*-sulphani-

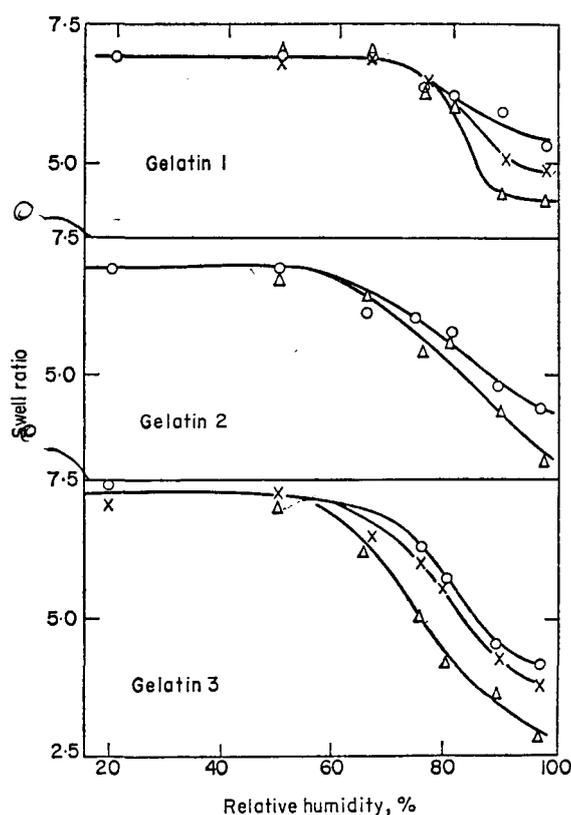


FIG. 1. Swelling in distilled water at 20°C of three isoelectric gelatins after conditioning at 20°C in various humidities for the periods indicated: ○, 1 day; ×, 2 days; △, 7 days.

Gelatin no.	Source	Isoelectric point
1	Alkali-extracted bone	5.0
2	50% Alkali-extracted bone + 50% Alkali-extracted hide	5.1
3	Acid-treated bone (Jopling, 1956).	7.4

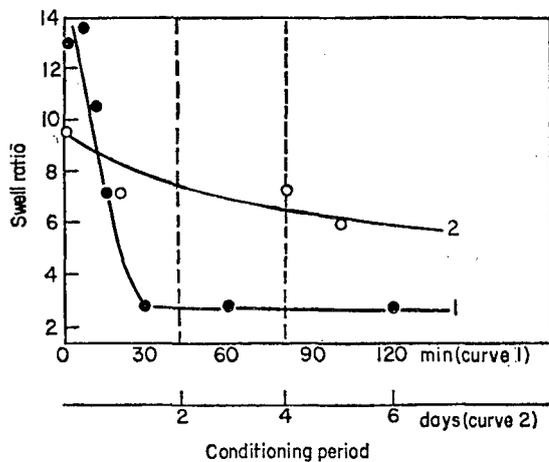


FIG. 2. Swelling in distilled water at 20°C of isoelectric gelatin no. 2 after conditioning at 90% RH and 20°C. ○, layer dried at 20°C; ●, layer dried at 70°C (Jopling, 1956).

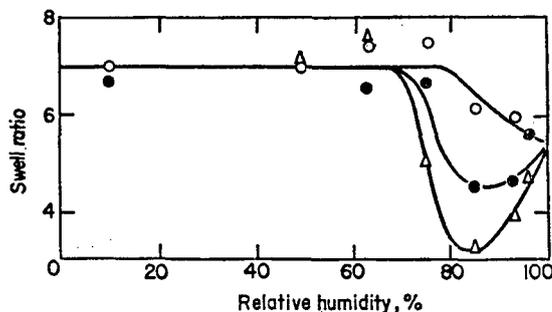


FIG. 3. Swelling in distilled water at 20°C of isoelectric gelatin no. 2 after conditioning at 50°C and various relative humidities for the periods indicated: ○, 1 day; ●, 5 days; △, 7 days (Jopling, 1956).

lino-1,3,5-triazine as crosslinking agent) of various thicknesses were studied by Libicky and Bermann (1972) using a modified Wallace indentation tester to measure thickness (Taylor and Kragh, 1970). The swelling curves obtained could, as a first approximation, be expressed by a second order rate equation with equilibrium swelling, h_{∞} , and the initial velocity of swelling, V_0 , as parameters.

$$dh/dt = V_0 (l - h/h_{\infty})^2$$

The temperature dependence of V_0 , in the range 10–30°C is independent of dry thickness, whilst the limiting value of the swell ratio, S_{∞} , varies linearly in the range of 4–24 μ of dry film thickness for non-crosslinked gelatin.

The penetration of ice in gelatin gels—of interest in food freezing (Callow, 1950)—varies enormously (from 700 cm/hr to <1 cm/hr at -3°C) and depends on pH, and the concentration of gelatin and salts. In general, factors which affect osmotic pressure of a gel similarly affect the rate of ice penetration. The effect has been studied in detail by Luyet and his co-workers (Rapatz and Luyet, 1959; Gehenio and Luyet, 1959; Persidsky and Luyet, 1959). When gelatin gels coagulate slowly at concentrations $>12\%$ at low temperatures, there is a sharp division into ice crystals and a more concentrated gel (Moran, 1926). Temperatures down to those of liquid air have been used; a 1% gelatin solution becomes a white mass, which upon warming slowly separates into a clear gelatin-free liquid surrounding a white gel with a concentration of approximately 13% of gelatin (Rousselot, 1949). This behaviour was observed both with technical and with deionized gelatin.

The nature of the ice structures in aqueous gelatin gels was studied by X-ray diffraction by Dowell, Moline, and Rinfret (1962), who found that hexagonal ice occurred predominantly for all cooling rates of 10–20% gels. Various amounts of vitreous, cubic, and hexagonal ice were found in 20–50% gels, depending on the concentration, cooling rate, and temperature during subsequent warming, although the cubic is predominant: vitreous ice occurs mainly above 50% concentration gels, changing on warming to cubic between -65°C and -25°C , and to hexagonal ice near -7°C for 50–60% gel.

In commercial practice, the cold-water solubility of gelatin has been improved by methods which suggest an alteration of the surface properties of the gelatin powder to improve dispersibility. Typically, gelatin solutions have been brought into contact with silicones, high-molecular weight polyethylene glycols, or polyoxyethylene derivatives of fatty acids at 50–70°C (General Foods, 1958a), when a gelatin is precipitated which has low hygroscopicity, high density, and is soluble in water at 1–20°C. Alternatively, a gelatin solution was aerated and dried as a 40 μ film at a high temperature (82°C; 40 sec) (Andel Colloid Corp., 1967). In this method a dispersing agent such as sugar or sodium chloride may be used. Other conventional surface active dispersing agents including silicones, polyethylene oxides, and polyethylene oxide fatty acid esters, have also been employed to coat (by ball-milling) spray-dried gelatin containing 1% of moisture. (General Foods Corporation, 1958b).

B. Swelling and Solubility in Solutions Containing Ions

Although the effect of ionic solutions on the swelling and solubility of gelatin is obviously important in photographic applications, reliable published information is relatively slender (Idson and Braswell 1957). Most globular

proteins can be dispersed in highly concentrated acids—typically gelatin can be dispersed in a mixture of 60–75% of acetic acid and 25–40% of phosphoric acid in practically anhydrous conditions (Caldwell, 1952). Such dispersions are stable for 10–15 days, with little or no chemical changes, and are suitable for coating purposes or for extrusion into a precipitating bath.

The curious phenomenon was observed by Larson and Greenberg (1933), that gelatin is soluble in 99.5% acetic acid only above certain concentrations, and precipitates again when the solution is diluted by addition of more acetic acid. The minimum amount of gelatin which gives a clear solution was 3.1 g/100 g of solution at 20°C, and was 2.7 g/100 g at 25°C. Above 80°C no gelatin fails to dissolve. This behaviour is not due to the presence of even as much as 5% of water.

The influence of cations on gelatin gels was studied by Nobel (1951), who dried a 17% aqueous gelatin solution and then swelled it in 1-N solutions of magnesium, calcium, strontium, and barium chlorides. The swelling increased irregularly with the equivalent of the cation in these cases, but was nearly the same with sodium, potassium and rubidium chlorides. It was greater with lithium and caesium chloride solutions.

An application of the insolubility of gelatin in sodium chloride solutions has been claimed (Swift & Co., 1957) as a method for the fractionation of proteins. Although the quantitative precipitation of proteins was thought to be possible only at the isoelectric point, or by lowering the pH of a salt-saturated solution (Jordon-Lloyd and Pleass, 1927; Flory and Rehner, 1943), it was claimed that both acid and lime-processed gelatin can be precipitated. A 0.56% gelatin solution starts to precipitate with 10–11% of sodium chloride, and only 0.1% of gelatin is left when the concentration reaches 16% sodium chloride. Such effects also occur with mixed solvents of water and polar organic compounds (see below).

C. Solubility of Gelatin in Non-Aqueous Solvents

Umberger (1967) has made a comprehensive study of solvents for gelatin and found that it is soluble in polar solvents of H-bond donor strength equal to or exceeding that of ethane-1,2-diol. Exceptions to this rule were formamide and other cis-amides which although less protic than ethane-1,2-diol dissolve gelatin apparently through a specific solvation or chelation with the polypeptide linkages. Lime-processed bone and acid-processed pigskin gelatins of photographic quality were used but the results were essentially independent of grade so it is probable that Umberger's observations (Table I) are generally applicable.

The solubility test was carried out by heating 5 g of gelatin and 100 g of solvent on a magnetic-stirrer/hotplate for 1 hour at 200°F (or higher for

26.95°C

TABLE I. Solubility of gelatin and solvent proticity (Umberger, 1967). H-bond donor activity (proticity) was measured semi-quantitatively by the quenching action on the dye erythrosin

Solvent in order of proticity	Erythrosin fluorescence lifetime (nanosec)	Relative fluorescence yield	Dissolve gelatin
Acetic acid		0	Yes
Trifluoroethanol	0.1	2	Yes
Water	0.1	3	Yes
Glycerol	0.7	8	Yes
Ethane-1,2-diol		8-10	Yes
Methanol	0.8	14	No
Formamide	0.9	15	Yes
Primary alcohols	1.0	15-16	No
Secondary alcohols	1.3	18-20	No
Glycol, mono ether		22	No
tert.-Butanol	1.7	23	No
Trichloroethylene	2.0	24	No
Chloroform			No
Methylene dichloride	2.5	30	No
Methylchloroform			No
Acetonitrile	2.8		No
Dimethylformamide	2.9	45	No
Acetone	3.5		No

solvents of m.p. > 200°F). Umberger also tested the compatibility of various solvents with a 20% w/w aqueous gelatin solution. 20 g of the aqueous gelatin were mixed with 80 g of solvent at 140°F (or higher for solvents of m.p. > 140°F) and the mixture observed immediately and after overnight cooling. The results are shown in Table II.

Dimethylsulphoxide was found to be a solvent for gelatin as were also a number of molten amides in addition to formamide, N-methylformamide, acetamide, thioacetamide, propionamide, 2-pyrrolidinone, N-ethylurea and other substituted urea and thiourea derivatives.

The solubility of collagen in aqueous alkylamine solutions has been applied to the manufacture of gelatin (Damschroeder and Kauffman, 1954). Collagen-containing sources were dissolved in aqueous solutions containing 1-30% of methylamine, dimethylamine, ethylamine, ethylenediamine, ethanolamine, propanolamine or propylene diamine with or without addition of a smaller amount of ammonia, and the extracted gelatin was insolubilized and washed.

Flory and Garrett (1958) studied solutions of collagen and gelatin in ethane-1,2-diol and Veis and Anesey (1959) used the mixed solvent systems

TABLE II. Compatibility of a 20% w/w gelatin solution with various solvents
(Umberger, 1967)
(1 part gelatin solution mixed with 4 parts solvent at 140°F)

<i>Solvent</i>	<i>Compatibility with aq. gelatin solution</i>
Acetic acid, glacial	Clear mixture, no gel at 50°F
2,2,2-Trifluoroethanol	" " " "
Formamide	" " " "
Water	" " " "
Ethane-1,2-diol	" " " "
Glycerol	" " " "
2,2-Thiodiethanol	" " " "
Diethylene glycol	Partial precipitation, gelled at 50°F
1,4-Butanediol	" " " "
Ethanolamine	Clear mixture, no gel at 75–80°F
Diethanolamine	Clear mixture, gelled at 75–80°F
Triethanolamine	Clear mixture, gelled at 75–80°F
Ethylenediamine	Clear mixture, no gel at 50°F
Diethylenetriamine	" " " "
Dimethylsulphoxide	" " " "
Dimethylformamide	Precipitated gelatin
Triethylphosphate	" "
3-Methyl-tetramethylene-sulphone (cyclic)	" "
Adiponitrile	" "
Dimethyl carbonate	" "

formic acid (FA)—dimethylformamide (DMF) and dichloroacetic acid (DCA)—ethylene dichloride (EDC). With a series of solutions of constant gelatin concentration but varying FA/DMF ratio, the specific rotation of the gelatin increased from $[\alpha]_D^{25} = -116^\circ$ in 100% FA to -58° in 5% FA/95% DMF. The latter value is similar to an $[\alpha]_D$ of -59.8° found by Steinberg *et al.* for a solution of bovine gelatin in a 12% FA/88% *n*-propanol mixed solvent.

Methods of solution of gelatin in polyhydric alcohols (especially glycerol) have been claimed (Tice, 1951a,b) for applications in the preparation of pharmaceutical products. Typically, a 50% solution of gelatin in glycerol is obtained by packing powdered gelatin in a jar or funnel, wetting evenly with glycerol by suction, and heating at 80–100°C (Tice and Moore, 1950).

III. MECHANICAL PROPERTIES

A. Mechanical Properties of "Solid" Gelatin

Since "solid" gelatin always contains some water, normally 10–15%, it can be considered as a sol or gel of very high concentration. The water in effect

acts as a necessary plasticizer since gelatin films of very low moisture content (less than 5%, say) are too brittle for almost all practical purposes. In many photographic and pharmaceutical applications, non-volatile plasticizers are added such as glycerol, and other polyhydric alcohols and there is an extensive patent literature which includes the use of polyethers (Kodak, 1959), polyethylene oxide (Union Carbide Corp., 1965), styrene-acrylic and acrylonitrile-acrylic copolymer dispersions (Eastman Kodak, 1957), and N-acylated caprolactams (Gevaert, 1959).

1. Effect of conditions of formation and drying

One of the most important studies of the mechanical properties of gelatin films is that of Bradbury and Martin (1951, 1952) previously mentioned in Section II. They measured the elasticity, tensile strength and elongation at break of gelatin films as a function of the temperature of drying the film and also the relative humidity during measurement. Films were prepared in two ways:

- (i) by allowing a 5% solution to gel and then drying initially with cool air (70% RH and 20°C) followed by warm air;
- (ii) drying above the gel point with air at 55–60°C and 70% RH. Specimens 5 × 6 cm were cut from the sheets and conditioned at known humidity and temperature before being submitted to a tensile test at a rate of loading of 25 kg/cm²/sec. The results are shown in Figs. 4 and 5.

At all humidities, the films prepared from the gel state had a greater tensile strength than those prepared from sol and up to 65% RH they also had a greater elongation at break although the reverse is true at RH of 75% and 85%. At these high humidities all films are very much extensible and, even

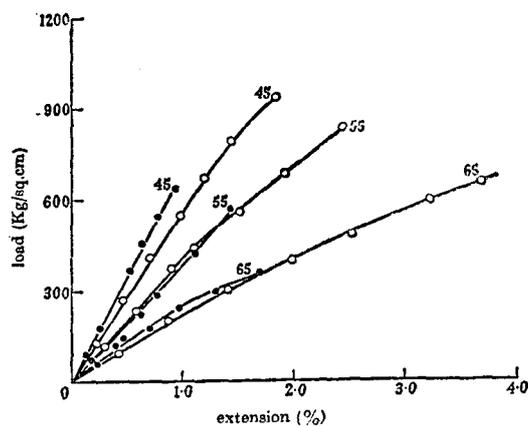


Fig. 4. Load-extension curves at relative humidities of 45 to 65%, indicated on the curves.

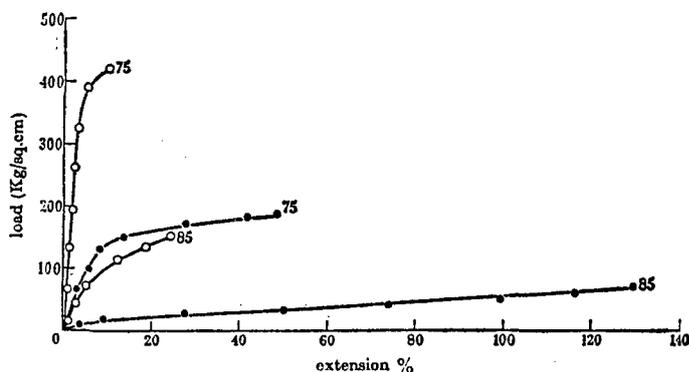


FIG. 5. Load-extension curves at relative humidities of 75 and 85%. ○ and ● as on FIG. 4.

near the breaking strain, the deformation is almost completely elastic at this relatively high rate of loading. There was no thermal degradation during the drying of the hot-dried films and similar results were obtained with films prepared at temperatures up to 100°C. It is clear that the two methods of drying produce films with quite different structures and, as indicated in Sections II and V, not only their mechanical properties but also solubilities in water, X-ray diffraction behaviour, and infra-red dichroisms, are markedly altered.

Similar studies have been reported by Kotina (1951), who also found differences in properties depending on the solution concentration from which the film was prepared, by Pchelin (1968), and by Norris and McGraw (1964). The latter studied films of three commercial photographic gelatins prepared at 10°C and 55°C and investigated also the effects of deionizing and tanning treatments.

2. Mechanical properties of drawn fibres

As with other fibre-forming polymers, mechanical properties can also be modified by mechanically-induced molecular orientation, e.g. fibre-drawing, and there have been several Russian studies of this effect and also that of subsequently tanning the drawn fibre (Anokhin and Kotov, 1960; Grigor'eva *et al.*, 1961; Burdygina *et al.*, 1969).

3. Internal stresses in films

A knowledge of the internal stresses developed in restrained gelatin films during drying is of importance in certain coating applications and useful studies are those of Sanzharovskii and Epifanov (1960), Shreiner and Zubov

(1960), and Zubov and Lepilkina (1961). A simple and effective technique of preparing such films is that of Coleman *et al.* (Coleman *et al.*, 1956; Weatherwax *et al.*, 1958) in which the film is formed as a supported disc within a glass cylinder. After conditioning at 50% RH, stresses of 4000–5000 pounds per square inch were observed in several films.

4. *Mechanical properties of plasticized gelatin films*

Yannas and Tobolsky (1964) have compared the torsion moduli of pure gelatin sheets with those of films plasticized with glycerol or dimethylsulphoxide over a wide temperature range. The results show the plasticized gelatin to have viscoelastic behaviour typical of a plasticized microcrystalline polymer such as polyvinyl chloride. Small protein crystallites are thought to be present even at very high plasticizer concentrations and to act as crosslinks which do not melt even at temperatures as high as 200°C. This interpretation is consistent with the claim that the glass transition temperature of pure gelatin is around 190°C. Castello and Goyan (1964) also explain their results of stress relaxation measurements on gelatin/glycerol films on the basis of crystallites but postulate that these dissolve at higher temperatures. Gupta (1964) has studied the effect of loading on the plastic flow and rigidity modulus of plasticized and unplasticized gelatin films.

5. *Creep*

Eliassaf and Eirich (1960) measured the equilibrium stress-strain curves at 26.5°C, 31.5°C, 36.5°C, and 46.5°C of gelatin films exposed to 100% RH. At 26.5°C, the amount of water absorbed (27%) plasticized the film to a sufficient extent that it showed essentially rubber-like behaviour whereas at 46°C the amount absorbed (15%) was insufficient to modify the glass-like behaviour of "dry" gelatin. Ueno and Ono (1963), in a study of creep behaviour of gels over a wide range of concentrations, found glassy state behaviour at concentrations above 85%. In this region, compliance curves at various concentrations were superimposable by a shift along the concentration axis.

B. Mechanical Properties of Gelatin Gels

1. *Introduction*

Gelatin gels exist over only a small temperature range, the upper limit being the melting point of the gel (which depends on gelatin grade and concentration) and the lower limit the ice point (at which ice crystallizes out). The mechanical properties are very sensitive to temperature variations and even at constant temperature, they vary with time and with the previous

thermal history of the gel and of its precursor solution. Meaningful measurements of the mechanical properties of gelatin gels require that all these factors are controlled.

In qualitative visual examination, gelatin gels appear to exhibit marked elastic behaviour and quantitative experiments confirm that for small strains applied for short periods, of the order of 30 sec, many systems are Hookean. However tests of longer duration result in the gel showing creep (at constant stress) or stress relaxation (at constant strain) with resulting irrecoverable deformation.

2. *Elastic behaviour at small strains*

(a) *Methods of measurement*

The static rigidity modulus of a gel can be derived quite simply by the application of classical formulae to the measurements of forces and strains in a controlled deformation in which the total strain is kept small. Most of the simpler deformation geometries have been reported—bending or stretching of bars or cylinders of gel (Leich, 1904; Poole, 1925), compression of cylinders (Hatschek, 1932), torsion of a cylinder (Sheppard *et al.*, 1920), torsion of a cylindrical annulus (Hatschek and Jane, 1926; Kinkel and Sauer, 1925; Schremp *et al.*, 1951; Cumper and Alexander, 1952b), shear between parallel plates (Ivanova-Chumakova *et al.*, 1956), distortion of a surface under air pressure (Kinkel and Sauer, 1925; Saunders and Ward, 1954; Tunson and Kelly, 1966), and uniaxial compression (Slonimskii *et al.*, 1969).

Dynamic rigidities from wave propagation experiments have been determined by Konno and Kaneko (1969), Yasunaga and Sasaki (1951), Ferry (1941), and Kudryavtsev (1960). The latter observed two completely separate types of propagation of 150–300 Hz longitudinal vibrations in 0.25% aqueous gelatin gels. The first type of propagation had a velocity of 1488–1600 m/sec comparable with the velocity through water whereas the second type had a velocity of 1.3–4.0 m/sec and was thought to be due to propagation along the actual colloid network. A similar phenomenon was observed in a rubber sponge filled with liquid.

(b) *Maturing conditions*

When a gelatin solution gels its rigidity modulus does not reach a constant value; even at constant temperature, the rigidity increases rapidly over a period of several hours after which the rate of increase moderates. Figure 6 shows this effect in the results of Ferry (1948a) for change over 30 hours of the dynamic rigidity at three concentrations of a calfskin gelatin. However he (Ferry, 1948b) found that an approximate constancy could be obtained after chilling the gel at 0°C for 24 hours then raising the temperature to 15°C for 5 hours. Hence in all measurements of gel rigidity or “jelly strength” it is

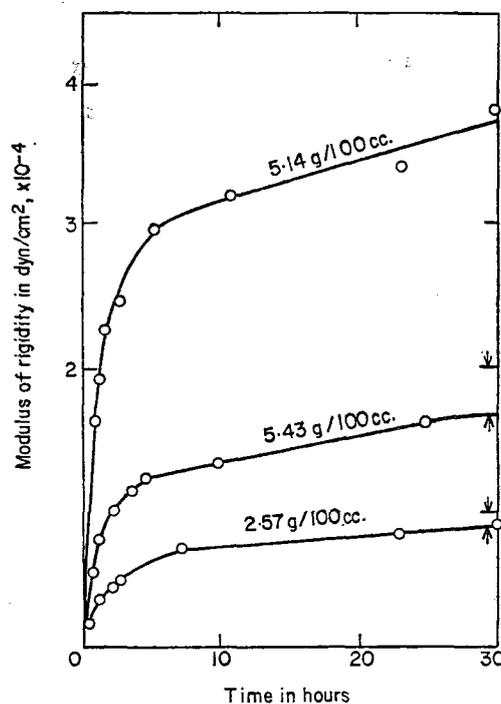


FIG. 6. Increase of rigidity of gelatin gels with time (Data of Ferry, 1948a).

essential to specify exactly the maturing conditions, and the time after gelation at which the observation is made.

(c) Effect of magnitude and duration of applied stress or strain

Sheppard and Sweet (1921) using a torsion method showed that their gels, ranging in concentration from 8.5 to 34 g/100 ml, were Hookean almost to the breaking point with no evidence for a yield point. However Poole (1925) found the Young's modulus of his materials to be a function of strain for strains of zero to 0.4. Hatschek (1932, 1933) confirmed Poole's results in similar extension experiments but found a decrease in Young's modulus with increasing strain in compression tests. Similar behaviour has been observed by Saunders and Ward (1954) for an alkali-processed calfskin gelatin ($pI = 5.08$) at a concentration of 5.5 g/100 ml tested at 10°C after maturation for 17 hr at the same temperature. The apparent rigidity increased rapidly with increasing strain for strains of 0.5 to 1.0 and moreover the strain-dependence of the rigidity differed from one gelatin to another. It follows that many industrial methods of measuring "jelly strength" in which high local strains are

induced are strictly only suitable for comparison of materials of similar rigidities.

Talwar *et al.* (1953) have reported that borate-buffered gelatin solutions can be induced to gel by the application of hydrostatic pressure. Solutions of less than 1% concentration do not gel under any pressure and at higher concentrations, there is a minimum and a maximum pressure for gelation. Above 36°C and concentrations of 10%, no gel is formed at any pressure. Inorganic electrolytes have an appreciable effect on the gelation pressure but pH is not critical.

It has been known for a long time (Rankine, 1906; Hatschek, 1921) that if a gel is deformed at constant strain for a period of longer than a few seconds, the stress required to maintain this strain relaxes with increasing time and some permanent deformation of the gel occurs. Similarly under constant load, the strain increases with time (creep). Ueno (1963) has made an extensive study of the creep of an alkali-processed hide photographic gelatin ($pI = 4.8$) over a concentration range of 5–87% and at temperatures of 10–50°C. He found three distinct types of mechanical behaviour which he attributed to different molecular mechanisms.

Ferry and his associates (Miller *et al.*, 1951) have used their concentric cylinder apparatus to study stress relaxation in gels and its dependence on temperature and thermal history. They used a degraded ossein gelatin ($M_n = 34,000$) at a concentration of 5.92 g/100 ml. The static rigidity measured over a period of several minutes was identical within the limits of experimental error with the dynamic rigidity measured at several hundred Hz. Hence in this particular system, there were no elastic mechanisms with relaxation times between 10^{-3} and 10^2 sec that were contributing to the rigidity. The stress relaxation data indicated a distribution of viscoelastic mechanisms with relaxation times between 10^3 and 10^6 sec. This distribution was shifted in the direction of shorter relaxation times with increasing gel temperature and to longer times with increasing time of ageing before application of stress. Similar studies have been reported by Arakawa (1962), Watase and Arakawa (1967), and Gray (1949) who also distinguished three types of behaviour.

The persistence of optical birefringence after stress decay is complete was noted by Hatschek (1921) and Ferry (1948a). Tobolsky (1955) has suggested that this can be explained if part of the stress decay in gelatin gels is due to further growth of oriented crystallites, possibly around existing nuclei.

(d) Concentration dependence

Early investigators of the dependence of concentration (c) on gel rigidity, G , concurred in finding that G was very nearly proportional to c^2 , for a variety of gelatins all other factors being kept constant (Leich, 1904; Poole, 1925;

Sheppard and Sweet, 1921; Kinkel and Sauer, 1925). However both Sheppard and Sweet and Kinkel and Sauer found highly-degraded gelatins to deviate from this rule, the concentration exponent being greater than 2. Cumper and Alexander (1952b) found the rigidity to increase more rapidly with concentration than would be predicted by the square law when the concentration was above 20 g/100 ml of solution.

Ferry (1948b) and Ferry and Eldridge (1949) using the method of transverse vibrations investigated the concentration-dependence of the rigidity of various well-defined gelatins at constant ionic strength (0.15 M sodium chloride), pH (7.0), and fixed temperature. Ferry (1948b) reported that, under these conditions G was proportional to c^2 in the gelatin concentration range 2.3 to 5.75 g/100 ml and the square law relation was also found by Szczesniak and MacAllister (1964). The later study of Ferry and Eldridge (1949) indicated some deviation from the square-law at lower concentrations. Saunders and Ward (1954) working with an alkaline-treated calfskin gelatin over the concentration range from 0 to 20% found that no part of the plot of G against c^2 was linear, even after a deionizing treatment. They conclude (Ward and Saunders, 1958) that from the considerable amount of work which has been done on this subject, it is unlikely that there is a general relationship which can be applied to all gelatins.

The rigidity of a mixture of two gelatins can be calculated from the expression:

$$\sqrt{G_{\text{mix}}} = (\sqrt{G_1} + \sqrt{G_2})/2$$

which is widely used as a basis for industrial blending of gelatin (Miller *et al.*, 1951).

Instead of preparing gels of different concentrations by setting of the corresponding solutions, Northrop and Kunitz (1926) varied the concentration by allowing the gel to swell in water. By this method they found the rigidity modulus to be very nearly proportional to concentration.

(e) Temperature dependence

Gel rigidity is extremely sensitive to the temperature at which it is measured. Ferry (1948b) has shown that a plot of G/c^2 against temperature is approximately linear over the range 0–30°C, the points for a degraded ossein gelatin ($M_n = 45,000$) falling on the one curve for all concentrations studied. However a similar analysis of results for an ossein of even lower molecular weight ($M_n = 34,000$) gave separate curves for each concentration indicating the non-applicability of the square law.

Pouradier (1967) from a study of the temperature variation of gel rigidity has deduced that intermolecular crosslinks in the gel have heats of formation ranging from 5 to 60 kcal/mole.

(f) Effect of pH and added substances

The variation of gel rigidity with pH has been studied by Gerngross (1926), Steigmann (1957), and Cumper and Alexander (1954). Konno and Kaneko (1969) have measured dynamic rigidity at 100 Hz as a function of pH and temperature (10–50°C). The pattern of behaviour found by Cumper and Alexander is typical with a maximum rigidity at pH~9, a slight falling away to pH~5 and a marked drop below pH 4 and above pH 10. They used an alkali-precursor gelatin (Davis) with pI = 5.0 at a concentration of 27 g/100 ml, a constant ionic strength of 0.15, and the rather high temperature for gel studies of 25°C at which temperature also the gels were matured.

A lowering of the rigidity modulus due to the presence of electrolytes has been demonstrated by Bundenberg de Jong and Hennemann (1932) for a series of potassium salts and by Cumper and Alexander (1954) for sodium chloride. The latter observations could be expressed as a linear relation between rigidity and the square root of the ionic strength except at the highest salt concentrations. Of the non-electrolytes which affect rigidity most attention has been devoted to glycerol because of the industrial importance of the gelatin/glycerol system (Sheppard and Sweet, 1921; Hatschek, 1932; Hirai and Kishimoto, 1953; Mindru and Ceacareanu, 1966; Nixon *et al.*, 1966; Carless and Nixon, 1970).

Addition of glycerol markedly increases the gel rigidity and the papers of Nixon *et al.* describe a full investigation of the effects of composition and gelatin grade. For gels containing 4–15% of gelatin and up to 40% w/w of glycerol, the rigidity modulus G is given by:

$$G = \alpha + \phi Z^2 + (\beta + \gamma Z)C$$

where α , β , γ , ϕ , are constants, numerical values of which are listed for various grades of gelatin.

Z is % gelatin concentration

C is % glycerol concentration

Ethyl alcohol also increases rigidity (Sheppard and Sweet, 1921) as does cane sugar (Sheppard and Sweet, 1921; Hatschek, 1932), formaldehyde (Hatschek, 1932), and other tanning agents.

Stress relaxation of a series of rubber-like gelatin compositions has been studied by Yannas (1968) who found that behaviour typical of brittle glasses, semi-brittle plastics, rubbers, and viscous liquids could be exhibited according to composition, temperature (–40°C to +40°C) and time scale of the deformation. A reduction in rigidity can be brought about by gel depressants such as phenol, urea, and ammonium nitrate (Watase and Arakawa, 1969; Zubov *et al.*, 1954).

3. Fractionated and degraded gelatins: gel rigidity and molecular weight

The rate of thermal degradation of a gelatin solution is appreciable above about 60°C and rapid at 100°C. When a solution which has been heated in this way is cooled to a gel again, the rigidity is found to have decreased irreversibly (Ames, 1947; Croome, 1953a). The more severe the conditions, the greater the loss of rigidity until in the limit the solution does not gel at all. Scatchard *et al.* (1944) prepared samples of gelatins of differing average molecular weight in the range 33,000 to 72,000, measured viscometrically and osmometrically, by degradation of an alkali-precursor ossein gelatin. Ferry (1948b) and Ferry and Eldridge (1949) measured the rigidity of 4% gels of these materials by the method of transverse vibrations at temperatures of 5°C, 10°C, 15°C, and 25°C. The results could be expressed by the empirical equation:

$$G^2/C = 1.22 \times 10^{-4} [M_w - 3.1 \times 10^{10} \exp(-7900/RT)]$$

However Ferry also noted that the rigidities of samples with M_w of 80,000 to 120,000 prepared by Sheppard by fractionation of a calfskin gelatin were almost independent of M_w .

The more general picture has subsequently been established by the work of the schools of Ward and Pouradier. Above a certain molecular weight the

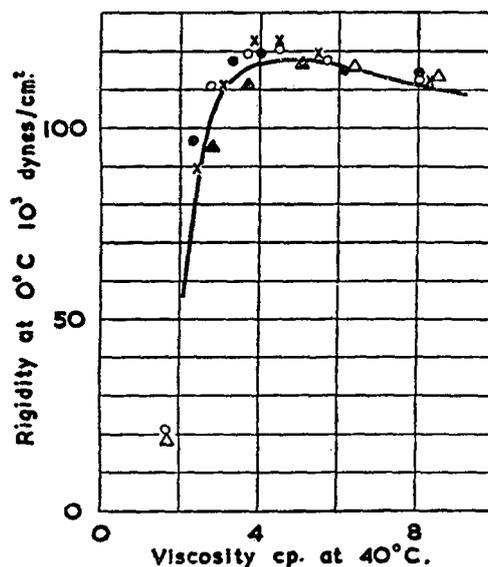


FIG. 7. Degradation by the proteolytic enzymes papain, trypsin, chymotrypsin, and pepsin in comparison with acid degradation. Δ , \blacktriangle , papain (0-20h); \circ , trypsin (0-23h); \bullet , chymotrypsin (0-23h); \times , pepsin (0-9h); \circ , acid-degraded (pH 1.7, 60°C); (Saunders and Ward, 1958a).

rigidity is broadly independent of molecular weight with minor qualifications as to the temperature and gel concentration of the rigidity measurement and the individual characteristics of the particular gelatin. This rigidity plateau is well illustrated in Fig. 7 from the observations of Saunders and Ward (1958a) on a series of degraded gelatins prepared by various routes from a common precursor gelatin. A similar pattern was found using series of gelatins obtained by fractionation (Stainsby *et al.*, 1954; Pouradier *et al.*, 1954). Courts (1959) determined the average chain weights, C_n , of similar fractionated and degraded gelatins by end-group analysis. In this C_n range (55,000–70,000) low temperature (10°C) rigidity was practically independent of C_n and of intrinsic viscosity (range 0.4–0.92). Gelatins of different origins had different rigidity plateaux but these also could not be correlated with C_n or viscosity.

It follows that there must exist some further structural feature ("the rigidity factor") which affects the rigidity of gelatin gels. Todd (1961) has shown that this is the facility of molecules of a particular gelatin to reform partially the origin collagen triple helix in the gel. Such randomly-oriented lengths of triple helix (formed from portions of three gelatin molecules) are what bind the molecules into a network and confer stiffness on the structure. These conclusions follow from Todd's discovery of a general linear relationship between specific rotation of a gel and square root of the rigidity modulus.

4. *Elastic behaviour of gels at high strains and rupture*

Cobbett (1961, 1968) has devised a technique for subjecting dumbbell-shaped specimens of gelatin gel floating on a thermostatically-controlled mercury surface to unidirectional extension under load to the point of rupture. Near the origin, the load-extension curves are linear with a slope which correlated closely with the rigidity modulus measured by other methods. At high strains, the extension is less than that predicted by the straight-line relation. The breaking load is closely correlated with the rigidity modulus and jelly strength.

Cobbett's work has been extended by Marrs and Wood (1972) who have investigated the variation of breaking load with pH and with added salts.

A rather different type of large strain is that which occurs in a gel subjected to a high centrifugal field. Under such conditions, a gel/solvent interface is formed which then sediments further. A significant fraction of the gelatin remains in the sol phase and itself then sediments behind the gel interface (Johnson and Metcalfe, 1963; Johnson and King, 1968). Thus gel ultra-centrifugation affords yet another method of fractionating a gelatin.

5. *Cross-linked gels*

Saunders and Ward (1958b) have studied the behaviour of formaldehyde-crosslinked gels under conditions where the normal gelling forces were absent

either because of the high temperature (above 40°C) or because of the addition of a H-bond breaker (potassium thiocyanate). As required by the kinetic theory of rubber-like elasticity, the rigidity modulus increased with increasing temperature with a coefficient of 0.002 per degree C. A similar conformity with theory was found by Jopling (1958) using gelatin films crosslinked with formaldehyde or difluorodinitrobenzene and then swollen in alcohol-water mixtures. The load-extension curve had the theoretical form even for extensions of 300%. In the range 50–70°C the temperature coefficient of the stress at constant extension was positive and proportional to absolute temperature. Between 50°C and 0°C, the elastic modulus increased greatly with decrease in temperature due to the formation of additional crosslinks by gelation.

C. Mechanical Properties of Gelatin Solutions

1. Introduction

The viscosity of dilute gelatin solutions and the influence of factors such as pH and added electrolytes are fully discussed in Chapter 4. This section will consider the mechanical properties of more concentrated solutions. It may be helpful at the outset to describe these in general terms as follows:

A concentrated solution of a high molecular-weight gelatin at a temperature just above that at which it gels will be quite strongly viscoelastic and moreover the viscosity and related properties will be time-dependent for several hours after the solution has been brought to this temperature. A change in the direction of increasing temperature, decreasing molecular weight, decreasing concentration or a combination of these leads to the flow behaviour becoming more closely or even completely Newtonian (see also Levi, 1960). It follows that any meaningful study of the rheology of concentrated gelatin solutions necessitates the use of a viscometric technique which can spot the onset of non-Newtonian flow i.e. the least requirement is that the rate of shear must be well defined. Wiegand (1963) has shown how the presence of viscoelastic flow in a gelatin solution may be demonstrated qualitatively by the Weissenberg effect.

2. Temperature- and concentration-dependence of viscosity

The temperature and concentration dependence of gelatin solutions is illustrated in Figs. 8 and 9 for a 190 Bloom commercial acid ossein gelatin. The slight S-shaped log (viscosity)—concentration isotherms and the rectilinear relationship between log (viscosity) and the reciprocal of absolute temperature in the temperature range 50–90°C have been found to be universal for a very wide range of materials from very low grade (30 g double Bloom) animal glues to top quality gelatins (Jobling *et al.*, unpublished). Measure-

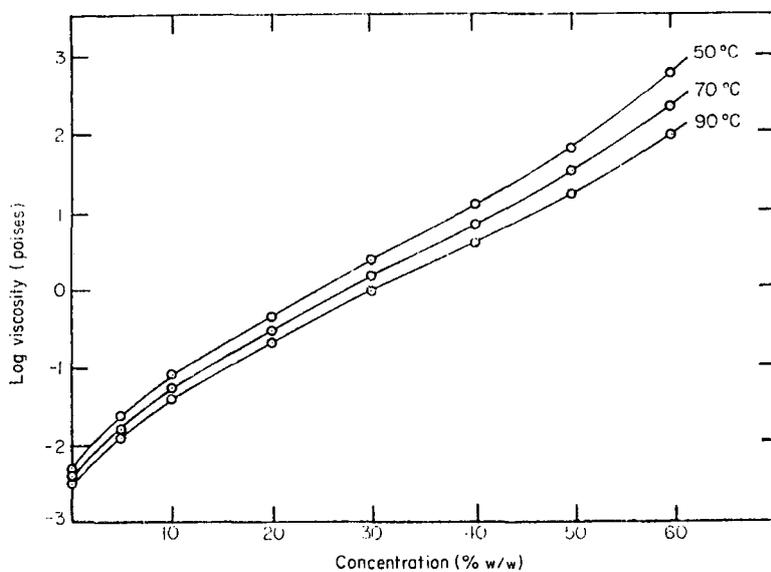


FIG. 8. Viscosity of solutions of a 190 g 28 mp (at 6 $\frac{2}{3}$ %) acid ossein gelatin as a function of concentration and temperature (previously unpublished data of Jobling and co-workers).

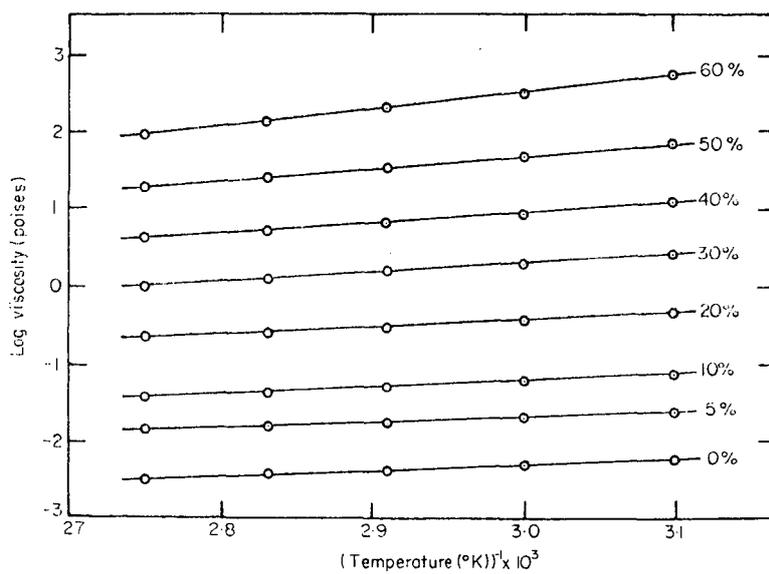


FIG. 9. Log (viscosity) as a function of reciprocal absolute temperature at constant concentration (data of Fig. 8).

ments at 20% concentration and higher were made with a Ferranti concentric cylinder viscometer and at 5 and 10% concentration with a glass capillary viscometer with careful precautions against thermal degradation during measurements at the higher temperatures. Variations of viscosity with rate of shear in the range 5–200 sec^{-1} were within the overall limits of experimental error. The straight-line dependence of $\log(\text{viscosity})$ on reciprocal absolute temperature has previously been reported by Croome (1953b) and Cumper and Alexander (1952a) have found the approximately exponential increase of viscosity with concentration (10–50% range) exemplified by the central portions of the S-shaped curves in Fig. 8. The audio-frequency (100 Hz) dynamic rigidity and viscosity of aqueous gelatin solutions have been measured by Konno and Kaneko (1969) as a function of temperature (10–50°C) and pH.

3. *Ultrasonic irradiation of gelatin solutions*

The behaviour of ultrasonic waves in gelatin solutions has been studied by Mikhailov and Tarutina (1950), Mikhailov (1964), and Wada, Sasabe, and Tomono (1967). The last-named team measured the velocity of longitudinal waves at 3 MHz in gelatin solutions by the sing-around method. (For description of apparatus, see Tschiegg and Greenspan, 1956.) The velocity increased with increasing pH reflecting a decrease in adiabatic compressibility. Dynamic shear viscosity and shear modulus were measured at 50 kHz by a torsional crystal technique. The shear viscosity measured as a function of pH passed through a minimum at the isoelectric point of the gelatin.

4. *Mechanical degradation in gelatin solutions*

As is to be expected, sufficiently powerful ultrasonic irradiation of gelatin solutions can cause chemical breakdown as evidenced by reduction of viscosity and gel strength (Zhukov and Khenokh, 1949; Morel and Grabar, 1950; Khenokh and Lapinskaya, 1951). Loss of nitrogen occurs, particularly below pH 7. Morel and Grabar found that the nature of the cleavage was different from that resulting from acid hydrolysis. Mechanical breakdown of polymers under continuous shear is a well-known general phenomenon. Hewitt and Jobling (unpublished) obtained a > 20% irreversible reduction in viscosity in a 65% low-grade (100 Bloom) gelatin solution sheared for 3 minutes at 40°C in a small rubber masticator.

IV. DIFFUSION THROUGH GELATIN FILMS AND GELS

An understanding of the diffusion of various materials through swollen gelatin layers is of basic importance in the photographic process and most

studies of diffusion in gelatin have been carried out for this reason. However Allison and Humphrey (1959, 1960) have proposed the use of gelatin gels of varying concentration (4–40%) as molecular sieves for estimating the effective size of virus antigens and claim a reproducibility to within +30% after calibration with reference antigens.

Diffusion of water and aqueous solutions into gelatin films and gels has been followed by radioactive tracer methods using tritiated water (Aussel *et al.*, 1965), or ^{32}P -labelled phosphoric acid (Cetini and Ricca, 1958). Staaf (1967) has used an optical method—measuring the critical angle of reflection at the glass-gel interface and an electrical conductivity method has been used by Amelina and Kovalichev (1957) for water and by Fink and Furrer (1957) for a ferric chloride etching solution alone and with various additions. The practical application of the latter system has also been discussed (Cartwright, 1949). Aussel *et al.* found the diffusion coefficient of water into gelatin to decrease with increasing gelatin concentration and with decreasing temperature and adduced a purely mechanical explanation of these effects. Cetini and Ricca (1958) using aqueous phosphoric acid and Cordier (1955) using various electrolytes have both reported deviations from regular behaviour as zero gelatin concentration is approached.

In the diffusion of the $(\text{HPO}_4)^{--}$ ion of Na_2HPO_4 in gelatin gels, in an homogeneous chemical medium, values of the diffusion constant measured at various points of the gel are not constant, but tend to a limit with increasing distance from the separation line between the solution and the gel (Marignan and Crouzat-Reynes, 1956).

Diffusion of radon gas through dry gelatin films has been studied by Vigneron *et al.* (1955), who impregnated photographic plates (Ilford C_2) 100 μ thick with a radium salt for 30 minutes, followed by air-drying for 1 hour and development for 3–13 days. They were led to postulate the existence of two types of film, one unstable and only slightly permeable to radon and the other with high permeability.

Diffusion of dyes into and from gels has been extensively investigated and Nixon *et al.* (1967) include a bibliography of earlier work. This paper reports a thorough study of the factors governing the diffusion of methylene blue in gelatin-glycerol gels including the effects of Bloom grade, temperature, and concentrations of gelatin, glycerol, and methylene blue. For all gelatins used, the diffusion coefficient passes through a maximum at a glycerol composition of about 10% w/w. Other data on the velocity of diffusion of other dyes (fuchsin and bromophenol blue) in gelatin have been related to the pH of the gel (Rastelli, 1951). The curves show a characteristic bend at the isoelectric point of the gelatin.

V. OPTICAL PROPERTIES

A. Colour and Clarity of Solutions

The visual appearance of aqueous solutions or gels of gelatin is of great importance in many applications: in other cases, the colour of the dry film may be important. Methods for measuring both colour and clarity have been described by Saunders and Ward (1952, 1953) in which the optical densities of solutions, before and after filtration by a bacterial filter, are measured either by spectrophotometer or by a photoelectric absorptiometer, using standard colour filters. The method was described in British Standard Specification 757: 1944 but was omitted from later revisions.

It was first observed by Kraemer (1926) that the turbidity of a gelatin solution was a function of pH, maximum turbidity occurring at the isoionic point in a salt-free solution. Later studies by Janus *et al.* (1951), Boedtker and Doty (1954), and Veis *et al.* (1958) have confirmed that, for alkali-precursor gelatins, isoionic points determined by the maximum turbidity method are sharply defined and in excellent agreement with those determined by deionization or titration methods. For acid-precursor gelatins, the turbidity maxima are broad and not always coincident with the deionization pH due to a tendency to bind alkali ions.

B. Optical Rotation

Studies of the optical rotation of gelatin solutions, gels and films have proved a powerful tool in the elucidation of their molecular structure. The gelation of gelatin is accompanied by a rapid decrease in the optical rotation, which gradually slows its rate of decrease but never attains a constant value. The specific rotation, $[\alpha]_D$ reduces from approximately -120°C at 40°C to up to -350° at 0°C . This change is reversible.

C. R. Smith (1919) observed that $[\alpha]_D$ of gelatin solutions at 33°C or above was independent of time. Below 33°C , $-[\alpha]_D$ decreased rapidly with time at first, but much less rapidly later. Smith found that if the gel was maintained at a temperature several degrees below the melting temperature, and then warmed up, a near equilibrium value was attained much more quickly. Smith also found that $-[\alpha]_D$ was temperature-independent above 35°C in the 1–7% range of concentration. This was confirmed by Kraemer and Fanselow (1925), Ferry and Eldridge (1946), and Robinson (1953) and it was also noted that $-[\alpha]_D$ increased with decreasing temperatures. Above 34°C , $-[\alpha]_D$ was independent of gelatin concentration, but at lower temperatures there was a significant dependence on concentration, increasing with lower temperature. Kraemer and Fanselow (1925) investigated the effect of pH on 0.5% gelatin solutions. At 50°C , $-[\alpha]_D$ was independent of pH in the range

pH 2–12, but at 20°C a maximum was observed at pH 5, which decreased with further increases of pH. Electrolytes also affect $-\alpha_D$: at 40°C increasing concentration of salt slightly increased $-\alpha_D$, irrespective of the salt used (Carpenter, 1938) but at 0.5°C, a marked increase in $-\alpha_D$ was observed when salts such as potassium thiocyanate or lithium bromide, which are known to reduce the rigidity of gelatin gels by affecting the hydrogen-bonding, were added to the solutions. Comparisons of $-\alpha_D$ for soluble collagen and gelatin solutions in the presence of lithium bromide with those of different forms of poly-L-proline have been made by Harrington (1958) and the results analysed. The configurational contribution to the observed $-\alpha_D$ was estimated to be 230°C for collagen, 30°C for gelatin in water and –40°C for gelatin in lithium bromide.

When gelatin films are prepared by drying a gelatin solution above 35°C, or by drying in the gel state, the specific rotations of the two films are different (Smith, 1919; Robinson, 1953). The former has $-\alpha_D$ of 120°C, like that of a gelatin solution at 35°C, whilst the cold-dried film has $-\alpha_D$ of 750°C, which is much greater than the specific rotation of a gel. Studies of the optical rotation, and especially of the optical rotatory dispersion of gelatin, have been greatly directed towards the elucidation of protein structure (Elliott, 1957; Pchelin, Izmailova, and Merzlov, 1963a, 1963b; Merzlov and Pchelin, 1965). The optical rotation of a series of relatively thick acid pigskin gelatin films prepared under a variety of drying conditions has been reported by Coopes (1968), who also measured changes during drying. The optical rotation is unaffected by temperature and humidity at wet bulb temperatures below 25°C, but above this temperature, the optical rotation drops sharply (Fig. 10). Films containing formaldehyde and mucochloric acid tend to have lower rotations than unhardened films. Various types of gelatin show differences in the gel-state. These differences tend to decrease on drying, a tendency which is inhibited only by stable chemical crosslinks or high drying temperatures.

The optical rotation of gelatin in solvent systems such as formic acid-dimethyl-formamide, and dichloroacetic acid-ethylene dichloride changes from $[\alpha_D] - 110^\circ\text{C}$ to $[\alpha_D] - 65^\circ$ by addition of a suitable non-solvent of low dielectric constant (Veis and Anesey, 1957).

C. Refractive Index

The refractive indices of amino-acids, proteins and related substances have been determined by McMeekin, Groves and Hipp (1964) who have shown that the refractive index is a unique characteristic of the protein. The solution refractive index is well established as a rapid and convenient method for determining gelatin concentrations (independent of quality and small amounts

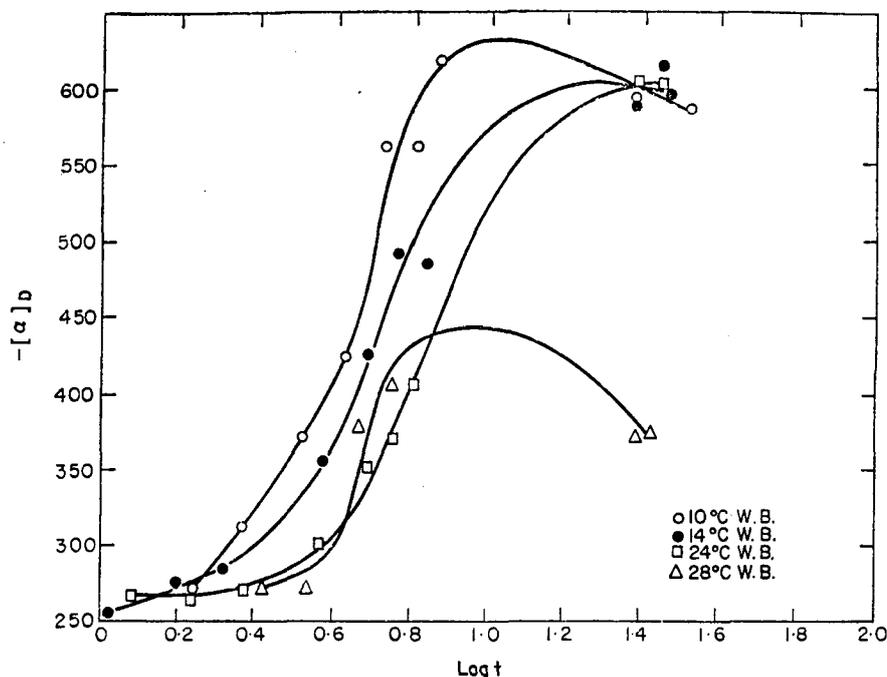


FIG. 10. Specific rotation, $-[\alpha]_D$, plotted against logarithm of time, t , in hours for gelatin films dried under various conditions of temperature and humidity. W.B., wet bulb (Coopes, 1968).

of inorganic salts). Kenchington (1952) showed that up to concentrations of at least 25%, the refractive index-concentration relationship at 34.5°C is given to an accuracy of $\pm 0.15\%$ concentration by:

$$n_s - n_0 = 0.0018c$$

where n_s = refractive index of gelatin solution at 34.5°C

n_0 = refractive index of water at 34.5°C.

c = concentration (g of anhydrous gelatin per 100 ml of solution).

D. Streaming Birefringence

Determination of the extinction angle, χ , of moderately concentrated colloidal solutions, which exhibit birefringence, Δ , under different physical and chemical conditions, allows changes in physical structure to be studied. This technique has been used to observe the molecular aggregation which precedes the solidification of gelatin gels, as a function of temperature, concentration, and molecular weight of gelatin, pH, and the concentration of added salts (chlorides and phosphates). Concentration and temperature are

the main factors affecting the speed of the transformation. Molecular weight has a negligible effect (Joly, 1949a; Bourgoïn and Joly, 1952, 1954). The gelation mechanism appears to consist first of the formation of fibrils, not necessarily in rectilinear alignment, within the gelatin. For a 1.5% gelatin solution at pH 6.8, the length of these fibrils increases from 165Å after 15 minutes to 345Å after 6 hours. Addition of small amounts of sodium dodecyl sulphate increases the rate of formation of these fibrils, but larger quantities cause a shortening, probably due to formation of a monolayer, followed by a second layer of sodium dodecyl sulphate molecules. Addition of gum arabic at pH 6.75, when coacervation does not occur, retards gelatin when freshly prepared, but after 2 days causes acceleration, indicating a slow interaction between the two colloids (Joly, 1949b).

The successive stages of gelation may be followed by observation of χ and Δ (see Fig. 11) which indicates that changes occur in gelatin solutions before

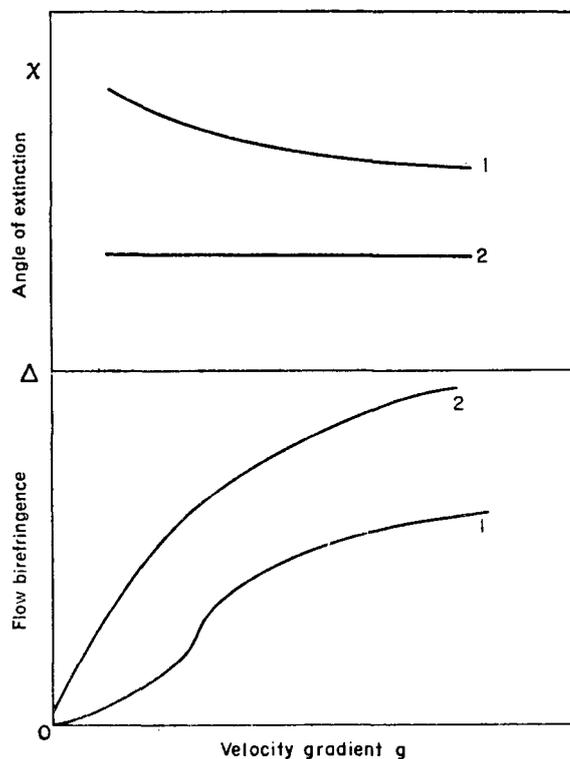


FIG. 11. Schematic representation of the variation of the angle of extinction χ , and the flow birefringence, Δ , as a function of the velocity gradient, g (Bourgoïn and Joly, 1958).

1. First phase of pre-gelation
2. Second phase of pre-gelation

hardening takes place. These modifications of structure occur in several different stages, indicated by breaks in the curves of χ and Δ . Similar and related breaks are also observed in the viscosity-shear curves (Bourgoin and Joly, 1957). Calculation of the theoretical relations on which the curves for viscosity and Δ are based (Bourgoin and Joly, 1956) suggests that during the pre-gelation more or less labile bonds are established between the gelatin molecules in solution, the number of which increases with time.

E. Light Scattering

Light scattering studies of the aggregation of gelatin during gelation (Beyer, 1954) have shown that an average of between one and nine gelatin molecules are contained in the aggregates found as a function of gelation time. It has been suggested (Katti, 1949) that two distinct stages occur in the sol-gel transition: (i) development of a cloud of water molecules around each solute particle, leading to a viscosity increase without a corresponding change in the light-scattering behaviour, and (ii) an increase in the size of the solute particles. At a later stage, aggregation occurs. This pattern agrees in outline with the conclusions mentioned above, based on flow birefringence and viscosity studies.

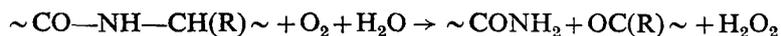
F. Miscellaneous Optical Properties

Although the X-ray diffraction and infra-red absorption spectra of gelatin are, strictly speaking, optical properties, they are more conveniently discussed in relation to other structural studies in Chapter 5.

VI. ELECTRICAL PROPERTIES

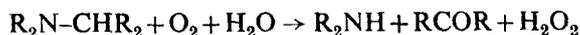
A. Irradiation of Gelatin

The effects of irradiation on gelatin have been studied from two principal aspects—the chemical reactions induced, and changes in the physical properties of the polymer. Several chemical reactions have been noted, but in such a complex system it is unlikely that all possible changes have been elucidated. Bennett and Garrison (1959) suggested that a principal reaction during radiolysis of aqueous gelatin in the presence of air was:



followed by oxidation of terminal and/or side-chain amino-groups to give ammonia. In the absence of oxygen, aspartic acid was liberated (Garrison, 1959). The same group (Garrison, Jayko and Bennett, 1962) studied the effects of ionizing radiations on proteins in terms of related mechanisms

involving the N-C linkage. Major radiation-chemical changes may be interpreted in terms of the overall reaction:



where R_2N-CHR_2 can represent any of the possible N-C linkages of protein structure.

The intrinsic viscosity of gelatin decreases with the dose of ionizing radiation, but the degree of chain scission is small enough not to cause measurable reduction in Bloom strength at doses up to 2 Mrep (Mateles and Goldblith, 1958). However, doses of 2 Mrad and 6 Mrad in dry gelatin were stated to decrease both Bloom strength and viscosity: the presence of nitrogen and low moisture did not minimize this radiation damage, although the heat emitted by the radiation did not affect the physical properties (Bolaffi *et al.*, 1959). It is claimed (Kučera, Šácha, and Strelba, 1959) that irradiation with only 0.08-0.1 Mrep is sufficient to sterilize glue or gelatin completely, if it is stored for at least 3 months after irradiation, as the micro-organisms die during this time. For complete immediate sterilization of gelatin, 1.6 to 2.3 Mrep is necessary, but the quality is impaired above 2.0 Mrep.

The effects of γ -irradiation, using a ^{60}Co source, have been studied by Japanese workers. Irradiation of an aqueous gelatin solution (^{60}Co source, 2000 curies, dose rate 1.98×10^5 Röntgen/hour) caused crosslinking when nitrogen was passed through the solution, whilst degradation took place in an oxygen atmosphere (Tomoda and Tsuda, 1961). Empirical equations relating changes in viscosity and the number of inter- or intra-molecular crosslinks were obtained. Dry gelatin, γ -irradiated in air, nitrogen, and ether, did not show any change in viscosity or melting point (Tomoda, 1960), whilst non-linear increases as a function of dose were shown in the viscosity and melting point of irradiated 0.3-7% photographic gelatins, indicating that water plays a significant part in these changes. The higher the initial viscosity, the greater was the viscosity increase. At higher irradiation doses, warm-water insoluble gelatin gels were formed. More recently, Hopp and Wundrich (1964), γ -irradiated acid-processed pigskin powder of 13% moisture content with doses of 0-100 Mrad, and found that gel strength, viscosity, and setting point decreased with increasing irradiation doses, starting at 0.2 Mrad, indicating chain scission of the protein molecule, whilst the conductivity increased and the isoelectric point decreased, indicating changes in the functional groups. Irradiation of 20% gels, under nitrogen, was carried out at 0.1-0.8 Mrad, since higher doses gave insoluble gels containing many gas bubbles. At 0.1-0.8 Mrad, the product showed increased viscosity and setting point, decreased gel strength, and unchanged conductivity and isoelectric point. These effects were more marked when gels were prepared from air-free water. After γ -irradiation with doses over 0.7 Mrad, the gels were water-insoluble,

even at 60°C. Lowering of the pH of gels to 2.0 before irradiation caused increased drop of gel strength and viscosity upon subsequent testing after readjustment of pH to the original value.

γ -Irradiation of gelatin (0.9% in water) from great blue shark-skin collagen resulted in gel formation at 10°C. Both viscosity and optical rotation of collagen solutions decreased following irradiation, the former more than the latter (Kubota, Kimura, and Ohashi, 1968). Amongst earlier work on the irradiation of gelatin, the effect of X-rays (Woodward, 1932; Kawamura and Hirota, 1951), electrons (Perron and Wright, 1950), and other radiation (Pavlovskaya and Pasynskii, 1955a, 1955b, Bovey, 1958) have been reported.

B. Electrical Conductivity

Data on the electrical properties of gelatin in solid, gel, and solution are fragmentary, and only certain aspects have been studied. The d.c. conductivity of polar polymer adsorbates has been measured for gelatin (and also for keratin and nylon) over a wide range of concentrations using water and formic acid as adsorbates (King and Medley, 1949), and the effect of temperature and the presence of salts investigated. If it is assumed that the conduction process is ionic, the Bjerrum relation adequately describes the phenomena. The presence of proteins and non-electrolytes, including gelatin (and also albumen, globulin, glucose and glycerol) increases the conductivity of electrolyte solutions: the effect is employed commercially in electroplating. There is no relation between the conductivity effect and the viscosity of the protein solution (Llory, 1958).

The temperature coefficient of the electrical conductivity of gelatin at room temperature is $\sim 10\%$ (Gross, 1959). Changes in conductivity of gelatin under visible and infra-red radiation are due to thermal effects, and are not connected with photosensitivity of photographic materials. The effect is related to humidity—with an optimum conductivity at 50–80% RH, which is observed when the resistance of the sample and the input resistance of the d.c. amplifier are equal (Kamiya, 1954). This effect has also been studied in gelatin films humidified with deuterium oxide vapour (Niven, 1961). Riehl (1955, 1957) has found an increase in conductivity in gelatin on irradiation at 366 nm wavelength. The electrical resistance of thin gelatin films increases with length of time of exposure to a strong electric field, but does not return quickly to the normal value after reversal of the field (Niven, 1958). This effect is related to the semiconduction phenomenon observed in gelatin (and also in albumen and in gelatin-dye complexes). The semiconduction activation energy of dry films on glass and quartz was 1.13 ± 0.05 eV for albumen at 40–100°C and 1.48 ± 0.05 eV at 110–140°C. By extrapolation, the resistivity values at 21° are 1×10^{17} and 2×10^{22} ohm cm. Addition of a small amount of

rhodamine or methylene blue to gelatin reduces resistivity by 1×10^4 and 5×10^7 , and activation energy to 1.15 and 0.66 eV respectively (Peerson and Ore, 1963). The temperature dependence of these properties is complex.

The melting and gelation behaviour of dialysed gelatin solutions has been studied by specific conductivity measurements (Rudenko and Levi, 1968). At low gelatin concentrations the electrical specific conductivity showed two changes, at the melting point and at 32°C, related to the two-stage mechanism of melting. A hysteresis effect was noted.

An investigation of audio-frequency and microwave conductivity of ash-free and potassium chloride-containing gelatin solutions at 10–50°C (Masszi and Orkenyi, 1967) showed that, with ash-free gelatin, hydrated water and proton migration play an important role in microwave conductivity. The electric field of the polyelectrolyte changes at the sol–gel transition. Also at the sol–gel transition, the microwave conductivity of potassium chloride dissolved in gelatin changes, which indicates a decrease in the hydrated water.

C. Dielectric Properties

In studies of the dielectric properties of collagen and gelatin, Saiki and Okamoto (1966) found two kinds of dielectric absorption (α - and β -) at –70° to 200°C and 30–10⁶ Hz. The dipoles which contribute to the molecular motion for the α - and β -absorption lie on the polypeptide main chain. The activation energies of the absorptions are 24–28 kcal/mole and 12–19 kcal/mole respectively.

At low frequencies, the dielectric constant of gelatin gels (and also those of agar, carboxymethyl cellulose (CMC) and maize starch) is much greater than that of water. It decreases continuously as frequency increases, tending to level off at about 10⁸ Hz (Masuzawa and Sterling, 1968). The dielectric constant is lower the higher the polymer concentration and increases in the order CMC < agar < starch < gelatin, which may be explained by assuming that the lower the dielectric constant, the more solid the water construction. The nature of these structures has also been studied by nuclear magnetic resonance by the same authors (Sterling and Masuzawa, 1968).

At 3.2 cm wavelength, the dielectric constant depends on the water content of gelatin, and may be used to determine free and bound water (Sevcik and Vetterl, 1965). In measurements with gelatin gels containing glycerol between 100 MHz and 15 KHz, the complex dielectric constant, ϵ , of glycerol with 13% of water at 29°C was identical with the relaxation equation for pure glycerol. Measurements of ϵ for solidified glycerol gels with 0–20% gelatin in water containing glycerol dispersed at 10 KHz and 120 MHz at 20–60°C showed a decrease of static dielectric constant with increasing gelatin concentration (Pottel and Wuelfing, 1963).

D. Miscellaneous Electrical Properties

Some of the photoconductive (Gross, 1959; Kamiya, 1954) and semi-conductive properties (Peerson and Ore, 1963) of gelatin have been mentioned in the previous section. The triboelectric properties of various polymer films including gelatin have been correlated with the nature or the orientation of the groups on the surface, and with adsorbed substances or surface contaminants (Webers, 1963).

VII. THERMAL PROPERTIES

The effects of heat on gelatin in solid, gel, and solution form, have been examined from various aspects. Some of the reports are conflicting, and notable gaps in the literature may be noted.

A. Irreversible Changes on Heating

Gelatins from both acid- and alkali-processes are "denatured" when subjected to dry heat at 120°C, 150°C and 170°C (Tice and Moore, 1952). The changes produced are progressive, passing from an altered solubility and swelling in water, to that state in which pepsin and papain are not able to effect complete hydrolysis. The higher the temperature, the shorter the time required for denaturation. The denaturation effect is complex, and is not due to dehydration alone, since no such changes occur with complete desiccation in the cold *in vacuo*. The differential thermal analysis of gelatin (and also wheat gluten and egg albumen) shows that exothermic reactions occur up to 225°C. The general form of the differential thermal analysis curves has been reported (Laws and France, 1949). DTA shows that the heat of hydration of gelatin is intermediate between that of liquid water and water in a crystalline hydrate (water-oxalic acid) (Jahn and Witnauer, 1967).

The specific heat of the evaporation of water from gelatin has been determined (Kazanskii, 1964a, 1964b) and found to depend on the nature of the bonding of the water. The specific heat of isothermal evaporation, ΔH_{ev} of water from gelatin was measured as a function of the water content, $[H_2O]$, in the polymer, at 61.5°C for $[H_2O] > 15$ and at 110°C for $[H_2O] < 15$. ΔH_{ev} is slightly higher than that of free water for $[H_2O] \lesssim 18\%$, below which ΔH_{ev} rises sharply.

Other Soviet workers (Burdzhanadze, Privalov and Tavkheldze, 1962) have studied the heat capacity of gelatin solutions of different concentrations from 0–60°C. Addition of 5 M-urea eliminated all heat-absorption peaks, indicating a dependence of partial heat capacity on temperature, with curves parallel to those for dry gelatin. The heat capacity of the gelatin solutions

(in the range studied) is not a simple additive function of the heat capacities of the individual components.

Earlier work on the thermal expansion of gelatin gels and solutions (Neiman, 1954a, 1954b, 1956; Meerson and Puchkova, 1959) on changes in the X-ray spacings of gelatin films upon heating (Zaides, 1950), and on thermally induced crosslinking reactions (Bello and Riese-Bello, 1958) has been superseded by the careful work of Yannas and Tobolsky (1968) and that of the Russian school (Burdygina, Undzenas, Fridman, Koslov, and Kargin, 1968; Burdygina, Fridman, Koslov and Kargin, 1969). The former workers have discussed the possible melting of gelatin in the range 150–250°C. This is

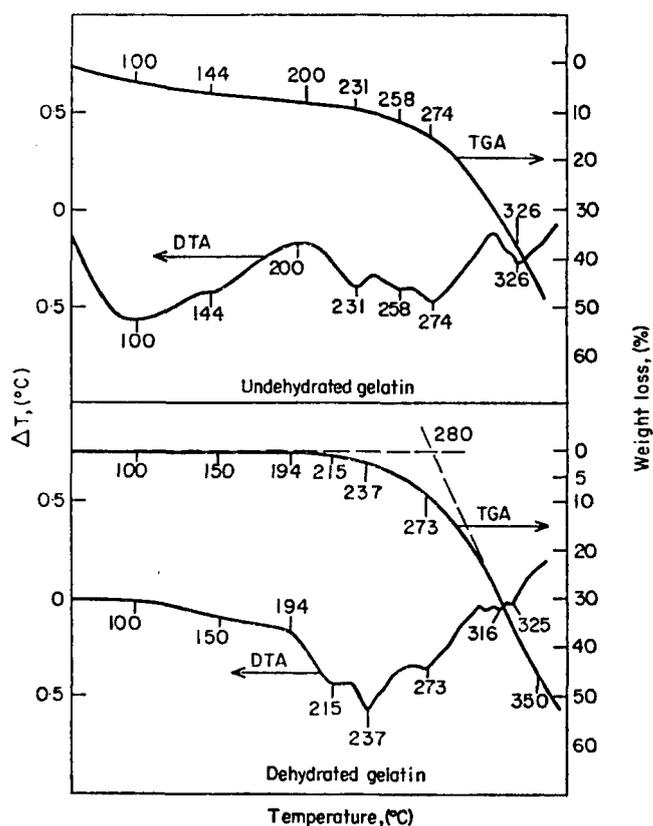


FIG. 12. Thermal properties of initially undehydrated (11.5% wt. water) and initially dehydrated (moisture content less than 0.2 wt. %) gelatin in the range 50–350°C. Results from both differential thermal analysis (DTA) and thermogravimetric analysis (TGA) are shown. Data obtained at a heating rate of 5°C/min under a nitrogen atmosphere (after Yannas and Tobolsky, 1968).

complicated by losses of residual tightly-bound water, and by chemical decomposition (Fig. 12).

DTA and thermogravimetric analysis of dehydrated gelatins under nitrogen indicate that non-oxidative pyrolysis begins above 200°C, whilst at about 230°C an endothermic transition occurs which reaches a peak at 241°C ± 4°C. This transition, although it resembles melting, includes an irreversible chemical transformation. The chemical nature of gelatin is unchanged below 200°C and the general behaviour is not affected by the source and method of preparation of the gelatin. On the basis of these studies, the thermal behaviour of gelatin is unique in the bulk state. The Soviet workers used thermal dilatometry and X-ray methods in the range -100°C to 230°C to study the molecular structure. A so-called "super-contraction" was observed at ~210°C, when a helix-to-random coil transition occurred, with loss of the mechanical properties characteristic of a fibrillar structure. At moderate temperatures (~20–120°C), the behaviour was determined by moisture content; increasing the moisture content increased the negative coefficient of linear expansion. At low temperatures (-100°C to +20°C) the properties of gelatin are similar to those of solids with a small positive coefficient of linear expansion. In the range 160–170°C the solubility of gelatin in water and hot urea solution is greatly diminished, apparently as a result of crosslinking of the molecules. The helix-to-random coil transitions caused by heating increase the hydrophilic properties if carried out below the crosslinking temperature.

The heat of crosslinking of gelatin gels was calculated by Pouradier (1967) by a thermodynamic analysis of earlier results on the variation of rigidity as a function of temperature. This indicates that the intermolecular crosslinks responsible for the cohesion of gelatin gels have variable energy, with heats of formation of 5–60 kcal/mole. It is suggested that the crosslinks are probably formed by the local association of at least two molecules joined by up to 12 neighbouring hydrogen bonds.

B. Effect of Plasticizing Additives

The thermal transitions (and also the viscoelastic properties) of gelatin incorporating glycerol, ethane-1,2-diol, or dimethylsulphoxide have been studied from -130°C to 250°C by Yannas and Tobolsky (1966) who showed that the glass transition, T_g , was independent of diluent over a wide range of diluent concentrations (Yannas, 1966). The gelatin-glycerol system was studied over the whole concentration range in both anhydrous and slightly hydrated states. In the former state, the gel was always crosslinked, with a T_g of 196 ± 3°C, whilst in the system containing traces of water, no crosslinking was observed. From data on slightly hydrated specimens, it was

concluded that the T_g for anhydrous un-cross-linked gelatin free of diluent is $175 \pm 10^\circ\text{C}$.

C. Thermal Conductivity of Gelatin Gels

The thermal conductivities of gelatin gels, and other related foods including meats and fats in the range of -25°C to $+5^\circ\text{C}$ have been reported by Lentz (1961).

D. Density of Aqueous Gelatin Solutions

The variation of density of gelatin and glue solutions with temperature has been reported by Eastoe, Fysh and Ward (1952), who found for hide gelatin and for several grades of glue that

$$d_{\text{gel}}^t = d_{\text{H}_2\text{O}}^t + 0.00255 [\text{gelatin}] + 0.000055 [\text{ash}]$$

where:

d_{gel}^t = density of gelatin solution at $t^\circ\text{C}$.

$d_{\text{H}_2\text{O}}^t$ = density of water at $t^\circ\text{C}$.

[gelatin] = concentration of gelatin (at 15% moisture) in g/100 ml of solution.

[ash] = ash content of gelatin (%).

VIII. SURFACE ACTIVE PROPERTIES

A. Introduction

Many practical applications of gelatins such as their uses in foams, emulsions, and coatings depend on the fact that they possess moderate surface activity. Unfortunately, the many investigations of the surface properties of gelatins which have been reported rarely include a full consideration of several factors which can markedly affect the results. The effect of pH was largely neglected in early work. Surface-active impurities such as fatty residues are almost universally present and commercial gelatins may contain bactericides, antifoams, and small but not insignificant amounts of detergents used for plant cleaning. Surface ageing effects are quite marked in gelatin solutions particularly under conditions where molecular aggregates can form and it is therefore important in considering experimental observations to know both the temperature and the time elapsed between the formation of the surface and the making of a measurement. For these reasons and because no two gelatins are identical, it is almost impossible to compare in detail the results of different workers. Nevertheless some generalities do emerge.

B. Surface Tension

Early work on the measurement of the surface tension of gelatin solutions by both static and dynamic methods has been summarized by Kragh (1956). A comparison of various studies of the effect of pH on surface tension showed that Jermolenko (1929), Johlin (1930), Ackerman (1934), Zhukov and Bushmakin (1927), and Bottazzi (1928) working with gelatin concentrations of 1% or below at temperatures of 10–45°C and on interfaces probably less than one hour old all agreed in finding a surface tension maximum in the pH region 2–3 and a minimum in the region of the isoelectric point. Both of these effects have also been observed more recently by Pchelin and Kuhlman (1962) using a sessile drop method. Pouradier (1945) found the surface tension minimum at the isoelectric point but no maximum. He also found a linear decrease of surface tension with temperature up to 30°C, a sharper drop between 30 and 40°C and a further fall with increasing temperature above 40°C, the rate of decrease being greater than that for pure water. Where ageing effects have been looked for, a decrease of surface tension with time is generally observed.

Coming to more concentrated solutions, Sauer and Aldinger (1939) report surface tensions of 44–48 dynes/cm for a 10% glue solution at 50°C by a maximum bubble pressure method and unpublished observations from the authors' laboratories are that animal glue solutions as used in industrial adhesives (40–50% concentration, say, at 60°C) may have dynamic surface tensions as low as 35 dynes/cm.

The surface tension of a gelatin solution is obviously relevant to its ability to form a stable foam, an important property in a number of practical applications. However "foaming power" and "foam stability" also depend on other factors, primarily viscosity of the solution. Kragh (1956) has also reviewed this subject.

C. Interfacial Tension

There is much less information available on the properties of the interfaces between gelatin solutions and other liquids or solids. The main interest has been to try to correlate interfacial tension of liquid/liquid systems with emulsifying power. Sheppard and Sweet (1922) measured the interfacial tension between toluene and a 1% solution of a purified ash-free gelatin at various pH's and temperatures above 30°C. The curve of interfacial tension against pH showed a broad maximum over the pH range 3–10 interrupted by a sharp downward kink at the isoelectric point of 4.7. This accords with the observation of Zhulov and Bushmakin (1927) of maximum stability at the isoelectric point of an emulsion of benzene in aqueous gelatin solution. Other studies of the stability of gelatin emulsions of various non-aqueous liquids

have been reviewed by Kragh (1956) but the observations are specific to the systems studied. Braudo *et al.* (1967) have studied the reverse situation of the wetting of gelatin films and gels by various liquids. The contact angle of water on the surface of a 15% gel of dialysed photographic gelatin was $123 \pm 3^\circ\text{C}$ and on an air-dry film $88 \pm 4^\circ\text{C}$. The first figure seems extraordinarily high for a supposedly hydrophilic substrate. Contact angles of various organic liquids on gelatin gel and dry film surfaces are also recorded.

D. Surface Films

A gelatin solution will spread on a clean water surface in a surface trough to form a stable film whose force-area behaviour, surface viscosity and so on can be studied by standard techniques. Pouradier and co-workers (1949) have been leaders in this field and have shown that compression isotherms for spread films of gelatin can be split into three distinct regions: a gaseous region in which it is suggested that Boyle's Law is applicable and molecular weight could be determined; a region where surface tension is dependent on pH of the supporting liquid and is a minimum at the gelatin isoelectric point; and a third, high-pressure region in which there is a linear relation between surface tension and concentration.

Pouradier *et al.* have also compared the surface properties of spread films with those formed spontaneously by surface adsorption from solution. At low surface pressures, spread films appear to be well-ordered monomolecular layers whereas the films formed by adsorption appear to achieve equilibrium only slowly and even then consist of an imperfectly orientated layer of non-uniform composition.

REFERENCES

- Ackerman, T. (1934). *Kolloid-Z.* **69**, 87-93.
Allison, A. C. and Humphrey, J. H. (1959). *Nature (London)*. **183**, 1590-2.
Allison, A. C. and Humphrey, J. H. (1960). *Immunology* **3**, 95-106.
Amelina, K. S. and Kovalichev, F. F. (1957). *Sbornik Nauch. Trudov Leningrad Inst. Tochnoi Mekh. i Optiki, Mat., Mekh. Khim.* No. 24, 127-133.
Ames, W. M. (1947). *J. Soc. Chem. Ind.* **66**, 279-84.
Andel Colloid Corp. (1967). *Fr. P.* **1**, 492, 386.
Anokhin, V. V. (1958). *Trudy Kiev. Tekhnol. Inst. Legkoi Prom.* No. 39-45.
Anokhin, V. V. and Kotov, M. P. (1960). *Izvest. Vysshikh Ucheb. Zavedenii Tekhnol. Legkoi Prom.* No. 5, 30.
Arakawa, K. (1962). *Bull. Chem. Soc. Japan.* **35**, 309-12.
Aussel, P., Chanal, J. L. and Marignan, R. (1965). *Trav. Soc. Pharm. Montpellier* **25**, 145-50.
Bayley, H. G. (1959). *Nature (London)* **183**, 1757-8.
Bello, J. and Riese-Bello, H. (1958). *Sci. et Inds. Phot.* **29**, 361-4.

- Bennett, W. and Garrison, W. M. (1959). *Nature (London)* **183**, 889.
- Beyer, G. L. (1954). *J. Phys. Chem.* **58**, 1050-1.
- Boedtker, H. and Doty, P. (1954). *J. Phys. Chem.* **58**, 968-83.
- Bolaffi, A., Mezzino, J. F., Lowry, J. R. and Baldwin, R. R. (1959). *Food Technol.* **13**, 624-8.
- Bottazzi, F. (1928). In "Colloid Chemistry" (J. Alexander, ed.) Vol. II, p. 121. The Chemical Catalog Co. Inc., New York.
- Bourgoin, D. and Joly, M. (1952). *J. Chim. Phys.* **49**, 427-36.
- Bourgoin, D. and Joly, M. (1954). *Kolloid-Z.* **136**, 25-36.
- Bourgoin, D. and Joly, M. (1956). *Kolloid-Z.* **146**, 121-33.
- Bourgoin, D. and Joly, M. (1957). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 204-8. Pergamon Press, London.
- Bovey, F. A. (1958). In "The Effects of Ionizing Radiation on Natural and Synthetic High Polymers" Vol. I, Polymer Reviews. Interscience Pubs., New York.
- Bradbury, E. and Martin, C. (1951). *Nature (London)* **168**, 837-8.
- Bradbury, E. and Martin, C. (1952). *Proc. Roy. Soc. (London) A.* **214**, 183-92.
- Braudo, E. E., Tolstoguzov, V. B. and Slonimskii, G. L. (1967). *Vysokomol. Soedin., Ser. B.* **9** (II), 796.
- Bundenberg de Jong, H. G. and Hennemann, J. P. (1932). *Kolloid-Beihefte* **36**, 123-77.
- Burdygina, G. I., Fridman, I. M., Koslov, P. V. and Kargin, V. A. (1969). *Vysokomol. Soedin., Ser. A.* **11**, 118-31.
- Burdygina, G. I., Fridman, I. M., Koslov, P. V. and Kargin, V. A. (1969). *Vysokomol. Soedin., Ser. A.* **11**, 912-19.
- Burdygina, G. I., Undzenas, A., Fridman, I. M., Koslov, P. V. and Kargin, V. A. (1968). *Dokl. Akad. Nauk SSSR* **178**, 1360-3.
- Burdzhanadze, T. V., Privalov, P. L. and Tavkhelidze, N. N. (1962). *Vysokomol. Soedin.* **4**, 1419-24.
- Caldwell, J. R., (1952). (to Eastman Kodak). U.S. P. 2,592,120.
- Callow, E. H. (1950). *Chem. & Ind.* 756-7.
- Carless, J. E. and Nixon, J. R. (1970). *J. Soc. Cosmet. Chem.* **21**, 427-40.
- Carpenter, D. C. (1938). *Cold Spring Harbour Symposia Quant. Biol.* **6**, 244.
- Cartwright, H. M. (1949). *Process Engraver's Monthly* **56**, 82, 84.
- Castello, R. A. and Goyan, J. E. (1964). *J. Pharm. Sci.* **53**, 777-82.
- Cetini, G. and Ricca, F. (1958). *Gazz. chim. ital.* **88**, 617-26.
- Cobbett, W. G. (1961). *BGGRA Research Report* No. A. 27.
- Cobbett, W. G. and Ward, A. G. (1968). *Rheol. Acta.* **7**, 217-22.
- Coleman, B., Tarkow, H. and Weatherwax, R. C. (1956). *J. Polym. Sci.* **19**, 380.
- Coopes, I. H. (1968). *J. Polym. Sci. A-1*, **6**, 1991-9.
- Cordier, S. (1955). *Trav. Soc. Pharm. Montpellier* **15**, 116-8.
- Courts, A. (1959). *Biochem. J.* **73**, 596-600.
- Croome, R. J. (1953a). *J. Appl. Chem.* **3**, 280-6.
- Croome, R. J. (1953b). *J. Appl. Chem.* **3**, 330-4.
- Cumper, C. W. N. and Alexander, A. E. (1962a). *Australian J. Sci. Res.* **A5**, 146-52.
- Cumper, C. W. N. and Alexander, A. E. (1952b). *Australian J. Sci. Res.* **A5**, 153-9.
- Damschroeder, R. E. and Kauffman, M. E. (1954). *Canad. P.* 502, 893.
- Dowell, L. G., Moline, S. W. and Rinfret, A. P. (1962). *Biochim. Biophys. Acta.* **59**, 158-67.
- Eastman Kodak Inc. (1957). U.S. P. 2, 772, 166.
- Eastoe, J. W., Fysh, D. and Ward, A. G. (1952). *BGGRA Bulletin* **3**, 4, 10-13.

- Eliassaf, J. and Eirich, F. R. (1960). *J. Appl. Polym. Sci.* **4**, 200-2.
- Elliott, A. (1957). In "Recent Advances in Gelatin & Glue Research" (G. Stainsby, ed.) pp. 267-8. Pergamon Press, London.
- Ferry, J. D. (1941). *Rev. Sci. Instr.* **12**, 79-82.
- Ferry, J. D. (1948a). *Advances in Protein Chem.* **4**, 21.
- Ferry, J. D. (1948b). *J. Amer. Chem. Soc.* **70**, 2244-9.
- Ferry, J. D. and Eldridge, J. E. (1949). *J. Phys. Colloid Chem.* **53**, 184-96.
- Fink, P. and Furrer, E. (1957). *Ugra-Bull.* No. 3, 28-32.
- Flory, P. J. and Rehner, J. Jr. (1943). *J. Chem. Phys.* **11**, 521-6.
- Flory, P. J. and Garrett, R. R. (1958). *J. Amer. Chem. Soc.* **80**, 4836-45.
- Garrison, W. M. (1959). *U.S. At. Energy Comm. UCRL-8719*, 20pp.
- Garrison, W. M., Jayko, M. E. and Bennett, W. (1962). *Radiation Res.* **16**, 483-502.
- Gehenio, P. M. and Luyet, B. J. (1959). *Biodynamica* **8**, 81-4.
- General Foods Corporation (1958a). U.S. P. 2,834,683.
- General Foods Corporation (1958b). U.S. P. 2,819,970.
- Gerngross, O. (1926). *Kolloid-Z.* **40**, 279-86.
- Gevaert Photo-Prod N.V. (1959). Belg. Pat. 565,861.
- Graham, T. (1861). *Phil. Trans.* **151**, 183.
- Gray, V. R. (1949). *Nature (London)* **164**, 584-5.
- Gross, L. G. (1959). *Zhur. Nauch i Priklad. Fot. i Kinematografii* **4**, 411-16.
- Grigor'eva, N. V., Pchelin, V. A. and Rebinder, P. A. (1961). *Doklady Akad. Nauk. SSSR* **137**, 889-92.
- Gupta, R. C. (1964). *Appl. Sci. Res. Sect. A* **13**, 61-4.
- Harrington, W. F. (1958). *Nature (London)* **181**, 997-8.
- Hatschek, E. (1921). *Kolloid-Z.* **28**, 210-3.
- Hatschek, E. and Jane, R. S. (1926). *Kolloid-Z.* **39**, 300-13.
- Hatschek, E. (1932). *J. Phys. Chem.* **36**, 2994-3009.
- Hatschek, E. (1933). *Trans. Faraday Soc.* **29**, 1108-31.
- Hirai, N. and Kishimoto, S. (1953). *J. Chem. Soc. Japan, Pure Chem. Sect.* **74**, 347-9.
- Hopp, V. and Wundrich, K. (1964). *Leder* **15** (12), 277-83.
- Idson, B. and Braswell, E. (1957). *Advances in Food Research* (E. M. Mrak and G. F. Stewart, eds.) **7**, 298-9.
- Ivanova-Chumakova, L. V., Rebinder, P. A. and Krus, G. I. (1956). *Kolloid Zhur.* **18**, 682-90.
- Jahn, A. S. and Witnauer, L. P. (1967). *J. Amer. Leather Chem. Assoc.* **62**, 334-45.
- Janus, J. W., Kenchington, A. W. and Ward, A. G. (1951). *Research, Lond.* **4**, 247.
- Jermolenko, N. (1929). *Kolloid-Z.* **48**, 141-6.
- Johlin, J. M. (1930). *J. Biol. Chem.* **87**, 319-25.
- Johnson, P. and Metcalfe, J. C. (1963). *J. Phot. Sci.* **11**, 214-224.
- Johnson, P. and King, R. W. (1968). *J. Phot. Sci.* **16**, 82-88.
- Joly, M. (1949a). *Kolloid-Z.* **115**, 83.
- Joly, M. (1949b). *Bull. Soc. Chim. Biol.* **31**, 105-7.
- Jopling, D. W. (1952). *Sci. et Industr. Photog.* **23**, 253-6.
- Jopling, D. W. (1953). *Research, Lond.* **6**, 27S.
- Jopling, D. W. (1956). *J. Appl. Chem.* **6**, 79-84.
- Jopling, D. W. (1958). *Rheol. Acta* **1**, 133-7.
- Jordon-Lloyd, D. and Pleass, W. B. (1927). *Biochem. J.* **21**, 1352-67.
- Kamiya, I. (1954). *J. Chem. Soc. Japan, Pure Chem. Sect.* **75**, 788-91.
- Katti, P. K. (1949). *Proc. Indian Acad. Sci.* **30A**, 35-48.

- Katz, J. R., Derksen, J. L. and Bon, W. F. (1931). *Rec. Trav. Chim. Pays-Bas* **50**, 725-31.
- Kawamura, F. and Hirota, G. (1951). *Kagaku (Science)* **21**, 469-70.
- Kazanskii, V. M. (1964a). *Dopovidi Akad. Nauk Ukr. RSR* (2), 226-8.
- Kazanskii, V. M. (1964b). *Inzh.-Fiz. Zh. Akad. Nauk Belorussk. SSR* **7**, 53-6.
- Kenchington, A. W. (1952). *BGGRA Bulletin* **3** (1), 2-4.
- Khenokh, M. A. and Lapinskaya, E. M. (1951). *Doklady Akad. Nauk SSSR* **80** 921-4.
- King, G. and Medley, J. A. (1949). *J. Colloid Sci.* **4**, 9-18.
- Kinkel, E. and Sauer, E. (1925). *Z. Angew. Chem.* **38**, 413-21.
- Kodak Ltd. (1959). Brit. Pat. 908,777.
- Konno, A. and Kaneko, M. (1969). *Rep. Prog. Polym. Phys. Japan* **12**, 65-6.
- Kotina, V. E. (1951). *Kolloid Zhur.* **13**, 444-9.
- Kraemer, E. O. (1926). *Colloid Symp. Monograph* **4**, 102.
- Kraemer, E. O. and Fanselow, J. R. *J. Phys. Chem.* **29**, 1169-77.
- Kragh, A. M. (1956). *BGGRA Literature Review*, series D 2.
- Kubota, M., Kimura, S. and Ohashi, T. (1968). *Kikaku Kagaku* **14** (3), 105-11.
- Kučera, E., Šácha, F. and Střelba, F. (1959). Czech Pat. 93,078.
- Kudryavtsev, B. B. (1960). *Primenenie Ul'traakust. k. Issled. Veshchestva*. no. 12, 31-69.
- Lapin, P. M. (1955). *Moscov. Zaoch Poligraf. Inst. Nauch. Trudy* **2**, 27-106.
- Lapin, P. M. and Lipatov, S. M. (1939). *Kolloid Zhur.* **5**, 683-90.
- Larson, C. E. and Greenberg, D. M. (1933). *J. Amer. Chem. Soc.* **55**, 2798-9.
- Laws, W. D. and France, W. G. (1949). *Anal. Chem.* **21**, 1058-9.
- Leich, A. (1904). *Ann. Physik* **14** (4), 139.
- Lentz, C. P. (1961). *Food Tech.* **15**, 243.
- Levi, S. M. (1960). *Kolloid Zhur.* **22**, 599-605.
- Lewis, E. J. and Soper, A. K. (1950). *J. Sci. Instrum.* **27**, 242-3.
- Libicky, A. and Bermane, D. (1972). In "Photographic Gelatin" (R. J. Cox, ed.) pp. 29-48. Academic Press, London and New York.
- Lipatov, S. M. (1939). *Kolloid Zhur.* **5**, 613-38.
- Llory, J. (1958). *Ann. Biol. Clin. (Paris)* **16**, 308-20.
- McMeekin, T. L., Groves, M. L. and Hipp, N. J. (1964). *Advan. Chem. Ser.* **44**, 54-66.
- Marignan, R. and Crouzat-Reynes, B. (1956). *Trav. soc. pharm. Montpellier* **16**, 171-5.
- Marrs, W. M. and Wood, P. D. (1972). In "Photographic Gelatin" (R. J. Cox ed.) pp. 63-80. Academic Press, London and New York.
- Masszi, G. and Orkenyi, J. (1967). *Acta Biochem. Biophys. (Budapest)* **2** (2), 131-42.
- Masuzawa, M. and Sterling, C. (1968). *Biopolymers* **6** (10), 1453-9.
- Mateles, R. I. and Goldblith, S. A. (1958). *Food Technol.* **12**, 633.
- Meerson, S. I. and Puchkova, N. N. (1959). *Kolloid Zhur.* **21**, 613-7.
- Merzlov, V. P. and Pchelin, V. A. (1965). *Dokl. Akad. Nauk. SSSR* **163**, 147-50.
- Mikhailov, I. G. (1964). *Ultrasonics* **2**, 203-8.
- Mikhailov, I. G. and Tarutina, L. I. (1950). *Doklady Akad. Nauk. SSSR* **74**, 41-4.
- Miller, M., Ferry, J. D., Schremp, F. W. and Eldridge, J. E. (1951). *Phys. and Colloid Chem.* **55**, 1387-1400.
- Mindru, I. and Ceacareanu, D. (1966). *An. Univ. Bucuresti Ser. Stiint. Natur.* **15** (i), 25-31.

- Moran, T. (1926). *Proc. Roy. Soc.* **A112**, 30–46.
- Morel, J. and Grabar, P. (1950). *Bull. Soc. Chim. Biol.* **32**, 630–42.
- Neiman, R. E. (1954a). *Kolloid Zhur.* **16**, 201–3.
- Neiman, R. E. (1954b). *Kolloid Zhur.* **16**, 280–3.
- Neiman, R. E. (1956). *Kolloid Zhur.* **18**, 731–4.
- Niven, C. D. (1958). *Trans. Faraday Soc.* **54**, 441–5.
- Niven, C. D. (1961). *Can. J. Phys.* **39**, 657–61.
- Nixon, J. R., Georgakopoulos, P. P. and Carless, J. E. (1966). *J. Pharm. Pharmac.* **18**, 283–8.
- Nixon, J. R., Georgakopoulos, P. P. and Carless, J. E. (1967). *J. Pharm. Pharmac.* **19**, 246–52.
- Nobel, P. C. (1951). *Rec. trav. chim. Pays-Bas* **70**, 720–4.
- Norris, T. O. and McGraw, J. (1964). *J. Appl. Polym. Sci.* **8**, 2139–45.
- Northrop, J. H. and Kunitz, M. (1926). *J. Gen. Physiol.* **8**, 317.
- Pavlovskaya, T. E. and Pasynskii, A. G. (1955a). *Kolloid Zhur.* **17**, 305–14.
- Pavlovskaya, T. E. and Pasynskii, A. G. (1955b). *Dokl. Akad. Nauk SSSR* **101**, 723.
- Pchelin, V. A. (1968). *Dokl. Akad. Nauk. S.S.S.R.* **180**, 402–3.
- Pchelin, V. A. and Kuhlman, R. A. (1962). *J. Polym. Sci.* **59**, S 26–7.
- Pchelin, V. A., Izmailova, V. N., and Merzlov, V. P. (1963a). *Vysokomolekul. Soedin.* **5**, 1429–35.
- Pchelin, V. A., Izmailova, V. N. and Merzlov, V. P. (1963b). *Dokl. Akad. Nauk. S.S.S.R.* **150**, 1307–10.
- Peerson, E. and Ore, A. (1963). *Phys. Norvegica* **1** (4), 205–15.
- Perron, R. R. and Wright, B. A. (1950). *Nature (London)* **166**, 863–4.
- Persidsky, M. D. and Luyet, B. J. (1959). *Biodynamica* **8**, 107–20.
- Pinoir, R. and Pouradier, J. (1948). *C. R. Acad. Sci., Paris* **227**, 190–2.
- Pottel, R. and Wuelfing, A. (1963). *Z. Angew. Phys.* **15** (6), 501–5.
- Poole, H. J. (1925). *Trans. Faraday Soc.* **21**, 114–37.
- Pouradier, J. (1945). *Sci. Ind. Phot.* **16**, 305–13.
- Pouradier, J. (1949). *J. Chim. Phys.* **46**, 627–34.
- Pouradier, J. (1950). *Compt. Rend.* **230**, 1466–7.
- Pouradier, J. (1967). *J. Chim. Phys.* **64**, 1616–20.
- Pouradier, J. and Aribat, A. (1949). In "Surface Chemistry", pp. 135–44. Butterworths Scientific Publications, London.
- Pouradier, J. and Aribat, M. (1950). *Bull. Soc. Chim. Biol.* **32**, 947–51.
- Pouradier, J., Venet, A. M. and Trigny, L. (1954). *27th Congr. Internat. de Chimie. Ind. Bruxelles* **3**, 769–71.
- Rankine, A. O. (1906). *Phil. Mag.* **11**, 447.
- Rapatz, G. and Luyet, L. (1959). *Biodynamica* **8**, 85–105.
- Rastelli, A. (1951). *Atti accad. ligure sci. e lettere (Pavia)* **8**, 294–8.
- Riehl, N. (1955). *Zhur. Fiz. Khim.* **29**, 1537–48.
- Riehl, N. (1957). *Kolloid-Z.* **151**, 66–72.
- Robinson, C. (1953). In "Nature and Structure of Collagen" p. 96. Academic Press.
- Rousselot, A. (1949). *C. R. Acad. Sci. Paris* **228**, 1334–5.
- Rudenko, S. V. and Levi, S. M. (1968). *Vysokomol Soedin., Ser. A.* **10** (3), 647–15.
- Saiki, K. and Okamoto, Y. (1966). *Japan. J. Appl. Phys.* **5** (10), 962–9.
- Sanzharovskii, A. T. and Epifanov, G. I. (1960). *Vysokomol. Soedin.* **2**, 1709–14.
- Sauer, E. and Aldinger, W. (1939). *Kolloid-Z.* **88**, 329–40.

- Saunders, P. R. (1956) BGGRA Research Report A.11.
- Saunders, P. R. and Ward, A. G. (1952) BGGRA Research Report Series B3.
- Saunders, P. R. and Ward, A. G. (1953). *J. Soc. Food Agric.* **4**, 523-7.
- Saunders, P. R. and Ward, A. G. (1954). In "Proceedings of the Second International Congress on Rheology" (Harrison, ed.) p. 284. Academic Press, New York.
- Saunders, P. R. and Ward, A. G. (1958a). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) pp. 197-203. Pergamon Press, New York.
- Saunders, P. R. and Ward, A. G. (1958b). In "Rheology of Elastomers" (P. Mason and N. Wookey, eds.) pp. 45-52. Pergamon Press, London and New York.
- Scatchard, G., Oncley, J. L., Williams, J. W. and Brown, A. (1944). *J. Amer. Chem. Soc.* **66**, 1980-1.
- Schremp, F. W., Ferry, J. D. and Evans, W. W. (1951). *J. Appl. Phys.* **22**, 711-7.
- Sevcik, F. and Vetterl, V. (1965). *Biofizika* **10** (3), 441-6.
- Simms, E. M. and Blake, J. N. (1960). BGGRA Research Panel Report RPP/39.
- Sheppard, S. E. and Sweet, S. S. (1921). *J. Amer. Chem. Soc.* **43**, 539-47.
- Sheppard, S. E., Sweet, S. S. and Scott, J. W. (1920). *Ind. Eng. Chem.* **12**, 1007-11.
- Sheppard, S. E. and Sweet, S. S. (1922). *J. Amer. Chem. Soc.* **44**, 2797-805.
- Shreiner, S. A. and Zubov, P. I. (1960). *Kolloid Zhur.* **22**, 497.
- Slonimskii, G. L., Alekseyev, V. F., Grinberg, V. Ya., Izyumov, D. B. and Tolstoguzov, V. B. (1969). *Vysokomol. Soedin.* **A10**, No. 2, 460-2.
- Smith, C. R. (1919). *J. Amer. Chem. Soc.* **41**, 135-50.
- Staaf, O. (1967). *Kolloid-Z. Z. Polym.* **219**, 30-9.
- Stainsby, G., Saunders, P. R. and Ward, A. G. (1954). *J. Polym. Sci.* **12**, 325-35.
- Steigmann, A. (1957). *Sci. et Ind. Phot.* **28**, 353-9.
- Steinberg, I. Z., Harrington, W. F., Berger, A., Sela, M. and Katchalski, E. (1960). *J. Amer. Chem. Soc.* **82**, 5263-79.
- Sterling, C. and Masuzawa, M. (1968). *Makromol. Chem.* **116**, 140-5.
- Subba Rao, K. and Das, B. (1967). *Curr. Sci.* **36**, 657-8.
- Szczesniak, A. J. and MacAllister, R. V. (1964). *J. Appl. Polym. Sci.* **8** (3), 1391-401.
- Talwar, G. P., Basset, J. and Macheboeuf, M. (1953). *Compt. Rend.* **236**, 2271.
- Taylor, D. J. and Kragh, A. M. (1970). *J. Phys. D. Appl. Phys.* **3**, 29.
- Tice, L. F. and Moore, A. W. (1950). *J. Amer. Pharm. Assoc., Pract. Pharm. Ed.* **11**, 565-7.
- Tice, L. F. (1951a). U.S. Patent 2,558,065.
- Tice, L. F. (1951b). U.S. Patent 2,584,307.
- Tice, L. F. and Moore, A. W. (1952). *J. Amer. Pharm. Assoc.* **41**, 631-3.
- Tobolsky, A. V. (1955). *J. Phys. Chem.* **59**, 575.
- Todd, A. (1961). *Nature (London)* **191**, 567-9.
- Tomoda, Y. (1960). *Kogyo Kagaku Zasshi* **63**, 1436-8.
- Tomoda, Y. and Tsuda, M. (1961). *J. Polymer Sci.* **54**, 321-8.
- Tschiegg, C. E. and Greenspan, M. (1956). *J. Acoust. Soc. Amer.*, **28**, 158.
- Tunson, W. J. and Kelly, W. D. (1966). *Phot. Sci.* **10** (5), 278-80.
- Tyazhelova, T. P. (1937). *Kolloid Zhur.* **3**, 631-42.
- Ueno, W. (1963). *Reports on Progress of Polymer Physics in Japan* **V1**, 175-8.
- Ueno, W. and Ono, I. (1963). *J. Soc. Materials Sci. Japan* **12**, 341-6.
- Umberger, J. Q. (1967). *Phot. Sci. Eng.* **11**, 385-91.
- Union Carbide Corp. (1965). U.S. Patent 3,164,560.
- Veis, A. and Anesey, J. (1957). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) p. 269. Pergamon Press, London.

- Veis, A. and Anesey, J. (1959). *J. Phys. Chem.* **63**, 1720-5.
- Veis, A., Anesey, J. and Cohen, J. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) p. 155. Pergamon Press, London and New York.
- Vigneron, L., Génin, J. and Chastel, R. (1955). *Compt. rend. Acad. Sci.* **240**, 1423-5.
- Wada, Y., Sasabe, H., and Tomono, M. (1967). *Biopolymers* **5**, 887-97.
- Ward, A. G. (1954a). *Chem. & Ind.* 502-5.
- Ward, A. G. (1954b). *Brit. J. Appl. Phys.* **5**, 85-90.
- Ward, A. G. (1955). *J. Phot. Sci.* **3**, 60-7.
- Ward, A. G., (1959). *Rev. Pure & Appl. Chem.* **9**, 87-103.
- Ward, A. G. (1961). *J. Phot. Sci.* **9**, 56-66.
- Ward, A. G. and Saunders, P. R. (1958). In "Rheology, Theory and Applications" (F. R. Eirich, ed.) Vol. 11, p. 342. Academic Press, New York.
- Watase, M. and Arakawa, K. (1967). *Nippon Kagaku Zasshi* **88** (3), 317-20.
- Watase, M. and Arakawa, K. (1969). *Nippon Kagaku Zasshi* **90** (7), 658-63.
- Weatherwax, R. C., Coleman, B. and Tarkow, H. (1958). *J. Polym. Sci.* **27**, 59-66.
- Webers, V. J. (1963). *J. Appl. Polym. Sci.* **7** (4), 1317-23.
- Wiegand, J. H. (1963). *J. Chem. Educ.* **40**, 475-6.
- Winther, C. (1954). *Acta Chem. Scand.* **8**, 1053-8.
- Woodward, H. Q. (1932). *J. Phys. Chem.* **36**, 2543-53.
- Yannas, I. V. (1966). *Diss. Abstr. B* **27**, 1873.
- Yannas, I. V. (1968). *J. Appl. Polym. Sci.* **12**, 1-8.
- Yannas, J. B. and Tobolsky, A. V. (1964). *J. Phys. Chem.* **68**, 3880-2.
- Yannas, I. V. and Tobolsky, A. V. (1966). *J. Macromol. Chem.* **1**, 723-37.
- Yannas, I. V. and Tobolsky, A. V. (1968). *Europ. Polym. J.* **4**, 257-64.
- Yasunaga, T. and Sasaki, T. (1951). *J. Chem. Soc. Japan, Pure Chem. Sect.* **72**, 915-6.
- Zaïdes, A. L. (1950). *Kolloid. Zhur.* **12**, 414-20.
- Zhukov, I. I. and Bushmakina, I. N. (1927). *J. Phys. Chem. U.S.S.R.* **59**, 1061-9.
- Zhukov, I. I. and Khenokh, M. A. (1949). *Doklady Akad. Nauk. SSSR* **68**, 333-6.
- Zubov, P. I., Zhurkina, Z. N. and Kargin, V. A. (1949). *Doklady Akad. Nauk. SSSR* **67**, 659-61.
- Zubov, P. I., Zhurkina, Z. N. and Kargin, V. A. (1954). *Kolloid Zhur.* **16**, 109-14.
- Zubov, P. I. and Lepilkina, L. A. (1961). *Kolloid Zhur.* **23**, 418.

Chapter 9

Raw Materials

RUDOLF HINTERWALDNER

8 München 90, Postfach 90 0425, Germany

I General Aspects	295
II Preparation of Bones	297
A. Assorting and Size Reduction	297
B. Degreasing the Bones	298
C. Demineralization	304
III Preparation of Hide Stock	306
A. Bovine Hides and Skins	306
B. Pigskin	306
IV Preparation for Extraction	307
A. General	307
B. Liming Process	307
C. Acid Process	309
D. The Enzymatic Process	310
References	313

I. GENERAL ASPECTS

Gelatin is a product of biological materials, the nature of which varies greatly. As is described in detail in Chapters I, II and V, the source material for gelatin is collagen, which is the principal protein of animal connective tissues. Commercially almost the only sources of practical importance are hides and bones. A large proportion of the collagenous raw materials from hides and skins is a by-product of tannery operation. The conversion of hides into leather leaves many valuable secondary products, comprised chiefly of splits (the inner non-grain layer of the hide) other trimmings of the hide itself and also fleshings which are fatty tissues scraped off the interior surface of the hide. These raw materials come either in limed, wet or dry condition, depending on the stage at which they are separated from the main part of the hide which is to be used for leather making and on the subsequent treatments which

the by-products receive. In North America, the pig skin is the chief raw material for edible gelatin production. Pig skins are brought in frozen condition from the large meat packing houses to the market or direct to the gelatin factory on a contract basis. In Europe, too, the amount of pig skins processed for gelatin is steadily increasing, because of their potential for high grade gelatin preparation, of their much shorter time of raw material preparation before extraction and the reduction in effluent problems which they make possible. In Europe pig skins are rarely brought to the market frozen, despite the advantages this confers in preserving skin quality.

At present, the chief raw materials used in Europe for edible and technical gelatins are bones and cattle hides. It is necessary, in considering industrial gelatin processes to differentiate between:

- Bone (ossein) gelatin
- Hide gelatin
- Pig skin gelatin

The limited use of sinew as a raw material does not require detailed separate treatment as it is processed very similarly to hide.

For the production of bone (ossein) gelatin the main raw materials used are, firstly, Indian crushed bones, which have lost attached flesh and fat and have been sun dried and, secondly, fresh bones which are a by-product of the meat industry. Indian bones are mainly hard bones i.e. tubular foot bones of cattle and calves, select head bone, cheek bone, shoulder blade bone, etc. Fresh raw bones, on the removal from the animal carcass, must be subjected to a degreasing process prior to further processing, to reduce their fat content to a maximum of 1%. This step is not necessary with Indian crushed bones, as this material has been degreased by natural weathering—by the action of the monsoon and hot sunshine. A further material of only small volume is horn pith which yields a singularly pure and high-class gelatin.

The composition of the different raw materials varies considerably. The qualities of the gelatins produced, and to some extent the processes that can be used, depend on such factors as the species, breed, age and manner of feeding the animals and the storage conditions to which the raw materials have been exposed. These variations make it difficult to give exact values for gelatin yield even for closely related types of raw material. The following Table I is an attempt to give the approximate yield of gelatin for several materials.

The pretreatment of these raw materials required for them to be adequately prepared for gelatin extraction can be done in many different ways. Which method is applied depends, partly on the state of the raw material and partly on the prospective use of the final gelatin product. The various pretreatment

TABLE I. Composition of raw materials and gelatin yields

Raw material	Gelatin yield (air dry) %	On raw material		
		Grease content %	Water content %	Residue %
Calfskin trimmings				
salted	7-16	2	30-40	9
limed	7-16	1	50-70	5-10
dry	35-52	1	10	5
Hide splits				
salted	7-16	2	30-40	10
limed	7-16	2	50-70	8
dry	30-50	1	10	5-10
Buffalo skin				
(Surrone) dry	45-50	1	10-20	10
Bone with sinew dry	35-40	—	10-15	30
Sinew dry	50-70	—	10-15	—
Pig skin (frozen)	18-22	7-25	55-65	8-15
Dried ossein	55-65	—	9-12	—
Dried cattle bone	14-18	0.5-4.0	8-11	—

methods are described in separate sections, although they have some points in common.

II. PREPARATION OF BONES

A. Assorting and Size Reduction

We have already differentiated between fresh bones, with any attached tissues, and Indian crushed bones. Fresh bones are classified into "collected bones" and "slaughterhouse bones". The collected bones are collected by bone dealers in small quantities and so include mixed types and sizes. They pass through the hands of various dealers and merchants before they reach the glue or gelatin factory, unless the factory organizes its own collections. These bones are usually accompanied by more or less large quantities of other carcass waste. Due to their frequently prolonged storage, the fat they contain may be partially hydrolysed to free fatty acids. The gelatin yielding substance may also to some degree suffer damage by decomposition caused in the main by bacterial attack, so reducing the yield of gelatin obtainable. For this reason collected bone material of this type is rarely profitably used for gelatin making. It can be used as raw material for bone glue production, where the end product is necessarily much degraded by the manufacturing process.

Slaughterhouse bones, in contrast, are a very valuable raw material for

TABLE II. Analyses of hard and porous pieces from bones

	Porous pieces %	Hard pieces %
Organic matter	28.6	23.7
Ash content	58.8	63.3
Moisture	9.5	9.5
P ₂ O ₅	23.10	26.3
CO ₂	3.10	3.5
Specific gravity	1.5	1.9

gelatin production, as they are free from excessive contamination and are delivered for processing while still in fresh condition. They must not, however, be first treated in an autoclave to produce fat or bone broth, as the high temperature would permanently impair the collagen as a gelatin making raw material as well as causing some reduction in yield. The fresh bones are first sorted and then crushed. To this end they are put on a vibrator that distributes them uniformly and passes them on to a sorting and conveying belt. On the conveyor they are picked over mostly by hand to sort out any foreign materials. A powerful electric magnet is used to attract any iron pieces they may contain. The slow conveyor belt takes them to the crusher where they are reduced to walnut size. Screening can follow if selection for size is required.

To degrease and demineralize the bones conveniently they must be reduced to dimensions between 1 and 8 cm. The bone crushers used are of special construction, in order to eliminate as far as possible the production of smaller pieces than the specified lower limit. A knife type crusher has been found to be very suitable. In this crusher, size reduction is brought about by a rotating bladed roller intermeshing with a fixed steel comb. The reduced material produced is most easily transported to a bin or silo by a mechanical conveyor, from which the bone degreasing plant is fed.

B. Degreasing the Bones

The presence of fat in the raw material may be harmful both during liming when lime soaps may be formed and if they are present in any quantity in the gelatin produced. The processing methods used to remove fat are however liable to do harm to the collagen. The last twenty years has seen extensive progress in developing improved degreasing techniques. The oldest and formerly the most used method of degreasing the bones was by extraction by solvents, although a simple degreasing could be achieved by hot water treatment. Solvents typically used are benzene and chlorinated hydrocarbons.

The extraction is carried out in specially designed extractors. The broken bone stock deposited on a screen is constantly permeated by fat solvent vapours. The escaping vapours are condensed in a cooler and the water separated out. The solvent-fat mixture is drained off into a fat collector and the solvent still remaining in the fat is distilled off, condensed on the cooling surfaces and returned to the tank. After extraction, the solvent residue still contained in the bone stock, is expelled by direct steam at pressures of about 56 to 84 psig. During expansion into the extractor the steam behaves as superheated steam and so adds no water to the bones. The bone stock removed from the extractor has a fat content of between 0.3 and 0.5% and is air-dry. Solvents in common usage are paraffins and the chlorinated hydrocarbons such as trichlorethylene and perchlorethylene. Chlorinated hydrocarbons have the advantage that they are not inflammable and have some decolourizing action. Their disadvantage is that they corrode iron equipment. The toxic effect of chlorinated hydrocarbons is also important, requiring care in use. The author knows of one fatal accident in an extraction plant operating on chlorinated hydrocarbons. Depending on the solvent used, the extraction temperatures range from 80 to 130°C.

The more significant papers concerned with fat extraction are briefly described below:

Mueller⁽³⁾ states that careful extraction of bone fat from crushed animal bones can be carried out at a bone temperature between the melting point of the fat and the coagulation point of the bone albumin, i.e. between 32°C and 80°C. According to his patented process, degreasing is performed by means of a high vacuum. In a different method Mueller⁽⁴⁾ describes a continuous degreasing plant in which, in a number of tanks, the broken waste bone stock from slaughterhouses or the meat canning industry is degreased by means of benzene/paraffin solvent mixtures. For example, he degreases 400 parts of bone stock with 1500 parts of solvent.

Chochlowa *et al.*⁽⁵⁾ also perform the degreasing by benzene/paraffin vapours, which simultaneously serve to dry the bones.

According to R. Decker and H. Holz,⁽⁶⁾ fat and glue-containing waste are mixed with organic fat solvents. This is followed by distillation—if necessary at reduced pressure—until no more water can be removed. Then the fat solution is drained off and the solvent residue removed by superheated steam. The solvents applied, preferably chlorinated hydrocarbons, have a boiling point of over 100°C. Kowal⁽⁷⁾ recommends degreasing bone stock by dichloroethane as this not only improves operational safety, it also produces a lighter coloured bone stock.

G. R. Blumenthal *et al.*⁽⁸⁾ degrease bones with low boiling solvents. The crushed bone stock is mixed with a low boiling solvent, whereby the extracting agent not only functions as fat solvent but also as water solvent. As

examples of such solvents, acetone and its higher homologues are referred to. The extraction proper is performed at very low temperatures, and the water contained in the bones is extracted as well as the fat. The low temperatures reduce the extent of collagen damage.

The solvent extraction method for the removal of bone grease cannot be regarded as a satisfactory procedure, as apart from the dangerous combustibility of many of the solvents used, there is also an adverse effect on the fat quality and on the bones themselves. The latter involves collagen damage from the long heating, and also contamination with solvent. The actual duration of the extraction, which may reach 14 to 18 hours, is a further disadvantage.

Alternative to solvent degreasing for bone stock are the so-called wet and dry rendering methods. Dry rendering consists of heating the bone stock in the presence of hot fat for at least 4 hours. This treatment causes extensive damage to the collagen contained in the bones and to sinews. A substantial proportion of the fat remains in the bones and bone stock is rarely obtained by this method that contains less than 8% of fat by weight. It is clearly unsuitable for the production of gelatin raw material. An improved reduction in fat can be obtained by squeezing the treated bones in a thermascrew.

In the wet processing method, the bones are gently boiled in hot water for 5 to 8 hours, either with or without previous heating by steam or they are digested for the same length of time with hot water and steam. In the cooking process about half of the fat in the bone is extracted, while the rest remains in the bone stock. Further extension of the simmering process causes loss of collagen as well as a reduction of collagen quality. The fat quality is also affected to some extent. The digester process makes possible a greater fat yield than in dry rendering, but the loss of gelatin producing substance is substantial. The processing conditions for some wet methods are described below:

The process of Efka-Werke A.G., Faupel & Haake⁽⁹⁾ is based on water as solvent or dispersing agent. The degreasing of the bone stock with water is done in a chamber, which is connected to a vacuum line. A drum with a heated jacket can be used and this is three-quarter filled with water and the bones heated to 80°C and more. Fat and water are removed by suction and the bones washed for 1 to 1½ hours and dried. By this process it is claimed that the bones are degreased down to 1% and less, but are still of useful quality for the production of glue and gelatin. Some loss of collagen properties will necessarily occur at 80°C.

On the other hand, the Schwarzkopf⁽¹⁰⁾ method first opens up the marrow and fat containing parts of cattle bones by mechanical means and subsequently heats the bone stock for about one hour at 91 to 93°C in a hot water bath. The loosened fat is melted out at reduced pressure while a further degreasing is done by using a water spray at not more than 68°C temperature.

In order to obviate the disadvantages of lengthy exposure to high temperatures, improved processing methods have been developed. They are claimed as suitable for degreasing hard bone stock and have been used commercially. The Sheppy Glue and Chemical Works Ltd.⁽¹¹⁾ begin with bone stock of suitable size produced by a double crusher mill. This stock is put into a centrifuge and hydroextracted at an acceleration of between 550 and 700 g. Prior to being subjected to the centrifugal action, the fat containing stock is heated to 60 to 90°C as rapidly as possible, in order to burst the fat cells. The stock is separated into two phases by the centrifuge, a solid mass of bone residue containing the collagen and a fat containing fluid. The residue has substantially fat-free hollow spaces or cells, with an overall fat content of 2 to 4% by weight. It is passed to a drier. The time of exposure to high temperature is brief in this process and the collagen is largely unaffected by the treatment.

The Cleveland Product Comp. Ltd.⁽¹²⁾ subjects the crushed bone stock to a multiple stage degreasing, which includes stirring in a large tank of hot water, at 60–90°C. The fat separates out on the surface while the remaining bone and sinews form a settled mass. The process is claimed to result in a bone material having less than 1% by weight of fat. The ossein of the bone stock is stated to be intact, i.e., not to have suffered any damage, although all exposure to high temperature must cause some damage in practice.

The Stauffer Chemical Comp.⁽¹³⁾ developed wet rendering into a continuous process, with a processing temperature of less than 72°C and a treatment time of less than 30 minutes. This process avoids the main disadvantages of wet rendering, leaving the collagen almost totally free of damage.

Wet processing methods have the potential disadvantage, minimized in their most fully developed forms, that the quality of the degreased bone stock is reduced by the heating. In some instances the residual fat in the bone is unacceptably high for a gelatin raw material. Solvent processes also cause damage to the collagen, especially in the early stages when substantial amounts of water are still present.

The "Chayen-Process"^(14–17) is a different form of wet processing method of great industrial significance for degreasing. In the Chayen-Process the problem of wet degreasing was approached from a different angle, by utilizing an impulse rendering technique, whereby a continuous degreasing of the bones is ensured without high temperatures. The advantages claimed for this process may be listed as follows:

- (a) The fat is extracted in the cold, so that the quality of neither the collagen nor the fat is affected.
 - (b) The extraction is efficient, the degreased bones containing generally no more than 2% of fat.
-

- (c) Passage of bone through the impulse renderer takes less than a second, so that the degreasing can be carried out as a continuous process.

Figure 1 is a flow sheet explaining the Chayen-Process.

The crushed raw bones pass through the impulse renderer, where they are subjected to the under water action of a high speed mill. The cell walls are ruptured and fat released from the cells. The mixture passes from there, as degreased bones, with the cold rendered fat, through the screen into a separating vessel. The bones settle under gravity and are continuously removed by a scroll conveyor on which they are washed with cold water. The cold washed bones pass through a squeezer and are briefly washed with hot

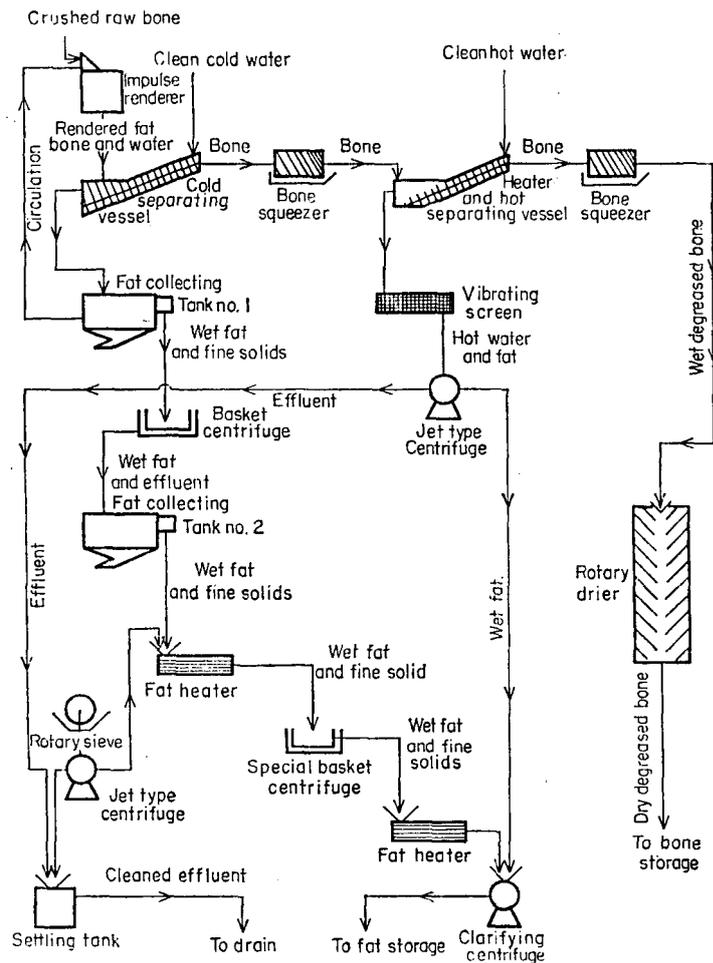


FIG. 1. Chayen-Process flow sheet.



FIG. 2. Bone degreased by the Chayen-Process.

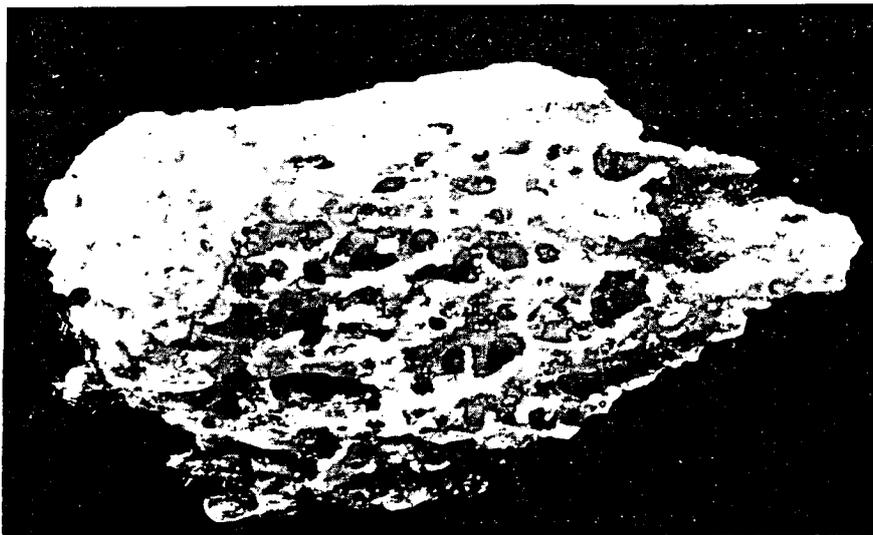


FIG. 3. Enlarged view of bone piece from Chayen-Process.

water (at 85 to 90°C) in another separating vessel. The mean retention time of the bones in this vessel is only about 2 minutes, so that the degradation of collagen is minimal. The treatment removes surface-held fat and shrinks the swollen sinew in the bones. The wet, degreased bones pass through another squeezer to reduce the moisture content to 36% and they are then dried in a rotary drier. Alternatively they pass directly to the gelatin manufacturing process. The fat, on emerging from the impulse renderer, floats on the top of the mixture in the bone separating vessel and passes from there into a fat collection tank. From here the fat is subjected to further treatment, see flow sheet (Fig. 1). The quality of the fat is so good that, in certain conditions, it may be used as an edible fat. Fig. 2 shows degreased hard bones and Fig. 3 an enlarged fragment of degreased bone, both from the Chayen-Process.

After the solvent extraction of bone fat the degreased, broken bones are further dried in a scrubbing and polishing drum. This process can also remove unwanted attached or free tissues which can be included in meat or meat and bone meals. This cleaning method is not necessary after wet degreasing. The degreased broken bones are then either passed on immediately for demineralizing or put in storage in dry conditions.

During the stage that the bone is stored dry, no deterioration of collagen takes place, since bacteria cannot multiply and enzymic changes are minimized.

C. Demineralization

The purpose of demineralizing the broken and degreased bone material is to remove the calcium and other salts in the bone, in order to release the collagenous gelatin producing substance "ossein". While this is mainly composed of collagen, it also contains mucopolysaccharides, small amounts of other proteins, etc. This calcium is present mainly as hydroxyapatite. The inorganic content of the bone pieces is removed by treatment in dilute hydrochloric acid solution, whereby the calcium phosphate is dissolved as acid phosphates.

This process is carried out in acid resistant containers using the counterflow principle. The fresh bone pieces receive the almost spent hydrochloric acid solution containing a substantial proportion of monocalcium phosphate and the almost completely demineralized bone pieces meet the fresh acid solution. Depending on the nature of the material, temperature and acid concentration, this demineralization process lasts from a few days to as much as 4 weeks. The acid concentration used is in the range 2 to 6% HCl. The demineralizing process must be carefully controlled, to avoid any harm to the raw material, arising from acid hydrolysis of the protein. Temperature is important in this respect and the exothermic character of the reaction requires appropriate conditions to avoid any heating up.

The demineralization of bones with hydrochloric acid is a diffusion process. R. J. Croome and F. G. Clegg⁽¹⁸⁾ interpret the extraction rate of calcium phosphate by means of Fick's diffusion expression. It is assumed that the acid diffuses under the gradient provided by the solution at the bone outer surface and the interface within the bone piece at which demineralization is occurring. The reaction there is assumed instantaneous giving zero acid concentration. As demineralization progresses the increased diffusion path slows the reaction rate but the higher acid concentration provided by the counter current technique partly compensates for this. In practice the termination of the acid treatment is determined by taking individual pieces of bone which are cut through and the cross-section examined. Any core of mineral containing tissue can be readily detected.

A satisfactory rate of leaching with minimal protein degradation is given by an initial acid concentration of 5%. The rate of leaching is dependent on the temperature, rising as the temperature is increased, as also is any hydrolytic degradation. Industrial practice has made it evident that demineralization is best performed in a temperature range between 0–15°C because, in this range, the degree of hydrolysis of the ossein remains minimal. The degree of hydrolysis can be measured by the nitrogen content of the acid liquor, as confirmed by R. Gorodetzka, M. Scheremet and Schachnasarowa.⁽¹⁹⁾ They suggest that where temperature increases are difficult to avoid owing to high ambient temperatures, the recommended hydrochloric acid concentration of 5% should be reduced to around 2.5%.

Other mineral acids can be used in place of hydrochloric acid, e.g. sulphurous acid (H_2SO_3), sulphuric acid (H_2SO_4) and phosphoric acid (H_3PO_4). The pH of the solutions of these acids for demineralization should also be 1 to 3. However, these mineral acids have not been extensively used in industrial practice, partly because of cost, but partly because of the need to recover phosphate in convenient form for use in animal feeds. This provides a valuable by-product.

The decalcified bone stock is separated from the spent acid liquor and washed with water to remove the bulk of the remaining acid. This washing may be followed by neutralizing with a weak lime suspension (0.5%) or with a diluted sodium hydroxide. The raw material, which retains the original skeletal structure of the bone, is then transported to the liming vat, which can be effected most conveniently by pumping or using a crane with gripping shovels. Where, as in Belgium, a cheap source of hydrochloric acid encouraged the production of ossein for sale, it was necessary to dry the demineralized bone for transport. Some changes occur in drying, so that it is preferable to avoid this stage and proceed to gelatin processing, using the washed ossein, in the same factory. The liquor obtained in demineralizing produces dicalcium phosphate when treated with milk of lime.

III. PREPARATION OF HIDE STOCK

A. Bovine Hides and Skins

The hide stock supplied to gelatin factories, such as splits, trimmings of dehaired hide, raw hide pieces, salted hide pieces and pig skins, is either taken into storage or else proceeds directly into processing. A first washing cleans the raw materials of adhering contaminants, such as dirt, preserving agents, salt or lime. Cattle and calf pieces in hair will need a dehairing stage using lime/sulphide mixtures as in the tannery. It is also possible to leave dehairing until the lime process, washing the pieces free of hair at an appropriate time before further liming. This is, however, rarely done today. They may need to be passed over a strong magnet to remove any metal pieces present. A conveyor belt can then conveniently transport them to equipment for reducing the piece size. Many proposals have been made in recent years for hide cutters, mincers, etc. In industrial practice a British and a German system have both proved to be effective. Both operate on a similar principle, i.e. employing an especially equipped meat chopper or mincer, which can also be called a hide chopper. It reduces the hides to pieces the size of the palm of the human hand. Special care is needed to exclude stones. Splits can also give trouble in the chopper because of their length. Disc cutters have also been fairly successful, although requiring frequent resharpening of the blades. A number of attempts have also been made to shred the raw hide material to the form of a pulp. Paper beating machinery has been used for this purpose. It became evident that the reduction was often carried too far, so that subsequent operations were made more difficult. Subdivision speeds up the various pre-treatment and extraction stages and may be desirable for the development of processes aimed at total extraction in a single liquor. Filtration of the liquor may become very difficult.

Some size reduction of most hide material is a fundamental necessity for gelatin making, because in this state the material is much easier to transport, the acid and alkaline treatments are more uniform and extraction is facilitated. If size reduction has been effectively carried out, pumping can be used to transport the material. A high water proportion is needed to aid pumping and motion through pipes. Grabs, skips and other forms of mechanized transport are also used.

B. Pigskin

In the U.S.A., pig skins are delivered in frozen condition and stored in this condition, in order that they remain fresh. Before processing they are either thawed in steam or washed for a few hours in cold water. A number of

methods have been suggested for degreasing raw hide materials. None of these suggested processes have been industrially accepted, as they are not readily applicable for the degreasing of pig skins. One process suggested by Armour and Comp.⁽²⁰⁾ uses dry steam on pigskins in a perforated drum, the fat melting out and flowing to a centrifuge. Any process causing thermal shrinkage of cattle hide (i.e. temperatures over 65–66°C) would make liming impossible to carry out. Pigskins and sheepskin both have high natural grease contents but suitably selected gelatin making processes avoid serious interference from the grease.

IV. PREPARATION FOR EXTRACTION

A. General

The prepared raw material of bone stock and hides can then be treated by two different methods, in order to prepare the collagen for conversion into gelatin. These are called the alkaline process and the acid process. The gelatin obtained from acid treated raw material has been called type-A gelatin and the alkaline treated raw material is referred to as type-B gelatin; terms which are used mainly in N. America. Whereas pigskin is always processed to type-A and cattle hide to type-B, ossein can be processed into both types of gelatin.

B. Liming Process

1. *The reaction*

The raw material is treated at ambient temperatures with a succession of liquors made up of lime in solution and in suspension. This is called the liming process. The alkaline preparation of ossein or of hide material is performed in a fresh suspension of hydrated lime in a concentration of 1 to 3°Be or 2 to 5% by weight. Depending on the nature, previous treatment and size of hides and liming temperature, the liming process may last from 6 to 20 weeks, normally from 8 to 12 weeks. For ossein, rather shorter time can be employed. The liming process is carried out in liming pits or vats. The material is mechanically moved about or stirred by air blowing during the process. According to the raw material and whether edible or photographic gelatin is being made, the used lime liquor will be replaced by fresh liquor more or less frequently. Ossein can readily be pumped from vat to vat and is much easier to treat uniformly.

The purpose of the liming process (see p. 161) is to destroy certain chemical crosslinkages still present in the collagen, that give the stock its firmness

as well as to remove unwanted material, other proteins, carbohydrates, etc. Fats still present are converted into insoluble lime soaps. The chemistry of the liming process deserves special attention (p. 163), because the action of the alkali is decisive for the desired change of structure and chemical composition of the collagen. It is thus decisive for solubilizing the collagen in hot water and influences the properties of the resulting extracted gelatins. Beside alkali concentration, liming temperatures should not exceed 20°C if excessive loss of collagenous material is to be avoided. If the temperature is too low then the action of the lime is slowed down despite increased solubility of calcium hydroxide. Where pits for liming are in the open, control of temperature is impossible. Reasonable control can be secured in covered buildings but for liming ossein in particular it is advisable to provide for temperature control in tanks, drums or pits.

During lime treatment some of the covalent crosslinks between collagen chains are broken as also, especially if liming is prolonged, are peptide links in the chains. Excessive prolongation of liming, while giving gelatin of good quality, causes losses of collagen in the lime liquors and so loss of gelatin yield. Release of ammonia from the amide groups of glutamine and asparagine residues releases carboxyl groups and reduces the isoelectric point of the polypeptide chains. The general reduction of linkages causes a marked progressive drop in the shrinkage temperature of the material. The reduction in crosslinkages progressively increases Donnan swelling at the high (as at low) pH, Courts.⁽²¹⁾ This is clearly visible as well as textural changes.

2. *Process control*

In industrial practice the liming process can be followed and controlled by the following measures:⁽²²⁾

- (a) Determining the degree of alkalinity of lime liquors by indicators and titration by acid.
 - (b) Determining nitrogen (amide-N) and reactive sulphur.
 - (c) Determining the total protein decomposition products and
 - (d) The degree of preparedness of the material by making test extractions.
- The shrinkage temperature and the isoelectric point have also been used.

3. *Accelerated methods*

Several methods have been suggested for accelerating the liming process, of which only a few have been industrially evaluated. The more interesting of these processes are described briefly.

The Industrial Patents Corp.⁽²³⁾ add peptizing substances, such as urea, to the lime liquor.

Eastman Kodak Co.⁽²⁴⁾ perform their alkaline treatment with ammonia or aliphatic amines. Mixtures of calcium hydroxide with alkali metal hydroxide

have also been suggested. Besides these, experiments with calcium chloride have been carried out. The Deutsche Gelatinefabriken⁽²⁵⁾ treat the collagen-containing material with strong acid or a weak alkaline liquor. A high concentration of sodium sulphate is used with sulphuric acid.

The concentration of the treatment liquors must be such that no tanning is effected. In these rapid processes, the treatment temperature is raised considerably. The collagen is converted into an easily extractable condition after washing out the acid, salt or alkali. It should be noted that fleshings, with their high fat content, do not respond well to liming and so are washed and neutralized in the condition they are received from the tannery.

4. Neutralization

Upon conclusion of the liming, the pretreated collagen is removed and subjected to a preliminary washing in normal water. The solid surface lime on the hides can readily be mechanically removed by water. The lime embedded in the pores of the raw material is more difficult to eliminate. For this reason a second washing follows the first with an acid solution, containing hydrochloric acid, phosphoric acid, etc. The acid treatment is made when the washing water of the preliminary washing has a pH value of 9.0 to 10.0. In the subsequent acidification over-acidifying must be avoided or excessive swelling may occur. A final washing is then given, to remove excess acid. For this purpose soft water or fully desalted water is to be preferred.

The washing process is carried out in mechanical washers. The two best known are the paddle washers and cone rollers (log roller) machines. Continuous agitation is essential during the entire washing process, requiring large quantities of water. The duration of the washing process depends on the nature of the material, the quantity of water available and the washing vessel equipment. In general it takes from 5 to 48 hours.

The washed and pretreated raw material is then forwarded to the extraction department for the production of gelatin.

C. Acid Process

The acid processing of collagen and ossein originated in the U.S.A. and, in recent times, has increasingly gained significance in Europe. It is especially suitable for less fully crosslinked materials such as pigskin and the ossein from the bones of young cattle. The reasons for the increasing interest paid to acid treatment of gelatin raw material are as follows:

- (a) The duration of the raw material preparation only requires 10 to 48 hours, in contrast to liming.
 - (b) In contrast to liming, the waste water involved is greatly reduced.
-

- (c) The newer degreasing processes have made available osseins for gelatin making which respond well to acid processing.

The increased interest in acid treatment is thus mainly directed to cost reduction. In addition, due to the short raw material preparation times, less raw material needs to be kept in stock. It must be remembered, however, that the gelatins produced by this process differ in important respects from that from the lime process so they cannot be regarded as equivalent. Acid processing is best adapted for pigskins, but is also used for ossein and for rabbit skins.

The washed and reduced raw material is soaked in diluted mineral acid solutions whose concentration should not exceed 5% and whose pH should lie around 3.5 to 4.5. The mineral acids used may be hydrochloric acid, sulphurous acid, sulphuric acid, phosphoric acid. Mixtures of these acids are often also used. In the diluted mineral acid solutions the hides swell, without showing any signs of solubility. This acid treatment is continued until the raw material is fully acidified or has attained a maximum of swelling. The temperature of treatment is usually the ambient temperature of the factory and so is about 15 to 20°C. The duration of acid treatment in turn again depends on the nature of the raw material, the temperature and the concentration of the acid, and the ranges from 10 to 48 hours.

Once the required condition has been reached, the acid solution is removed and the treated material is washed with cold water to remove any adhering acid. Washing is continued in non-corroding washing vessels until the pH has adjusted to above 4.⁽²⁶⁾ As the isoelectric point of some of non-collagenous proteins present in the raw material is around pH 4 to 5, they are least soluble in this range and may coagulate during extraction. The wash water is changed frequently. The acid-free washed raw material at pH 4 is ready for extraction.

The acid pretreated collagen or ossein material and the acid extracted gelatin are characterized by their isoelectric point and differ in this respect from such gelatins produced from alkaline pretreated raw materials. Alkali-precursor gelatins have an isoelectric point in the region of pH 5 (± 0.2). They have been investigated in detail by W. M. Ames.⁽²⁷⁻³⁴⁾ Acid-precursor pigskin gelatins on the other hand have an isoelectric point of around pH 9. It is clear however (p. 88) that the difference of isoelectric point difference is only one of the important structural differences between these two groups of gelatins.

D. The Enzymatic Process

The preparation of collagen-containing material by biological or biochemical means is relatively new. The first methods applied were made public

around 1954 and were chiefly developed by American and Japanese workers.

Armour & Co.^(35, 36) treat the hides with micro-organisms such as *mycoderma* and *torulopsis*. After about 21 days; microbiological decomposition has reached a stage at which it is interrupted after adjusting the pH to 1.5 to 3.5. The action of the micro-organisms is substantially accelerated by inoculating with cultures from other hide materials.

The work of the Japan Leather Co. Ltd.,⁽³⁷⁻⁴⁰⁾ especially of T. Fujii⁽⁴¹⁾ have established the fundamentals for an enzymatic method for producing gelatin. To solubilize insoluble collagen fibres, collagen-containing material was treated with the enzyme pronase in the presence of 0.4 M-CaCl₂. Pronase is a commercially available mycelial enzyme, whose principal component is a proteinase which has been isolated in the pure state by Nomoto and Nara-hashi.⁽⁴²⁾ The duration of the treatment with pronase is substantially shorter than in the liming process and even shorter than is sometimes used in the acid process. After inactivating the enzymes and eliminating the inorganic salts, the gelatin can be obtained by warm water extraction.

According to the Japan Leather Company this method has the following advantages, in contrast to preparation by lime.⁽⁴³⁻⁴⁷⁾

- (1) As compared with the former liming method, only about one-fifth as many hours are required for manufacturing.
- (2) The yield is almost 100%.
- (3) The physical properties such as jelly strength, setting point and melting point are better than the product of the previous method, in which only about 30% of the total product extracted is of high quality.
- (4) A concentration process is not required; therefore the cost of evaporation and steam is eliminated; while in the previous method concentration after extraction is essential. (In practice, filtration can only be carried out from a dilute liquor state. From this aspect it would be inadvisable to extract gelatin in too concentrated a form.—Eds.)
- (5) The molecular weight distribution is very sharp, in the neighbourhood of 120,000, while the gelatin manufactured in the alkali process has a broad distribution of molecular weights.
- (6) Purity is much higher than in the gelatin from the previous method, since according to the invention the collagen fibre is dispersed in a molecular state as a solution and is reconstituted as a fibre before conversion; so the manufacturing process includes a stage comparable with recrystallization. The gelatin is of special value for certain photographic purposes.

The physico-chemical properties of the gelatins produced by the enzyme method are given in Table III.⁽⁴¹⁾

Undoubtedly, the enzymatic pretreatment of collagen-containing material should gain importance in the future of gelatin manufacture.

TABLE III. Physical properties of gelatin made by the enzyme method⁽⁴¹⁾

Gelatin No.	Raw material	Enzyme	pl*	[n] (dl/g) in 0.15 M citrate plus 1.2 M KCNS, pH 3.7	Viscosity**	G(dynes/cm ²) × 10 ⁻⁴	Jelly strength**	Melting point**	Setting point**
46	Kip	Pepsin	8.60	0.49	7.8	7.1	230	—	29.8
11-B	Steer	Pronase	9.15	0.43	6.0	8.1	255	31.6	27.0
5	Calf	Pronase	9.30	0.34	5.0	8.4	290	32.4	—
27-C	Steer	Pronase	9.35	0.36	5.9	9.8	360	31.3	26.4
31	Pig	Pronase	9.40	0.29	4.2	9.5	375	29.6	24.9
34-A	Kip	Pronase	9.40	0.34	5.3	9.0	350	30.9	26.0

* Isoionic point measured by the ion exchange method.

** PAGI method.

REFERENCES

1. Sauer, E. (1958). "Tierische Leime und Gelatine", Springer-Verlag, Berlin.
2. Gerngross, O. and Goebel, E. (1933). "Chemie und Technologie der Leim- und Gelatinefabrikation", Dresden und Leipzig.
3. U.S. P. 2,395,590: "Degreasing from animal bones" by Mueller, C.
4. U.S. P. 2,397,973: "Bone glue" by Mueller, C.
5. Chochlowa, S. *et al.* (1953). "Improvement in the extraction of bones", *Fleisch-Ind. UdSSR*, 24, pp. 30-31.
6. German P. 813 187: "Treatment from wastes bearing fat and glue." by Stöbe, R. Appl.: Decker and Holz.
7. Kowal, W. (1951). "Preparation of bones." *Fleisch-Ind. UdSSR*, 22, pp. 18-21.
8. Brit. P. 555,299: "Extraction of fat and water from bones", Appl.: R. G. Blumenthal and the International Estate & Co.
9. Belg. P. 451,651: "Degreasing from bones", Appl.: Faupel & Haake, Efka-Werke AG.
10. German P. 751,071: "Treatment and extraction from bones to gelatin and glue", by Schwarzkopf, M.
11. German P. 1,006,559 and Brit. P. 775,003: "Improvements in or relating to a method of and Apparatus for Testing Fat-containing Material", Appl.: Sheppy Glue & Chemical Works Ltd.
12. German P. 1,072,768 and U.S. P. 3,087,945: "Process for cleaning and degreasing bones", Appl.: Cleveland Product Comp. Ltd.
13. Brit. P. 1,007,002: "A process for removing fat from bones", Appl.: Stauffer Chemical Comp.
14. Brit. P. 714,617 and German P. 836,982: "An improved Method for the Degreasing of Bones", Appl.: British Glues and Chemicals Ltd.
15. Brit. P. 722,311: "Improvements in and relating to Recovery of Fat from animal fat-containing products", Appl.: British Glues and Chemicals Ltd.
16. Chayen, I. H. and Ashworth, D. R. (1953). "The Application of Impulse Rendering to the Animal-Fat Industry", *J. appl. Chem.*, 3, 529-37.
17. Chayen, R. (1960). "Impulse Rendering, Chemical and Process Engineering, pp. 14-17.
18. Croome, R. J. and Clegg, F. G. (1965). "Photographic Gelatin", Focal Press, London-New York, pp. 22-23.
19. Gorodetskaja, R., Scheremet, M., and Schachnasarowa, M. (1952). "Reserven der Gelatine-Industrie", *Fleisch-Ind. UdSSR*, 23, pp. 45-49.
20. U.S. P. 2,820,804: "Method of Defatting Bacon Skins", Appl.: Armour & Comp.
21. Courts, A. (1960). *Biochem. J.*, 74, p. 238.
22. Kinkel, E. (1958). "Fabrikation der Gelatine in Tierische Leime und Gelatine", E. Sauer, pp. 120-33, Springer-Verlag, Berlin/Göttingen/Heidelberg.
23. U.S. P. 2,384,673: "Extraction of Glue and Gelatin", Appl.: Industrial Patents Corp.
24. U.S. P. 2,460,809: "Gelatin", Appl.: Eastman Kodak, Co.
25. Austrian P. 175,326 and Swedish P. 134,560: "Manufacture of Gelatin or Glue from Raw Materials of Collagen", Appl.: Deutsche Gelatine-fabriken.
26. Veis, A. (1964). "The Macromolecular Chemistry of Gelatin", pp. 186-218, Academic Press, New York-London.
27. Ames, W. M. (1944). *J. Soc. Chem. Ind.* (London), 63, pp. 200, 234, 277, 303.

28. Ames, W. M. (1945). *J. Soc. Chem. Ind.* (London), **64**, 242.
29. Ames, W. M. (1947). *J. Soc. Chem. Ind.* (London), **66**, 279.
30. Ames, W. M. (1952). *J. Sci. Food Agr.*, **3**, 454.
31. Ames, W. M. (1952). *J. Sci. Food Agr.*, **3**, 579.
32. Ames, W. M. (1957). *J. Sci. Food Agr.*, **8**, 169.
33. Ames, W. M. (1947). *J. Soc. Chem. Ind.* (London), **66**, 270.
34. Ames, W. M. (1949). *J. Soc. Leather Trades Chemists*, **33**, 407.
35. French P. 1,061,553: "Production or extraction of glue from salted skins", Appl.: Armour & Comp.
36. German P. 1,029,968, U.S. P. 2,908,615 and Brit. P. 817,367: "Method of Preparing Gelatin or Glue", Appl.: Armour & Comp.
37. Nishihara, T. and Miyata, T. (1962). "The Effects of Proteases on the Soluble and Insoluble Collagens and the Structure of Insoluble Collagen Fibre", Collagen Symposium, Vol. III, pp. 62-93.
38. Kubota, A. (1963). "The Structure of the End Region of Collagen Macromolecule", Annual Report of Nippon Hikaku Kenkyujo No. 9, pp. 1-11.
39. Miyata, T. (1963). "Renaturation of Acid soluble Collagen Macromolecule", Annual Report of Nippon Hikaku Kenkyujo No. 9, pp. 13-33.
40. Nishihara, T., Kubota, A., Nishizowa, M. and Miyata, M. (1965). "Enzymic Solubilization of Collagen Fibre", Annual Report of Nippon Hikaku Kenkyujo No. 10, pp. 21-37.
41. Fujii, T. (1966). "An Enzymatic Method of Producing Gelatin", *Bull. Soc. Sci. Photog. of Japan*, No. 16, pp. 30-37.
42. Nomoto, M. and Narahashi, Y. (1959). *J. Biochem.*, **46**, 1481.
43. Brit. P. 903,975: "Improvements in and relating to the Solubilization of Collagen and Reconstitution thereof", Appl.: Nihon Hikaku Kabushiki Kaisha.
44. Brit. P. 929,137: "Improvements in and relating to Colloidal Solutions", Appl.: Nihon Hikaku Kabushiki Kaisha.
45. Brit. P. 1,090,967: "Method for Solubilization of Collagen Fibers with Proteolytic Enzymes", Appl. Nihon Hikaku Kabushiki Kaisha.
46. Brit. P. 1,062, 083: "Method for Solubilization of so-called Insoluble Collagen Fibers", Appl. Nihon Hikaku Kabushiki Kaisha.
47. Canada P. 784,404: "Method for Solubilization of so-called Insoluble Collagen by Proteolytic Enzyme", Appl.: Nihon Hikaku Kabushiki Kaisha.

Chapter 10

Technology of Gelatin Manufacture

R. HINTERWALDNER

8 München 90, Postfach 90 0425, Germany

I Special Methods of Gelatin Manufacture	315
A. Introduction	315
II General Operating Procedures of Commercial Manufacture of Gelatin	320
A. Gelatin Extraction	320
B. Acid Extraction	322
C. Filtration and Clarification of Gelatin Liquors	326
D. The Evaporation of Gelatin Liquors	332
E. Sterilization	343
F. Drying	347
G. Grinding and Sifting of Gelatin	358
H. Storage of Gelatin	360
I. Secondary Departments in Gelatin Production	360
References	361

I. SPECIAL METHODS OF GELATIN MANUFACTURE

A. Introduction

The ultimate aim in gelatin production is the conversion of material containing collagen of different degrees of insolubility into a maximum quantity of soluble and highly purified gelatin with good physico-chemical properties such as gel strength, viscosity, lack of colour and high clarity. The fundamental production process is based on three stages:

preparation of the raw material, i.e., the elimination of non-collagenous components from the stock material with or without the reduction of crosslinkages between collagen components;

the conversion of the purified collagen into gelatin, and

refinement and recovery of the gelatin in dried form.

The preparation of the raw materials has already been dealt with in some detail in Chapter 9 on "Raw Materials". A number of processes have been published describing the conversion of collagen to gelatin and its subsequent refinement and recovery, up to its dry form. Many of these processes are only scientifically interesting and have never attained any significance in industrial practice. Not surprisingly, much of the information available is confined to the patent literature, from which it is impossible to assess the extent to which the processes have actually been used. A brief account of this literature follows, since a number of concepts have features of interest.

Most methods of converting pre-treated collagen into gelatin are based on similar extraction temperatures. This is determined by the need to exceed the shrinkage temperature of the collagen but not to damage the protein extensively. For example, Loeven,⁽¹⁾ Council of Scientific and Industrial Research,⁽²⁾ Kenyon and Silberstein⁽³⁾ extract between 50 and 100°C. The Russian gelatin factory Kasan⁽⁴⁾ employs extraction at 40–45°C in order to avoid the degradative high temperatures, so as to obtain a gelatin with better properties. The pH values for extraction differ in the various methods.^(5, 6) Clearly to permit extraction at temperatures as low as 40–50°C, pretreatment has to be carried to the point where rupture of hydrogen and other secondary bonds, together with assumption of a random coil configuration is all that remains to be done to give a gelation solution.

The Council of Scientific and Industrial Research⁽⁵⁾ recommends extraction at a pH of 6, whereas Kernot⁽⁶⁾ adjusts to pH 4 to 5 by means of phosphoric acid. The General Foods Corp. process⁽⁷⁾ substantially acidifies the collagenous raw material and extracts with circulating hot water. Acidification in this process is of such extent that the extracted gelatin solution has a pH of 1.5 to 3.5. The gelatin produced is immediately neutralized to about pH 4. This may give rise to rather high salt concentrations but ion exchange resins can, at the cost of an additional process, be used to adjust the pH without salt addition or may be used to remove unwanted salt. In another method referred to by General Foods Corp.⁽⁸⁾ the raw material is first stored in a strong acid and the extraction is then effected by circulating hot water as previously mentioned and the pH of the gelatin solution obtained adjusted, as before, to 4.0–8.0. Eastman Kodak Ltd. describe extraction in an alkaline medium.⁽⁹⁾ The raw material, pretreated by ammonia or by aliphatic amines, is extracted at 50°C to 65°C so that the extraction medium is buffered at pH 9 to 10. The amines can subsequently be eliminated from the gelatin liquor by vacuum evaporation.

A different approach was taken by Alexandrescu and Suszer⁽¹⁰⁾ who accelerated the hydrolysis of the collagen by small additions of hydrotropic agents. Extraction is performed in the presence of calcium chloride, urea or acetamide. Extraction takes about from 16 to 20 hours. It is claimed that

treatment of the raw material with lime suspension becomes unnecessary and that the gelatin yield is substantially improved. In their work Küntzel and Heidemann⁽¹¹⁾ discuss the desirability of carrying out the gelatin conversion in alkaline and acid media. They report experimental results using the two-stage process, i.e., lime pretreatment and subsequent thermal extraction and also using the essentially one-stage process in which acids are applied and are present during extraction. The differing effects of acid and alkali with the single-stage method is dealt with in detail, using swelling and conversion to gelatin at temperatures above 40°C in the presence of acids and alkalies. They have found that the swelling affect of alkali at elevated temperatures is considerably smaller than that of acids. This, they claim, is one reason why alkali is not suitable for the one-stage method of converting collagen into gelatin. It is clear, however, that the differing effects of acid and alkali on gel forming power (as studied by P. R. Saunders and A. G. Ward p. 185) is also of significance.

The General Foods Corp.⁽¹²⁾ has proposed a completely new approach, in contrast to the conventional improvements of processing methods. In order to obtain shorter extracting times with a higher yield, the collagen-containing material is bombarded with gamma rays. It is claimed that the physico-chemical properties are improved. In view of the opposing results of protein irradiation—rupture of bonds and the formation of crosslinks, which have been demonstrated for gelatin itself, this process must be viewed with caution.

Besides these improvements in processing technique, the equipment used is also of significance. For example, Hill,⁽¹³⁾ Rutskiij, Epsktein and Virnik⁽¹⁴⁾ have reported improvements in equipment.

In order to make the extraction processes more economical and, at the same time, to ensure a more rapid, but controlled conversion into gelatin, a number of researchers have investigated the problem of continuous processing. Frechlin⁽¹⁵⁾ describes an apparatus for continuous gelatin extraction. Hide chips are exposed to a counterflowing current of the hot aqueous medium which, in the extended extraction zone, extracts the gelatin, under pressure if desired. This processing method is also claimed to be suitable for ossein and for other collagen-containing materials. Mueller⁽¹⁶⁾ also applies the countercurrent extraction principle to producing glue and gelatin from animal waste materials, incorporating a multi-zone extraction system as the main feature.

Frechlin⁽¹⁷⁾ on the other hand converts the collagen containing material into noodle form, or at least into small pieces. It is passed through a tube countercurrent to the aqueous extraction liquor which melts the gelatin out of the material. In the course of this process the material, as it passes through extraction zone at elevated temperature, is first subjected to an increasing then to a falling pressure. The temperature gradient is so chosen that the

zone of highest pressure coincides with that of the highest temperature. The extraction chamber proper is U-shaped, so that changes in pressure are effected by the hydrostatic head. The material is moved along by an endless chain, which, at minor intervals, has perforated plates normal to the direction of movement. In this way small chambers filled with material for extraction are formed, through which the extraction fluid is forced. The double-walled tube is steam heated to about 100°C. The method makes provision for a bleaching with sulphur dioxide (SO₂).

Improved extraction conditions given by Hill and Hill⁽¹³⁾ involve heating the counter current extraction fluid by steam injection, the moving material being converted into gelatin or glue. By this method uniform heating of the collagen material is ensured, shortening the extraction process and giving a higher gelatin or glue concentration. This method can be used for ossein as well as for hide chips. In order to eliminate long pretreatment processes, Armour & Co.^(18, 23) have suggested a new approach. The collagen-containing material is comminuted and mixed with water to form a sludge. The further processing is carried out continuously. A quantity of the subdivided material is passed through a cylindrical heating zone in which the sludge is heated by live steam to about 100°C. A steam jet can create turbulence. The flow rate is around 90 kg per minute. After separating out the residual solid particles, e.g., by hydroextraction, the gelatin liquor is subjected to the usual filtration, concentration, etc. Where pigskin was used in this process, the fat melted out required to be separated.⁽²³⁾ A dry rendering process for degreasing has been described by Armour.⁽²⁴⁾

The method proposed by Coudun and Coudun⁽¹⁹⁾ is a development of the use of alkaline pretreatments, such as liming. The pretreated material is pressed, causing a sticky fluid to be exuded which can be converted by heat into gelatin. The residue from pressing is disintegrated, left standing in an open vessel for some time and is then converted into glue. There must be doubt whether the proportion converted in this way to gelatin is sufficient to make the process economic. In the colloid mill, hide particles can be reduced to colloidal size (< 10 microns). Then the material can be converted into glue and gelatin by hydrolysis. This has been suggested by the Charles E. Ely method and results in a shortening of processing time.⁽²⁰⁾

Pigskin as a raw material for gelatin, offers opportunities to use various methods of extraction for gelation production. A number of these techniques are not applied in industrial practice but they often show new points of view, which could perhaps be developed.

Armour & Co.⁽²¹⁾ desalt the pigskins at around 45°C to 50°C. This is followed by hydrolysis with oxalic acid (COOH)₂, whereby the major portion is hydrolysed. After neutralizing the liquor, the material is subsequently processed in the usual way.

A novel approach was taken by Wilson & Co. Inc.,⁽²²⁾ based on the claim that gelatins of high gel strength are obtained if dry collagen is cooked for 15 to 60 minutes at a temperature of 60°C to 70°C. Pickled hides, pigskins, sinews and bones are heated at a pH within the range of 3.0 to 8.5, at a temperature of less than 38°C, to reduce the moisture content to below 50%. The temperature is then raised to about 60°C and the moisture content reduced to 16 to 18%, when it appears substantially dry. This material is then ground so finely that it will pass through a screen (20 mesh/cm). The "pulverized" collagen is exposed to a temperature of 60°C to 70°C and is claimed to be converted in about 30 minutes to a gelatin of 225–300 g bloom. The yield and the quality of the gelatin obtained corresponds to that obtained with 5 extraction liquors with the conventional methods. The ground collagenous material cannot only be used for gelatin production, but can also be employed as such in the production of foodstuffs incorporating gelatin.

Armour & Co.⁽²⁵⁾ have applied a sterilizing agent (hydrogen peroxide) and yeast for preparing the pigskins and converting them to gelatin. With other raw materials a liming stage is needed. The peroxide sterilized material is subjected to the action of living yeasts to condition them and then extracting. Hydrogen peroxide is used as the sterilizing agent but it should be noted that its action may also modify the collagen. Among the micro-organisms that have been suggested for conditioning, are strains of *Saccharomyces*. Once the liquor has been obtained, there are a number of processes, described in Section B, to be carried out. In particular purification can be carried further, for instance by employing ion exchange resins to reduce ash content.

A. Rousselot⁽²⁷⁾ studied ash removal from gelatin by means of cation exchangers. Using a 10% solution at 60°C he showed how ash contents of 1 to 2% could readily be reduced to below 0.1%. The Sharples Corp.⁽²⁸⁾ recommend desalting gelatin solutions by combining a cation exchanger with an anion exchanger. Veb Farbenfabrik Wolfen⁽²⁹⁾ give details of how to remove fatty residues from gelatin with synthetic resin exchangers. Eastman Kodak & Co.⁽²⁶⁾ describe the elimination of protein and mucoprotein components. To an aqueous gelatin solution at 32–40°C pH value 4.7 to 4.8, they add a neutral volatile dehydrating agent such as acetone or alcohol, until turbidity sets in. The addition of dehydrating agents is continued until an optical density of 0.4 to 1 in a 2 cm thick cell is obtained. The protein impurity is then separated off.

Attempts have been made to dry gelatin using both spray drying^(30–34) and freeze drying. Commercial methods being used successfully have not been described. Further details on various processing methods are contained in Hinterwaldner's "Monographie".⁽³⁵⁾

II. GENERAL OPERATING PROCEDURES OF COMMERCIAL MANUFACTURE OF GELATIN

A. Gelatin Extraction

Gelatin is produced from pretreated raw materials by extraction with water under appropriate temperature conditions. This extraction process, which normally comprises several stages, is one of the most significant processing steps in gelatin production. The extraction or conversion methods applied, together with the state of the raw material, influence the duration of the extraction process and the quality of the extracted gelatins. The ease with which gelatins can be extracted from the collagen fibres varies greatly, according to type and age of the animals, to a lesser degree also according to the tissue involved. A. G. Ward⁽³⁶⁾ has stated the following rough rule "The more mature a fibre is, the harder it is to dissolve." As has already been made clear the preferred raw materials are demineralized bones (ossein), cattle hides and pigskin.

As is discussed in Chapter 5, if the pretreated raw material is brought to a high temperature in contact with water at neutrality, a gelatin solution will gradually be obtained that has moderate gel forming properties, low viscosity and poor colour. If, on the other hand, the extraction conditions are varied by cautious addition of acid, then the conversion of collagen into gelatins will be accelerated and, at moderate temperatures, will not promote substantial undesirable secondary reactions to the same extent. Heating the collagen in an alkaline medium likewise speeds up conversion, but, at the same time it also promotes other not yet fully defined degradation processes, whereby the gel forming properties are impaired. For this reason, all industrially used processes are based on neutral or acid pH values.

Pretreatments reduce the extent of crosslinkages, especially when lime or other alkaline treatments are employed. Materials like pigskin which can be processed without alkaline pretreatment are presumed to have lower degrees of crosslinkage and also less colour forming impurities. During extraction crosslinkages and peptide bonds continue to be broken. The final step in the conversion of collagen into gelatin involves the breakage of hydrogen bonds which are a key stabilizing factor in collagen structure. This action is needed even though the preparation of the raw materials has already adequately disrupted the crosslinks and, to some extent broken peptide bonds.⁽³⁷⁾ Table I shows the effect of a good raw material pretreatment on extraction conditions on the one hand and on the quality of the yield on the other hand. It must be remembered that commercially the quantitative yield is also important.

TABLE I. Effect of the pretreatment and extraction upon C_n at ossein

	C_n	Yield (%)	Jelly strength (Bloom)	Viscosity (centipose at 40°C)
<i>2 weeks in lime</i> pretreated ossein	107,000			
1. Extraction ossein residue	↓ 39,000 93,000	12	—	—
2. Extraction ossein residue	↓ 57,000 94,000	22	199	5.9
3. Extraction	↓ 44,000	43	130	7.3
<i>2 months in lime</i> pretreated ossein	64,000			
1. Extraction ossein residue	↓ 51,000 69,000	28	285	7.1
1. Extraction ossein residue	↓ 51,000 69,000	28	285	7.1
Ossein residue	↓ 43,000 64,000	40	195	10.4
3. Extraction		14	153	9.3
<i>6 months in lime</i> pretreated ossein	69,000			
1. Extraction ossein residue	↓ 50,000	28	288	10.5
2. Extraction	↓ 58,000	21	195	13.2

Pretreatment by lime at 20°C.

1. Extraction 2 h at 60°C.

2. Extraction 3 h at 80°C.

3. Extraction 2½ h at 93°C.

Two methods are available for breaking the hydrogen bonds in gelatin extraction, namely,

1. Raising the temperature of the collagen until the shrinkage point is reached;

2. Treating the collagen in a concentrated solution of a hydrogen bond breaking agent at room temperature.⁽³⁸⁾

The first method is universally used in the gelatin industry, the latter having only theoretical interest.

B. Acid Extraction

As already explained, the gelatin industry uses acid extract conditions extensively but the degree of acid varies with different processes. Table II gives information on the course of gelatin extraction of alkali-pretreated ossein at near neutrality. Table III gives corresponding figures at an acid pH.

The figures given in Tables II and III can be regarded as typical. At near neutral pH's, the gel forming properties of the gelatin decrease with increasing time and temperature. The viscosity of the first extract is less than

TABLE II. Gelatin extraction of alkali pretreated ossein at pH 6.4*

Extract No.	Time of extraction hr.	Temperature (°C)	Yield (%)	Jelly strength* in dyn/cm ² (10°C)	Viscosity* (cP 40°C)
1	2	60	22	87,400	7.9
2	2.5	70	17	76,000	9.6
3	4	80	27	67,700	14.1
4	4	95	13	51,700	8.4
Residue			3		
Total Yield			82		

* At 5.65% gelatin solution after 16-18 hours jelling at 10°C.

TABLE III. Gelatin extraction of alkali pretreated ossein at acid pH

Extract No.	Time of extraction hr.	°C	pH	Yield (%)	Jelly strength* in dyn/cm ² (10°C)	Viscosity* (cP at 40°C)
1	0.33	50	2.0	24	96,000	7.5
2	0.42	50	2.0	19	93,000	9.2
3	1.5	50	2.5	12	99,000	16.2
4	1.25	50	2.5	8	98,000	10.3
5	3.5	55	2.8	14	100,000	10.0
6	4.0	60	3.0	3	80,000	4.2
7	7.0	60	3.3	5	56,000	—
Rest				2		
Total Yield				87		

* At 5.65% gelatin solution after 16-18 hours jelling at 10°C.

that of the immediately succeeding extracts and is also of lower molecular weight. In the last stages of extraction degradation in the unextracted material and secondary hydrolysis of extracted gelatin gives a reduced viscosity, as well as reduced gel strength.

With extraction at pH2, the extraction process proceeds so rapidly, even at low temperatures, that the successive extracts must be very rapidly removed, to avoid secondary changes and a loss of the desired gelatin properties. The viscosity of the gelatins obtained depends on the extraction times selected. It is interesting to note that a decline in the gel forming properties, as measured in the conditions noted, only occurs in the last stages on extraction when the molecular weight of the gelatin is being rapidly reduced.

Interesting as extraction conditions with an acid pH under 4 are, they are not easy to adapt for use in large scale industrial gelatin production. Extraction conditions must be selected that can be closely controlled at every stage. This is why preference is given to neutral or weakly acid extraction conditions from pH 7.0 to pH 4.0. Up to the present, the most usually used extraction methods are discontinuous with a number of discrete liquors. If, therefore, extraction conditions were created, as is the case in highly acidic extraction say, where the first extract is drawn off after say 60 minutes, the time required for drawing off is significant in relation to the extraction time itself. This would therefore increase the incidence of a secondary hydrolysis. At neutral or slightly acid extractions, the processing and handling of liquors at the various temperatures can be controlled and more time is available without rapid hydrolysis. Immediate restoration of pH's from acid liquors towards neutrality offers means of controlling breakdown but, unless done by ion exchange resins, cause unwanted rises in ash.

In industrial practice⁽⁴⁰⁾ gelatin is thus conventionally extracted from alkali-treated and acid-treated precursors by successive applications of water at gradually increasing temperature. The first extraction is in general made at 50°C to 60°C and, for the following extractions, the temperature is successively increased in steps of 5°C to 10°C until the last extraction can be made at boiling temperature. This procedure ensures the minimum thermal degradation of the extracted gelatins.

The extraction of gelatin from pretreated collagen is normally carried out in open, stainless steel vessels. These stainless steel vessels have a perforated bottom under which heating coils are arranged. This equipment must be installed in such a way that it can easily and quickly be removed for cleaning. To reduce the heating time, so as to shorten the overall extraction time, the water employed for extraction can alternatively be heated by means of a heat exchanger. It is then run off below the perforated plate and recirculated to the material being extracted after passing through the heat exchanger. Where the heat is derived from a steam coil the perforated plate is installed about 20 to

25 cm above the bottom of the vessel. The space in between accommodates the heating coils. The perforated plate separates part of the extraction liquor from the material being extracted. The extracted gelatin solution can then be easily and rapidly drained off from the extraction vessel and the heat transfer to the extraction liquor is made possible without damaging the skin or ossein.

When the extraction liquor reaches a concentration of 3–8% gelatin, the extract is drained off and pumped to the subsequent processing stages. The second and further extractions each involve addition of hot water. The temperature of the added water should approximate to the temperature at which the preceding extraction was performed. The temperature is then raised to the required extraction temperature. The number of extraction stages that can be gone through until the boiling point is reached depends on the raw material processed and on the qualities of gelatin to be produced. In industrial practice about three to eight extractions are made.

Opinions differ commercially in regard to extraction conditions and the processing techniques. One view point attaches great importance to forced movement of the liquor by circulation with pumps. A second view prefers gentle stirring or still extraction in which the liquor moves by convection. The use of a central funnel leading from the area near the steam coil to the surface of the liquor encourages convective circulation. In general, the greater the movement at this stage, the greater is likely to be the difficulty with filtration.

For the production of low ash gelatins, e.g. for photographic purposes, there are today two possible processing methods. The first, which is less used in practice, consists of a final washing of the pretreated collagen in deionized water and extraction also in deionized water. The second method, which is more commonly used, incorporates ion exchange. The dilute filtered gelatin liquor passes through anion and cation exchangers (or a mixed bed) and so is demineralized. Considerable care has to be exercised in this procedure, especially as substances other than salts may be removed by the resins. The process itself adds significantly to costs,^(41–43) but can now be regarded as relatively small in view of the rapidly rising raw material costs.

The gelatin liquors obtained require to be subjected to other forms of treatment, such as bleaching, decolourizing and related processes. For bleaching gelatin there are two possibilities:

- (a) reduction bleaching
- (b) oxidation bleaching.

Reduction bleaching is based on the addition of reducing agents, such as sulphur dioxide or sulphites. Evaporation removes subsequently much of the sulphur dioxide. Too high a level in edible gelatin can influence the flavour of the gelatins significantly, depending upon the use to which it is put. The food

laws of a number of countries set limits to the addition of sulphites to edible gelatins.⁽⁴⁴⁾ The most frequently applied oxidizing agent is hydrogen peroxide (H_2O_2). The method developed by R. Hinterwaldner⁽⁴⁵⁾ is very effective. The bleaching agent in solution is sprayed into the gelatin liquor by means of ceramic filter candles and using air, or oxygen as the pressure source. The high degree dispersion of the bleaching agent obtained in this manner ensures an effective and uniform bleaching of the gelatin liquor.

The early extracts, made at the lower temperatures, contain, for well pretreated materials, the bulk of the gelatin produced as well as the highest quality gelatins. The later extracts, in contrast, result in turbid and more intensely coloured gelatin liquors of lower gel strength and viscosity.

These last extracts require more heat and power for their production. If we break down the costs of the entire gelatin extraction, it is evident that these last extraction stages cost more for power and, because of the poor quality of the product, also give the highest costs for further processing, such as clarification, bleaching, filtering. As gelatins of low gel strength and viscosity are less valuable and are not saleable in such large tonnage quantities, other forms of utilization of residual materials are being developed to improve the economic position. This means that gelatin extraction is carried only to the point where gelatin extracts of good physico-chemical properties can still be obtained. The remaining residue is then greater than for a total extraction, but this allows residue utilization to be more profitable. The most interesting and economical method of residue utilization employs the protein for the animal feed industry. It must be noted that collagen has, mainly through the absence of S-containing amino acids an unfavourable content of essential amino acids, which limits its nutritional value. The processing needed involves separation of fat and other ingredients. The purified protein is then dried. Among others, Hefermehl, S. A., Vevey, Switzerland, are reclaiming the residue from gelatin extraction. Residues from pigskin gelatin production first showed practical and economic results.⁽⁴⁵⁾

In industrial gelatin practice the attempt has frequently been made to convert discontinuous gelatin extraction into a continuous process. Partial success using acid extraction of slightly limed ossein has not been widely exploited and other attempts have not been very successful. The present author has examined this problem. There are hopes that a method can be developed by which continuous commercial extraction of gelatin from collagen material can be carried out.

The extracted dilute gelatin liquors are subjected to the various processing stages described in detail in the following sections. An important prerequisite for these stages is rapid processing until the dry product is reached. Too protracted manipulation results in thermal decomposition, involving a drop in quality and risk of bacterial growth.

C. Filtration and Clarification of Gelatin Liquors

Filtration and clarification of colloidal solutions rank among the most difficult processes in industrial practice. This is true for gelatin liquors.

1. "Chemical" clarification

"Chemical" clarification of gelatin liquors is today relatively unimportant, not only because the processes are expensive, but also because gelatin quality may be damaged in the process. Some producers, however, occasionally subject their last gelatin extraction to "chemical" clarification. This involves producing a flocculated precipitate that adsorbs all fine particles, including colloidal substances. After the precipitate settles the gelatin liquor is perfectly clear. A floccular precipitate can be produced by means of lime, calcium phosphate or alum. This settles at the bottom of the tank. Not every flocculant system is suitable for clarification purposes. It seems advantageous not to produce precipitations by purely inorganic means. In early tradition eggwhite was extensively used for clarification of gelatins. The early commercial gelatins were marketed with instructions for clarifying by this means. Nelsons gelatin retained the telegraphic address "opaque" from its early history.

2. Preliminary clarification by means of separators

Gelatin liquors may be given a preliminary clarification by means of sludge separators. Today the separators used have multi-chamber drums and function in a self-clarifying way.

A built-in set of plates or discs allow the gelatin liquor to be clarified as thin layers. The separation of solid particles is accelerated and a high degree of clarification achieved. The discs are arranged at such an angle that the solids can slide off into the sludge chamber. The drum is cleaned of precipitates by a built-in sliding piston device that is hydraulically moved to and fro inside the drum. When the drum is full of accumulated solids the water chamber above the piston is filled, the piston rapidly moves downwards, the discharge is opened and the solids discharged.

This method of preliminary clarification of gelatin broths is not without its hazards. The best clarification is obtained by working in conditions quite close to foam-formation. The gelatin liquor must not develop foam, because the enlarged surface not only prevents separation but gelatin quality can also be substantially affected.

Preliminary clarification by separation eliminates coarsely dispersed particles and fat globules from the gelatin liquor. If the fat content of the gelatin liquid is high, then separation will normally be necessary. The author,

however, doubts whether the elimination of coarsely dispersed solid sludge is always of advantage. Research has shown that while the coarse particles are eliminated, some medium and finely dispersed particles are produced by the centrifugal forces. As a consequence, subsequent filtration becomes more difficult. Only practical experience can serve as a guide as to the most appropriate procedure.

3. Filtration

Technical progress has also been made in the important unit operation of filtration of the gelatin liquors. New filtering devices and new filtering auxiliaries have made this possible. These developments have somewhat reduced the importance of the widely used filtration with cellulosic substances. Although these substances (e.g. cotton) are economical, and can be used several times with a reclamation cycle, they also have many disadvantages. Reclamation, together with the necessary sterilization and formation of pressed filter cakes is expensive and labour intensive. This is why filters using cakes of cellulosic substances are less used today.

As a consequence of the development of centrifugal purification filters the inexpensive filter aid "Kieselguhr" is now important in gelatin filtration. In his reports^(46, 47) Hinterwaldner deals in detail with this problem, reporting on the results of his own researches.

The centrifugal self-cleaning filter comprises a closed pressure vessel in which round filter elements are mounted on a hollow axial shaft akin to a number of plates. This filter arrangement is horizontal in vertical filters and vertical in horizontal type filters. In practice gelatin filtration uses only the horizontal filter element arrangement. The filter elements proper are equipped with metal twist fabric that allows particles of 100 μ to pass.

The filters are fed by a FRISTAM-stainless steel centrifugal pump. The centrifugal self-cleaning filter is linked to a kieselguhr mixing and dosing device having an agitator and an automatic dosing arrangement. Only certain types of kieselguhr (diatomaceous earth) are suitable as filter aids. Mixtures of different types are normally used, for it is only in rare cases that one type of kieselguhr produces an optimal filtrate.

The filtration process requires, if it is to be efficient, a clear understanding of the behaviour and the physico-chemical properties of gelatin and of the sludgy or turbid particles. The gelatin is in different states of aggregation according to its temperature. Below the setting point (in the region of 32°C) gelatin forms a rigid gel in which the rigidity increases with increasing solid contents. The rigidity is referred to as "jelly strength" (see p. 379). Above the setting point or melting point (these points are not identical) the system passes through a stage with molecular aggregates to one which is molecularly disperse, after which a normal fall of viscosity with rising temperature will

occur. The whole operation is concentration dependent and also dependent on the particular gelatin used.

Degradation must be avoided or minimized, so limiting the temperatures which can be used. So it is not possible to reduce the liquor viscosity by using very high temperatures. It may however be necessary to filter moderately concentrated liquors to eliminate haze on concentrating.

So the filtration of gelatin is a complex processing technique and the person skilled in the art must utilize a wide range of experience in order to be able to determine the optimal filtering conditions in each filtration he is required to control.

The suspended particles in the liquor may be divided into coarsely dispersed, medium and finely dispersed, all the range normally being found in gelatin liquors. As has already been explained the coarse particles are removable in a centrifugal separator, but with the disadvantage of some further breakdown and dispersion of particles to give a very fine suspension. The coarser particles may also act as a partial filter aid in the filtration process. If a gelatin liquor contains greater quantities of fat, centrifuging is usually impossible.

The medium size dispersed particles can be well eliminated with the help of a coarse kieselguhr, as was also possible with a cellulose based filtration. The most intractable problem is the elimination of the finely dispersed particles which give turbidity to the solution. To eliminate such particles, very fine kieselguhrs are required. If a layer of this type of auxiliary filtering material is applied to a vertical filter element, then the filtering layers are inhomogeneous in structure and will therefore not deliver a good filtrate. On a horizontal filter element, a homogeneous layer of auxiliary filtering agent can be deposited, which, in contrast to the vertical filter layer, is of looser, more permeable structure. This was recognized in earlier gelatin filter tests where too great a pressure on the auxiliary filtering layer clogged it. So the form of centrifugal self cleaning filter with horizontal filter elements is to be preferred.

Filtration is therefore best performed in two stages:

- (1) Main filtration, i.e. the filtration of the dilute gelatin liquor at a low concentration, and
- (2) fine or polishing filtration of the concentrated gelatin liquor.

As processing medium in preparing the preliminary deposit of filter aid, hot water or a dilute gelatin liquor can be used. Hot water has the advantage that it brings the filter to the proper operating temperature without affecting the filtering auxiliary agents. If, on the other hand, the preliminary depositing is effected with a dilute gelatin liquor, the operating temperature must first be reached using hot water or steam. Industrial practice shows that with direct preliminary depositing from a dilute gelatin liquor at around 3-5% conc., a

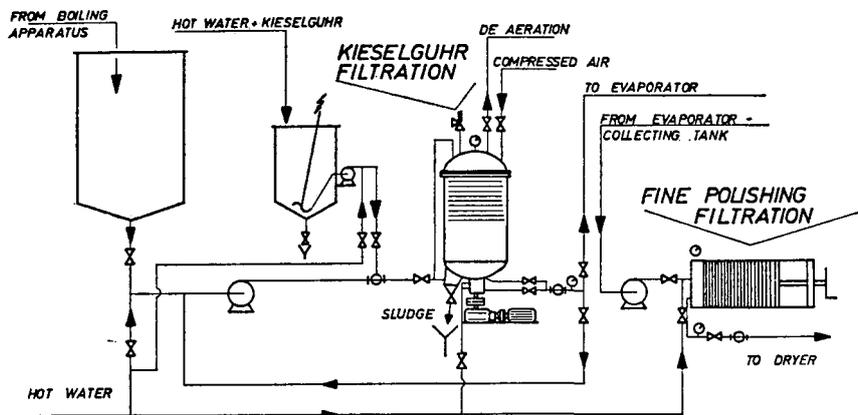


FIG. 1. Flow diagram—gelatin filtration system "Sohenk-Fitterbau."

clear filtrate is obtained more quickly. Figure 1 is a flow chart showing how gelatin filtration can be incorporated in a production process.

After preparing the filter, the filtration of the gelatin liquor can begin. The closed filtering circuit is maintained until the sight glass behind the filter shows a clear filtrate.

As continuous filtration of the liquor begins, an addition of the filter aid is required to ensure that the deposits on the filter elements remain porous,

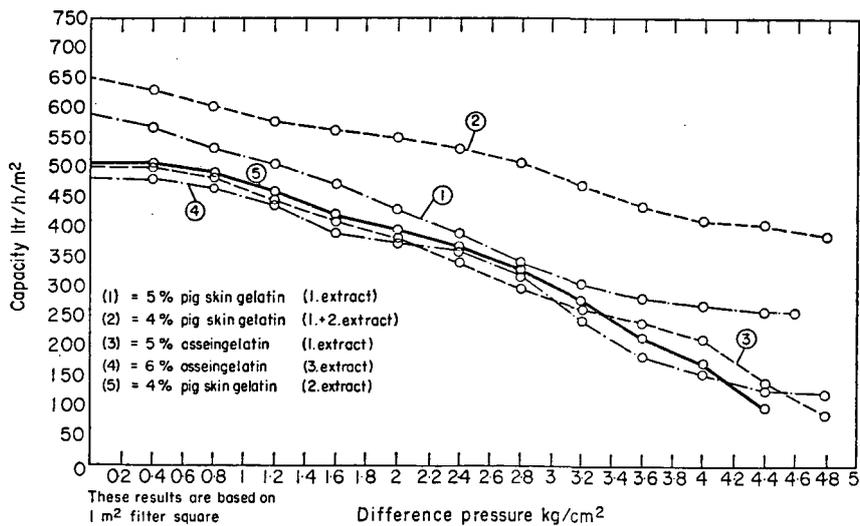


FIG. 2. Filter capacity from low concentrated gelatin.

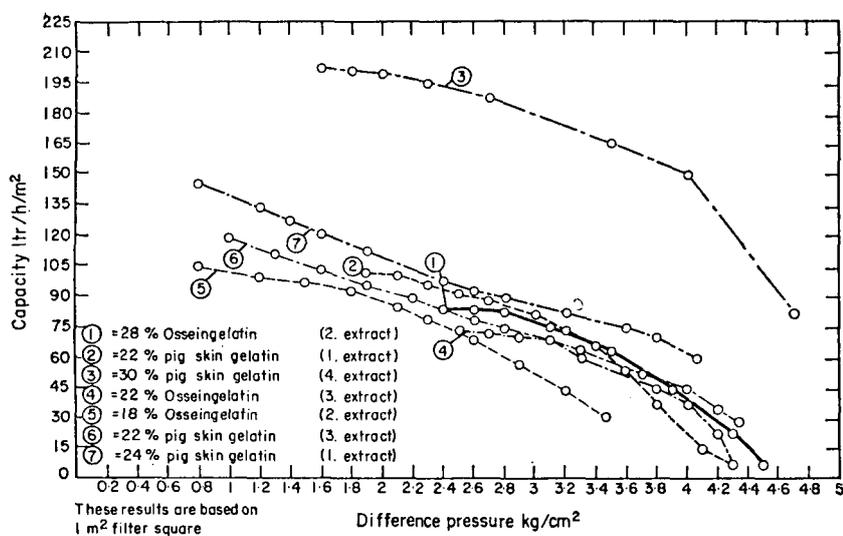


FIG. 3. Filter capacity from high concentrated gelatin.

i.e., to make sure that the permeability of the filter cakes remains sufficient over a high proportion of the period of filtration. Dosage of the filter auxiliary agents must be done exactly. If the dose of auxiliary agent is too small, pressure will rise rapidly and the filter elements will clog too soon. Too large a dosage will result in a slow rise in pressure, but the filter cake formation on the filter elements will take place too rapidly, resulting in increased expense, but as the Figs. 2 and 3 show, accurate dosage is necessary if an extended service life with constant filter efficiency is to be achieved.

In industrial practice the following average values for additional amounts of filter aids have been found:

approx. 1.4 to 1.8 kg filter aid per 100 kg of dry gelatin

These correspond to the following figures for liquors.

Gelatin liquor concentration in %	Filter aid required per 1000 litres of gelatin liquor in kg
5	approx. 0.7 to 0.9 (0.8)
10	approx. 1.4 to 1.8 (1.6)
15	approx. 2.1 to 2.7 (2.4)
20	approx. 3.0 to 3.4 (3.2)
30	approx. 4.4 to 5.2 (4.8)

The filtration of highly concentrated gelatin liquors poses additional problems in estimating the dose of filter aid required. A large number of tests made it evident that dry addition in a separate tank gave better results than so-called "wet dosing". Temperature is of special significance in highly concentrated gelatin liquors. If, for example, in a typical filtration, a 30% gelatin liquor is filtered at 40°C to 50°C, this gives an output of approx. 80 to 90 litres/m²/h. If the temperature is increased to about 60°C to 65°C, then the output is practically doubled, amounting to around 150 to 180 litres/m²/h.

Figure 2 shows the filtering capacity of various dilute gelatin liquors (solids content 4–6%) in relation to the differential pressure, whereas in Fig. 3 curves are plotted showing the filtering capacities for concentrated gelatin liquors (content of solids 18–30%). This demonstrates how the filtering performance is dependent on pressure. Filtering capacity drops as pressure increases. In other words, the filter cake becomes more and more clogged and loses permeability. Filtration of gelatin liquors should begin at as low a pressure as feasible, as the rise in pressure represents a scale of measurement for the drop in performance of the filter cake and thus of filtering capacity.

As soon as the main filtering cycle is complete, the gelatin remaining in the filter and filter aid is removed by means of hot water. So no loss of gelatin will be incurred. This filter cake cleaning is followed by cleaning the filter. More hot water is passed in, as a back flushing operation (see Fig. 1). The entire filtering aid deposit is brought into suspension, and in this manner the deposits from the liquor and the filter aid are removed as sludge. Cleaning takes 5 to 15 minutes, after which the centrifugal self-cleaning filter is again ready for operation.

The main filtration process needs to be followed by fine filtration as the last process in the liquid phase of gelatin production. The fine filtration plant should therefore be installed after the evaporator or the sterilization plant.

Why is fine or polishing filtration necessary at all? In many instances this manipulation could be carried out as part of the main filtration process, if the filter cake is given a different build up. But this would complicate and delay the main filtration, so that the output per filtration operation would be substantially reduced. In industrial practice, filtration may be divided into two processing stages, for economy and to give a better quality in the product. Fine filtration may be performed by means of filtering layers specially developed by SCHENK for gelatin processing. The same basic considerations apply to fine filtration as to the main filtration. As a filter element, once prepared, is often usable for several gelatin liquor filtrations, it is good policy to let hot water run very slowly through the filters during the idle times when it is not filtering, to keep the filters in operating condition.

In summary, the advantages of a kieselguhr filtration as compared with cellulosic filtration are substantial since:

- (1) due to the low price, no regeneration is required
- (2) this eliminates the danger of a possible reinfection of the gelatin liquor
- (3) operation is simpler and more economical.

D. The Evaporation of Gelatin Liquors

Dilute gelatin liquors after passing through the continuously operated filters reach the evaporator where they require rapid processing. As gelatin

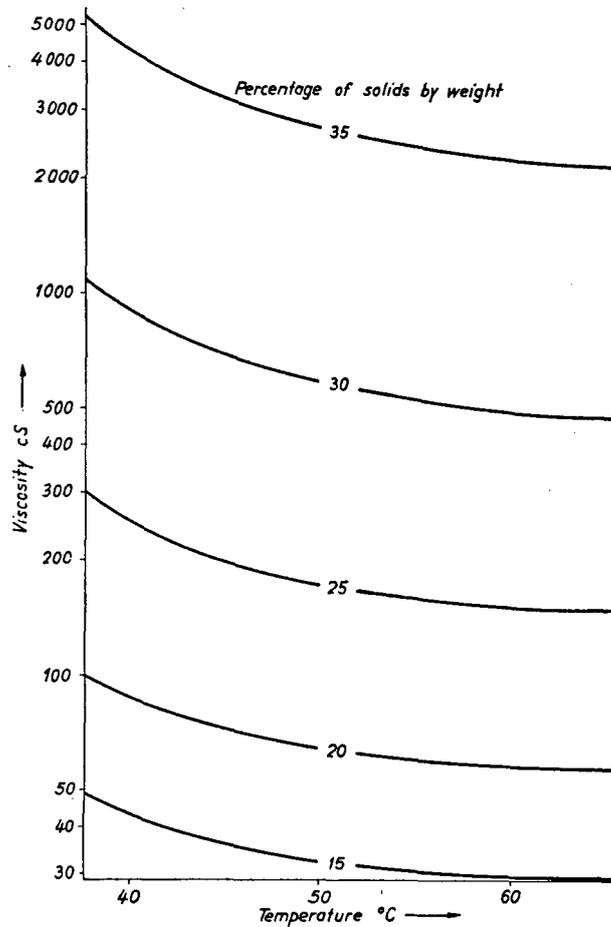


FIG. 4. Dependence of viscosity from solids and temperature.

liquors are thermosensitive fluids, certain basic principles must be observed in the evaporation process. It is also essential to minimize costs, in particular the cost of evaporating 1 kg of water and to keep in mind the difference between this cost and the similar cost in the subsequent drying operation.

As a rough comparison a single effect evaporator uses about 110–120% steam per kg of water evaporation compared with 240–350% of steam per kg of water evaporated in the drying plant. So a gelatin liquor ought to be concentrated as far as possible at the evaporation stage. The limit of such concentration is reached when the concentrated gelatin liquor can just be delivered by a pump or similar means (Fig. 4). The concentration of a gelatin liquor can be divided into three stages, namely:

- (1) preliminary concentration
- (2) main concentration
- (3) finishing stage for high concentration.

A gelatin evaporator must fulfil the following requirements:

- (1) low evaporating temperature
- (2) short contact time of the gelatin solution with the heating surfaces
- (3) suppression of foam formation, especially in the low solid content range of gelatin liquors.

Reduction of the temperature is effected by the application of a vacuum. A short dwell in the evaporator can be realized by continuous evaporation of the gelatin liquor by the evaporator. Suppression of foam formation during evaporation is today achieved in a most satisfactory manner by use of a centrifugal effect.

The application of a vacuum as such will not result in a saving of heat as against an evaporation under atmospheric pressure. Heat economy is a result of the multi-effect principle in which several evaporators are arranged in series so that the steam from the evaporating fluid of the first body heats the second body, etc. Such processing is only possible when, corresponding to the decreasing temperature of the steam from evaporation from stage to stage, the boiling temperature is reduced from body to body. This is possible by adjusting the vacuum. The boiling temperature range for the evaporation of gelatin liquors must lie between 80°C and 40°C. The temperature must be above the melting point of the gelatin and below temperatures at which degradation is rapid. The temperature interval available of 40°C is not large. So only double or triple effect evaporators can be applied, which will require very large heating surfaces. Large heating surfaces, however, mean substantial rinsing losses when changing from one gelatin quality to another. Modern gelatin evaporating plants are designed to obtain high heat transfer coefficients. Only in this way can the desired boiling temperatures be attained with a minimum of total heating area.

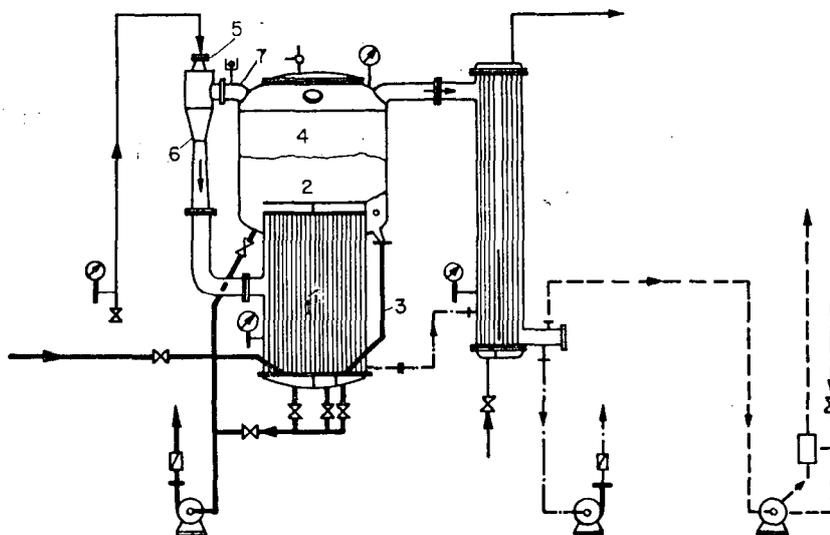


FIG. 5. One-stage vacuum evaporator with vapour compression.

In a multi-stage vacuum evaporator the boiling temperature in the last stage is determined by the efficiency of the condenser, the temperature in the heating chamber of the first body by the pressure of the steam and the other temperatures regulate themselves accordingly. In the idealized case a two-stage evaporator requires half, a three-stage evaporator one third of the quantity of the steam of a single effect evaporator. But these values cannot be obtained in industrial practice, as heat losses must also be considered. A saving in steam can be obtained by use of vapour recompression. But not every type of compressor is suitable for vapour compression. In a very simple manner and at a reasonable cost, vapour recompression can be performed by means of a steam jet vapour compressor. Figure 5 is a schematic representation showing vapour recompression.

The steam passes through the jet (5) into the steam jet vapour compressor (6). Through tube (7) it sucks in a substantial portion of the vapour from the liquid separator (4). In the jet vapour compressor the vapour is brought to a higher pressure: fresh steam and vapour produce a steam mixture that has a higher pressure and a higher temperature than the vapour drawn in and this returns to the heater of the evaporator. In this manner, a portion of the vapour is repeatedly brought into the heating circuit as steam effective in producing evaporation. The total quantity of the vapour cannot be utilized, since part of it must pass to the condenser. This approximately corresponds to the quantity of fresh steam that is continuously required for operating the jet vapour compressor.

Several different types of evaporating systems are applied for the evaporation of gelatin liquors and of these, the following have rendered the best service in industrial practice:

- (a) Tubular evaporator
- (b) Plate evaporator
- (c) Thin layer evaporator
- (d) Centrifugal evaporator

1. *Tubular evaporator*

The circulating evaporator is one of the oldest representatives of the tubular evaporator. The German Wiegand Apparatebau GMBH have developed plant especially suitable for use with gelatins and animal glues. In the tubular evaporator (Fig. 5) the tubes (1) are arranged vertically under the heater (2). The length and the diameter of the tubes are matched to the properties of the product to be evaporated and the operating conditions. The cross-section of the circulation tube (3) that enables the circulation of the boiling fluid in the evaporator must be carefully adjusted. The surprisingly high heat transfer coefficients needing only modest differences in temperature across the tube thickness, are a consequence of this arrangement. As long as the concentration, and hence the viscosity of the liquid to be evaporated, are not too high, it will only fill the bottom of the heating tubes, the middle and top sections of the walls of said tubes will only be covered by a film of liquor. The vapour rising in the tube from the boiling fluid drives the liquid film upwards at high speed. This results in a high rate of heat transfer. Foam bubbles that were formed at the lower sections of the tubes are destroyed, so that the liquid leaves the tubes more or less in drop form and free from foam. The separation of vapour and liquid takes place in the liquid separator (4). The higher the concentration, and with that the viscosity, of the liquid is, the more the tubes will be filled, i.e., the appropriate so-called "apparent liquid level" will adjust itself automatically. To separate the vapour from the liquid the Wiegand evaporator uses a centrifugal separator, as it is the least complicated and most efficient of available systems. The boiling chamber in a Wiegand evaporator is composed of several parts, with the following consequences. Since, in the evaporation of dilute gelatin liquors, a great increase in concentration must be achieved, the final concentration would have to be present in the entire apparatus, if the evaporator were not subdivided into several parts. The coefficient of heat transfer would be at the minimal value corresponding to the final stage at high concentration, whereas the boiling point would have to be at a maximum in the entire apparatus. Thereby evaporation efficiency would be extremely low. With separation into three chambers, where the liquid to be evaporated flows through the three chambers

successively, each provided with a circulation tube, the conditions can be separately chosen in each effect. The highest concentration only occurs in the last chamber. The lower concentrations in the previous chambers give much better heat transfer conditions and lower boiling points, so there is a substantial increase in the average evaporation efficiency of the entire plant. Some progress resulted from the introduction of the falling film evaporator, in which a very small operating liquid content can be evaporated to high concentration in a minimum of processing time. In the normal tubular evaporator, in which the movement of the liquids in the heating tubes is determined by the ascent of the steam bubbles formed, and the fluid rises from bottom to top, the liquid content cannot be decreased beyond a certain point. In the falling film evaporator both the liquid and the vapour flow from top to bottom. It is essential for the satisfactory operation of a falling film evaporator that the liquid feed to the heating tubes be properly designed. But the decisive feature is the radical reduction of the liquid content of the evaporator units. These are practically empty and the flowthrough time is at most one minute. It is important in the falling film principle that as much gelatin liquid as possible is brought into each heating tube, to ensure that the film in all the heating tubes is of the requisite thickness. This is easier to achieve with only a few heating tubes. Thus, long heating tubes must be installed, in order to obtain the necessary heating surface. As has become apparent in industrial practice, single effect falling film evaporators are only of limited serviceability, for the contact interval of the gelatin liquor is too short to ensure a substantial concentration change from evaporation. But where the preconcentration of dilute gelatin liquors is required, the falling film evaporator offered substantial progress in processing technique. The further evaporation of a preconcentrated gelatin liquor can then be performed in the normal circulation tubular evaporator. With the two systems combined into one, economical processing can be ensured.

Figure 6 is an illustration of a three-stage combined falling film-circulating evaporator plant with vapour compression, as is extensively used in the gelatin industry (manufacturer: Wiegand Apparatebau GMBH). The dilute gelatin liquor is delivered from the balance tank by the product pump for supply, through the preheater to the head of the evaporator body stage 1. On entering, the liquor is preheated to the boiling temperature of the first stage. It enters the chamber above the upper ends of the tubes and runs down the heated tubes as a thin film, evaporating violently in the process. The preconcentrated gelatin solution is pumped to stage 2 by the feed pump. The 2nd stage is designed as a circulation evaporator with natural circulation. As a result of the thermo-siphon effect of the generated steam bubbles in the heating tubes, the gelatin solution is delivered upwards and reaches the separator, for separation of vapour from the product. From the 2nd stage the

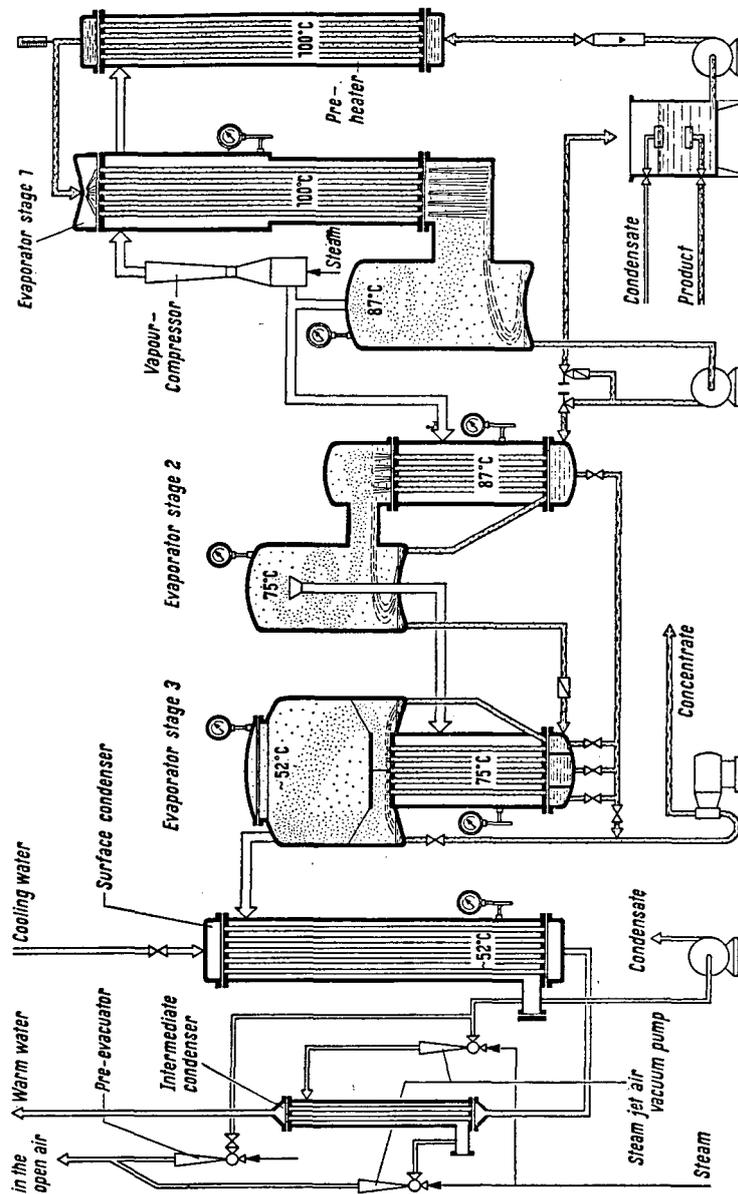


FIG. 6. Three-stage combined falling film/circulating evaporator plant with vapour compression (Wiegand).

product and the vapour pass to the 3rd stage for further concentration. This last stage has three divisions for the gelatin, whereas the heating chamber is not subdivided. The gelatin solution from the 2nd stage passes through 3 separate individual chambers with internal natural circulation until the desired final degree of concentration is reached, whereupon it is removed by a diaphragm pump. The vapour condenses in a cooled surface condenser which is drained by a condensate pump. The entire evaporating process is carried out under a vacuum that increases from stage 1 to stage 3, the vacuum being generated and maintained by a de-aerating plant.

2. Plate evaporator

In contrast to the evaporators equipped with heating tubes, the plate evaporator has its plates as heating surfaces. The development of a uniform film thickness is somewhat easier with the plate evaporator than with the tubular evaporator, because the gelatin liquor is compelled to travel in a prescribed path with a specific cross section. But the danger of scorching and encrusting the plates may be greater, which would prejudice heat transmission. The APV plate evaporator ensures a brief contact interval between the gelatin solution and the heating surfaces and also gives a low evaporating temperature. The development of a special plate for the gelatin evaporator has made it possible to obtain a special action. The effect of this plate is to increase the flow velocity through the evaporator at the final concentrating stage, when the viscosity of the gelatin solution is at its highest. The author has some

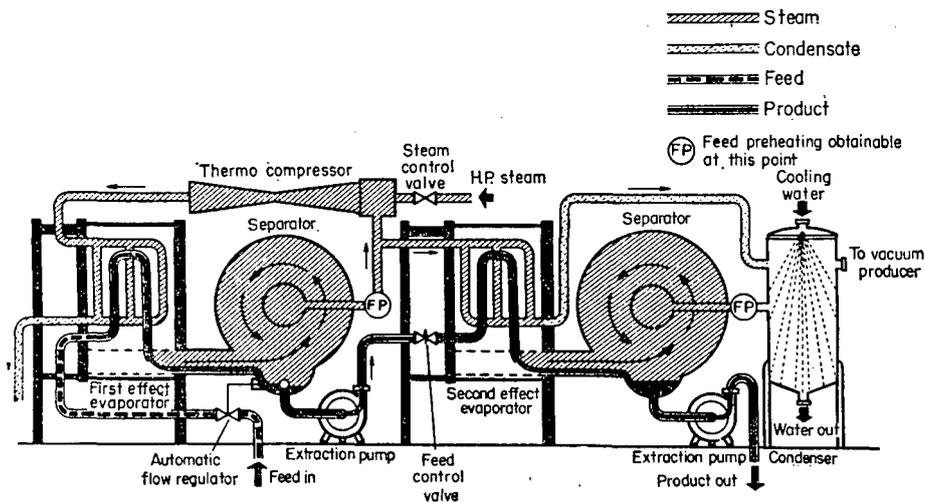


FIG. 7. Flow diagram of a double-effect plate evaporator.

criticism of the simultaneous performance of sterilization in the finishing stage, because the contact interval is too short to kill any spores present.

The plate evaporator can also be of multi-stage design, 2 or 3-stage types being most often used in the gelatin industry. Figure 7 shows a flow diagram of a double effect plate evaporator (The A.P.V. Company Ltd.).

The basic functioning of the plate evaporator is similar to that of the tubular evaporator. The thin gelatin liquor passes through an automatic flow regulator to the 1st stage where it is heated on the plate heating surfaces to produce evaporation and then passes to the separator. Here the liquid and the vapour are separated and the pre-concentrated gelatin solution reaches the 2nd stage. A part of the vapour is mixed with fresh steam and passes to the thermocompressor for recompression. This system can, thus, also be equipped with vapour recompression, to keep steam consumption within economic limits.

The Daniels-Parkson evaporator is also based on the plate system. In this patented system⁽⁴⁸⁾ a small volume of liquid is heated on a relatively large heat transfer surface. The continuously generated and expanding steam which results gives the small volume of liquid an extremely high velocity and turbulence. The viscosity of the gelatin solution is effectively reduced by this high velocity and turbulence in the presence of steam, so improving heat transfer.

The efficiency is often compared with that of a molecular still. The heating surfaces are corrugated plates that produce a high degree of turbulence so eliminating local overconcentration and overheating. A low temperature continuously operating evaporating plant is available from Parkson Industrial Equipment Co. Ltd.

3. *Evaporator finisher*

As already stated above, the trend today is to obtain the highest possible ultimate concentration by evaporation of the gelatin solutions. But as heat transmission to the product worsens with increasing concentration, so that evaporation diminishes, better forms of processing technique are needed. This was not possible with the traditional evaporators because they were liable to damage the gelatin properties too extensively.

One of the first serviceable machines developed for this final evaporation was the thin film evaporator (manufacturer: Luwa AG., Zurich). Figure 8 illustrates the mode of operation and the construction of such a plant. The concentrated gelatin liquor is fed from above the heating jacket (2) and distributed over the periphery. The impellers of the rotor (6) rapidly spread it as a thin film, with great turbulence, over the heated wall, from where the gelatin solution is gravity-fed downwards with simultaneous intense

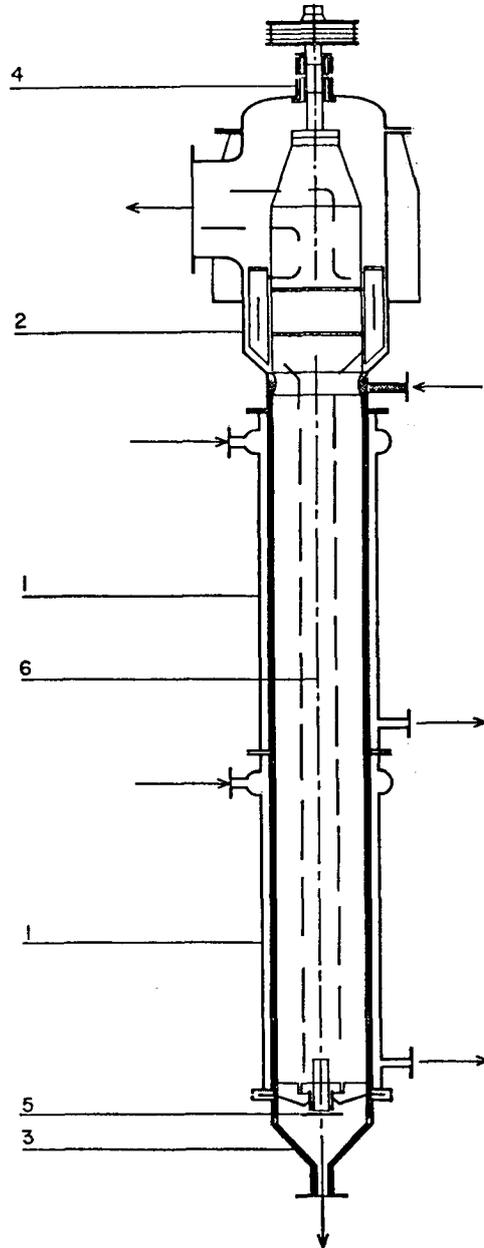


FIG. 8. LUWA-thin film evaporator.

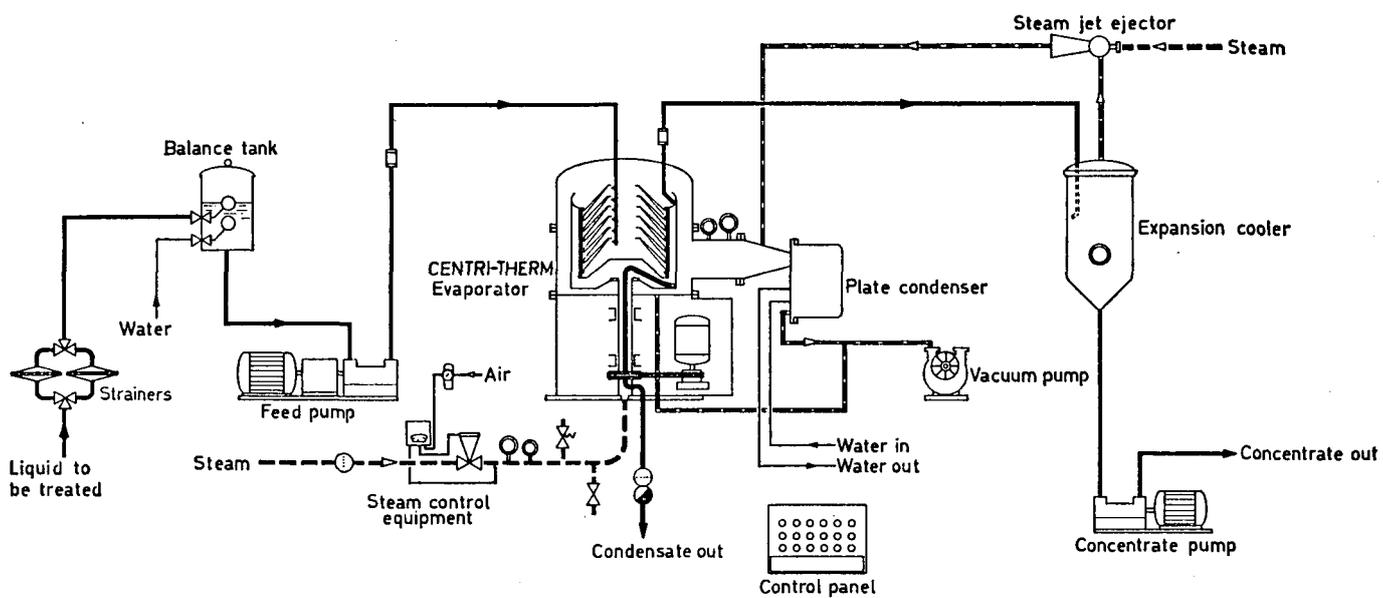


Fig. 9. Centri-therm effect.

evaporation. The heating jacket supplies the necessary heat. The vapours developed pass upwards through the apparatus, countercurrently, and pass the separator (1, 2).

The low liquid content in the evaporator results in a short dwell interval and, together with the high turbulence in the liquid film, it prevents any local overheating of the product. Here, too, as in the Parkson evaporator, turbulence is applied to obtain higher concentration.

Alfa Laval have employed an interesting approach with their Centritherm. The operating principle is a heating surface which consists of a rotating truncated cone whose inner surface is in contact with the product side and the outer surface with the steam. In addition there are separating walls, rotating gaskets and tube connections for the product and the steam. The cone requires an appropriate drive system. Figure 9 illustrates the Centritherm principle. The fluid is uniformly distributed over the interior of the heating surface and is driven by centrifugal force to form a thin layer towards the periphery. For concentrated gelatin liquors the layer is about 0.1 to 0.2 mm thick and the time of passage over the heated areas is about 0.5 to 2 seconds.

With the falling film evaporator as a starting point Wiegand developed a winding tube evaporator. Here the heating tubes are helically coiled and

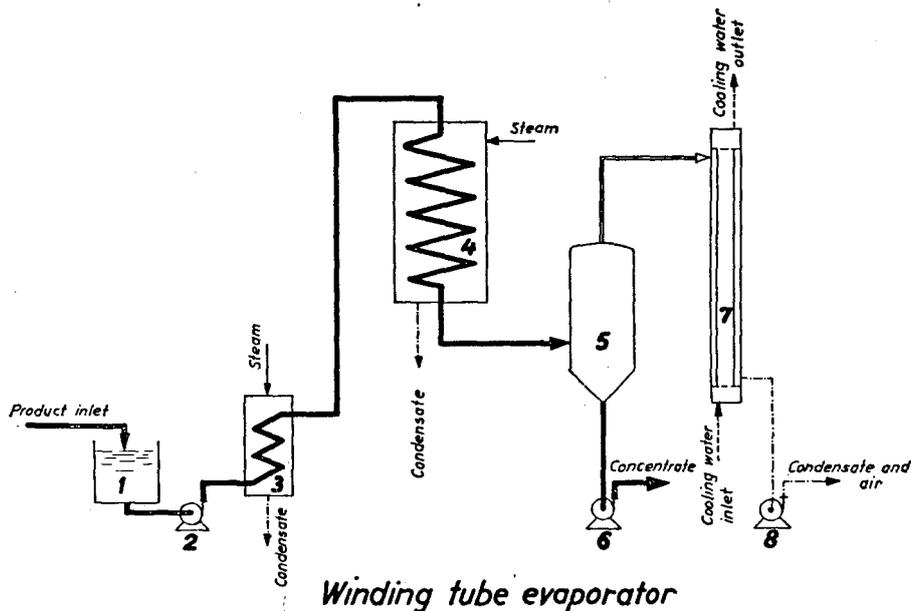


FIG. 10. Winding tube evaporator.

are arranged about a vertical axis. In comparison with the falling film evaporator, this makes a greater length of tube possible. The gelatin liquor also flows from top to bottom in this case. On the flow along the axis of a spirally shaped tubular coil is superimposed a secondary flow. The movement of the liquid film is decisively improved, so that good heat transmission is achieved, even with high viscosities. Figure 10 illustrates the principles of the winding tube evaporator. The APV paraflow plate heat exchanger which is especially designed as a finishing stage evaporator, to give a high final gelatin concentration, also deserves mention.

4. Technical Aspects and Economy

The question of which evaporating system or combinations to install in any production plant, depends on a number of factors. But one fact is very apparent, that the removal of water from gelatin is one of the most difficult problems encountered in evaporation technology, because gelatin has all those characteristics which normally make it unsuitable for such treatment. For this reason, only fully evaporating systems should be applied and caution should be exercised in the adoption of untried novelties.

The concentrations which in practice are attained with different evaporators depend on the mode of operation of the evaporator and on the quality and type of gelatin. Depending on the actual type of gelatin, the limitations on the concentration that can be achieved with the use of the finishing stage evaporators, are usually determined by the availability of a suitable extraction pump to handle the high viscosity liquor. The concentrations to be aimed at with the several complete evaporating systems also vary. Table IV lists tentative data for the several systems.

Steam consumption depends mainly on whether 1-, 2-, or 3-stage vacuum evaporators are applied and whether they operate simultaneously with vapour compression.

TABLE IV. Steam consumption per kg evaporated water

Steam per kg evaporated water	Without vapour compression	With vapour compression
1-stage Evaporator	1.2-1.4 kg	1.1-1.2 kg
2-stage Evaporator	0.6-0.9 kg	0.5-0.7 kg
3-stage Evaporator	0.4-0.5 kg	0.3-0.4 kg

E. Sterilization

Gelatin is a well known component in culture media in bacteriology, which depends upon its susceptibility to attack by introduced micro-organisms. This makes the sterilization of gelatin an important consideration in gelatin processing. The sterilization of gelatin is not however a purely nominal asset in a specification; the advantages of sterilization as a means of improving quality must be emphasized. We must strongly oppose the view that all problems of plant hygiene can be solved by a good terminal sterilization process. The sterilization of gelatins, especially gelatins for the pharmaceutical and food sectors, should only be regarded as an additional safety measure to counteract the spread of micro-organisms, above all, the spore forming type.

Techniques listed for materials generally under the term "Sterilization Processes" cannot be industrially applied to proteins such as the gelatins. Heating the solution for 8 to 10 minutes to 120–134°C in an autoclave is not practical for use with large liquor volumes and, in addition would cause severe degradation.

Gerngross⁽⁴⁹⁾ found that the drop in viscosity and the change in gel forming power in the initial phase of thermal degradation are not both linked to hydrolysis of the polypeptide chains. In more recent investigations Saunders and Ward⁽⁵⁰⁾ point out that conditions (pH, enzymes) employed influence the mode of thermal action, and the relative reduction in molecular weight or in the gelation factor^(51, 52) has confirmed these assumptions. The work of Ames⁽⁵³⁾ showed minimum viscosity breakdown occurs at neutral pH's (see Chapter 11).

There is little margin between the conditions for sterilization, as required by bacteriology, and the thermostability of the gelatins. A number of processes have become known in recent years for producing sterile or near sterile gelatins. Regulations prohibiting the addition of preservatives to food gelatins in a number of countries have increased the value of a satisfactory physical method.

Alle⁽⁵⁴⁾ attempted sterilization of food gelatins with ultraviolet radiation while the liquor was in turbulent flow. But the attempted penetration sterilization was only a prophylactic secondary sterilization. Alle showed that if the most abundant micro-organisms occurring in gelatins, including spore forming types, are exposed to repeated ultraviolet irradiation, then some of them are found to be resistant to the treatment as a result of mutation.⁽⁵⁵⁾ Thus only a bleaching effect and not sterilization is obtained.

Apart from sterilization in solution, a hot air sterilization method has been proposed for dry gelatins. The method of the Chemische Fabrik Roehrig⁽⁵⁶⁾ can be applied to commercial types of dry gelatin. The gelatin is heated for

several hours in a dry air current up to about 95°C and is subsequently sterilized at a temperature of 120°C to 125°C. This method eliminates virtually all the normal water content of the gelatins. So the gelatin has to be reconditioned to 10–15% water content, under sterile conditions, so as to be returned to its commercially customary water content. In industrial practice the Roehrig method has proved unsuitable, because the processing techniques involved are too time consuming, costly and complicated. The physico-chemical properties were also modified to a significant extent by the treatment.

Hot air sterilization after drying is still of some practical significance today. It can best be carried out in the batch drier, the dry gelatin being heated in an air current to 120°C to 130°C for an hour. The high steam consumption increases production costs considerably. Another disadvantage is that the water content drops below 5% and the characteristic swelling properties of gelatin are reduced. This can readily be detected. In its last drying zone, with a temperature of 90°C, the HAAG fluidized bed dryer produces a partial sterilization. This temperature is not high enough to kill spore forming bacteria, but it is sufficient to reduce the swelling properties of the gelatin. This may in part arise from the formation of crosslinks rather than be purely a result of dehydration.

R. Hinterwaldner and G. Elovsson⁽⁵⁷⁾ have developed a process for the continuous sterilization of gelatin liquors. They made the interesting observation that by indirect short-interval heating, e.g., in a plate heat exchanger, the process was defective both in bacteriological action and in causing a drop in the physico-chemical properties. Heavy incrustations form on the plates, especially when processing highly concentrated gelatin liquors, was the cause. This reduces heat transfer and so the contact time for sterilization is substantially extended. As a further approach to continuous sterilization, the Alfa-Laval-Vtis^(68, 59) high temperature short time system for milk was applied. This sterilizer for gelatin works using direct steam injection (Fig. 11). The gelatin liquor is fed to a float hopper from which it is passed by a centrifugal pump to a plate heat exchanger (5) and there preheated to about 75°C. A high-pressure pump (6) now forces the liquor through the steam injector head (7) into a holding tube (8). The injection of live steam instantly raises its temperature to 140–142°C, and it is retained at this temperature in the holding tube for 4 to 6 seconds. The liquor issues from the tube into a vacuum vessel, where the original concentration is restored by the flashing off of water vapour, while at the same time the liquor is cooled by expansion. The aseptic centrifugal pump (10) passes the product to a plate type cooler (1), where the temperature of the gelatin liquor reduces to about 40–50°C. The vapour from the vacuum vessel (9) is condensed in a plate heat exchanger (3, 4).

The gelatin sterilizer can be inserted at any stage of the manufacturing process between extraction and drying, but for maximum economy and

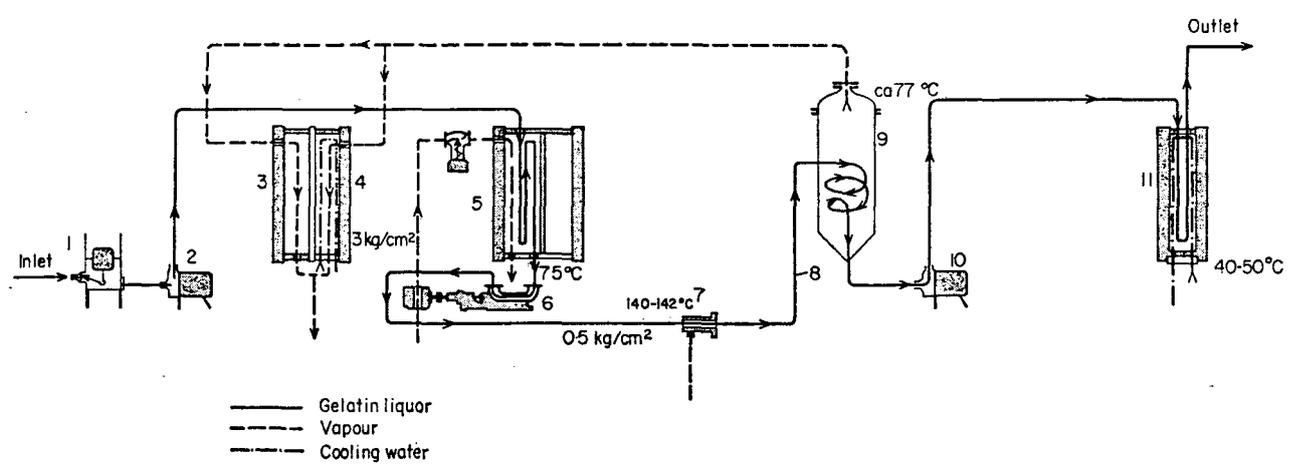


Fig. 11 Flow-diagram of gelatin sterilization.

TABLE V. Bacteriological analysis of an edible gelatin before and after sterilization

Edible gelatin	Total bacteria per g. Temp. 30°C	B. Coli per g. Temp. 37°C	Gelatin liquefier per g. Temp. 25°C	Anaerobes total per g. Temp. 37°C	Part of Clostridium per g. Temp. 37°C	Part of anaerobe spores per g. Temp. 55°C
Before sterilization	1600	0	11	45	10	40
After sterilization	0	0	0	0	0	0

minimum risk of reinfection it is best placed after the last stage of liquid phase treatment, i.e., between the final concentration and the chilling section. The rate of product flow through the sterilizer can be matched either to the capacity of the following chilling section, in which the liquor gels, or to the rate of feed of concentrated liquor. Preheating the gelatin liquor from 50–60°C to 75°C is necessary to avoid an unduly large interval in reaching the sterilization temperature of 140–142°C.

Since the sterilizer must be resterilized after every shut-down or interruption in the flow of product, it is advisable—especially for large-scale production—to regulate the feed of gelatin liquor in a continuous uninterrupted flow to the sterilizer for a full working week. Sterility of the gelatin liquor is maintained up to the aseptic centrifugal pump.

For a good sterilization effect the gelatin liquor must be rapidly heated by live steam. About 4 seconds are required to reach a temperature of 140–142°C. If this interval is extended to more than 20 seconds, then losses in the quality of the gelatin can be detected. With only 4–6 seconds heating no noticeable loss of physico-chemical properties such as jelly strength, viscosity, etc. occurs. The change in concentration is negligible as it is less than 0.5%.

The cost of this method relates to the volume throughput and not to gelatin concentration, so emphasizing the advantage of using concentrated liquors. Steam requirements lie between 10–12% of steam at 113.6 psig per 100 litres of gelatin liquor.

Gelatin liquors with around 30% solid content can readily be sterilized by this process. The main risk of lack of sterility rests on post sterilization recontaminations. This will not normally involve contamination with spores.

F. Drying

For most practical purposes, dry gelatin is obtained from concentrated sols by cooling to the solid gelatin gel and drying the gel.

Elimination of water from the liquid gelatin solution can only be achieved by spray drying or roller drying. But, in comparison with other protein containing liquids, it is possible to exploit the gelling power of gelatin to facilitate drying. For this reason drying processes today utilize the sol-gel transformation. In spray drying, the warm solution is dried directly. Heat economy of such a system would be more favourable than in the gel drying systems. However, spray drying has not succeeded in reaching widespread commercial use in the gelatin industry, for the reasons explained below:

- (a) significant degradation of the gelatin occurs
- (b) it results in a fine, light powder having a very low bulk weight and very large packaging volumes
- (c) it gives a very large specific surface area for the fine gelatin powder.

Since gelatin powder has to be re-wetted for most uses, the very large specific surface of the gelatin particles causes the water adsorption to be irregular. Unmoistened particles are left which hydrate slowly, so swelling is also irregular. When melting such a batch, lumps are formed in the gelatin solution which are hard to dissolve or do not dissolve at all.

Roller driers have not become popular as means of drying gelatin solutions, because of the danger of the dried substances suffering damage due to overheating on the metal surfaces. The strong foaming of the gelatin solution on the heated rollers is also a disadvantage. The form in which the roller dried gelatin is delivered, with a large specific surface, resulted in the rejection of this method of drying gelatin. A further interesting method is freeze drying. This can enable the instantly resolvable "sol" configuration of the gelatin molecule to be obtained in the dry state. The cost is high and can only be justified where the properties are essential.

1. *Fundamentals of gel drying*

The elimination of water from the gels is not effected as easily as with some other drying systems. The conditions of the step-by-step elimination of water are represented in the curve showing isothermal dehydration (Fig. 12).⁽⁶¹⁾

This curve makes evident two separate regions. The main quantity of the water (A to B) can be eliminated by only a minor reduction of water vapour pressure, the drying air being able to have a relatively high moisture content. This continues until the gel has a dry matter content of about 60-70%. The elimination of the remaining quantity of water (B to C) calls for a substantial progressive reduction of the water vapour pressure. In industrial practice, such reduction of vapour pressure of the drying air is not possible in the normal driers, so a water content of 10% is the minimum to be aimed at. These considerations imply an equilibrium between gel and drying air which would apply only to a fine powder through which the air is passing. In

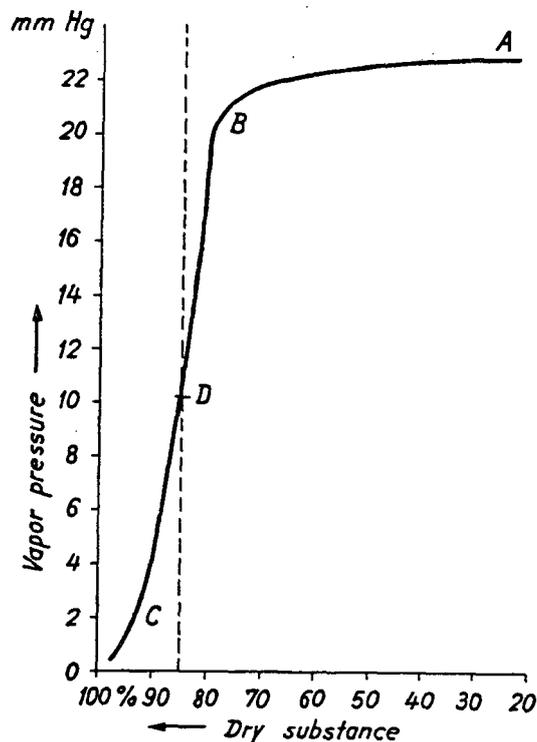


FIG. 12. Progress of water elimination of the gelatin gel.

practice the transport of water to the surface of the drying portions of gel constitutes the limiting factor in the later stages of drying. A further difficulty in drying gels has its origin in the effect of temperature on the gels. In normal drying practice water removal proceeds all the more rapidly the higher the temperature of the drying air is, because of higher rates of heat and moisture transfer and increased water absorbing capacity of the dry air. Gelatin gels represent a difficult case because, above all in the initial stages of drying, drying is not possible at too high a temperature, as the gel will liquefy. Advantage can be taken of the fact that in the early stages of drying, when the gel surface behaves essentially as a free water surface, the gel temperature approximates to the wet bulb temperature of the drying air, which will be very much below the actual air temperature. This is the "constant rate" stage of the drying. When this phase is over and the falling rate drying period takes over, the temperature of the more concentrated, partially dried gel rises towards the drying air temperature, reaching it as drying ceases and the water vapour pressure in the air is that of the dry gelatin.

As was said above, the rate of drying of gelatin gels depends on the evaporation of the water from its surface and its removal; then, in the later stages, on the speed with which the water migrates from the interior of the gel to the surface. The study of the swelling processes in gelatin gels shows that the rate at which water moves within very concentrated gels is limited and there is no actual porous structure, with sizeable pores remaining in the dry product. In the drying process it is at first only the so called "free" water that is eliminated. There is a tendency to form a sheath of partly dried gelatin round the particles ("case hardening"). As a consequence of the peculiar construction of the gels and the manner in which these gels bind water, there is only a relatively slow equalization of water distribution at this stage inside the gel. Once the free water is eliminated from the gel, the drying rate decreases sharply. Sheath formation and the raised gelatin concentration enables the gel to be exposed to higher temperatures without melting. Advantage can be taken of this in designing the drying process. The advantage of subdividing the gel to shorten the path from interior to surface will be evident.

Sauer and Veitinger⁽⁶²⁾ using a sheet of gel 2 mm thick, with 10% solids content, determined the course of weight reduction during drying in an air current at constant temperature. The air velocity was 1.5 litre/cm²/minute and the temperature was kept constant at 24°C. Figure 13 is a diagram showing the rate of water removal. This clearly shows the initial constant rate period during which the water loss is linear with time and the transition to the falling rate period. Similar tests made under otherwise identical conditions but with different temperatures, give curves with different gradients corresponding to different drying rates. The maximum usable air temperature must be such that the wet bulb temperature is below the gel melting point.

The size and thickness of the gel layers is critical in deciding the drying rate. In order to study the influence of layer thickness on drying speeds Sauer and Veitinger⁽⁶²⁾ performed a series of drying experiments with gel sheets of increasing thicknesses (from 4–16 mm) from a gelatin solution with 10% solids content. The tests were done with air at 18°C and a low air velocity, so that very long drying times were required. Under these conditions, which bear no relation to commercial drying, the drying rate is the same for all layer thicknesses, as shown by the parallel course of the curves.

There is no literature on detailed experimental investigations of the course of drying gelatin gels with different particle size and shape. Nevertheless, gel crumbs and gel leaflets will dry more rapidly and easily than sheets because they have greater specific surfaces and smaller paths for moisture to diffuse to the surface. The specific surface of gel crumbs is larger than that of the gel leaflets or gel noodles.

By analogy with the results and observations of Kamei⁽⁶³⁾ and Krischer-

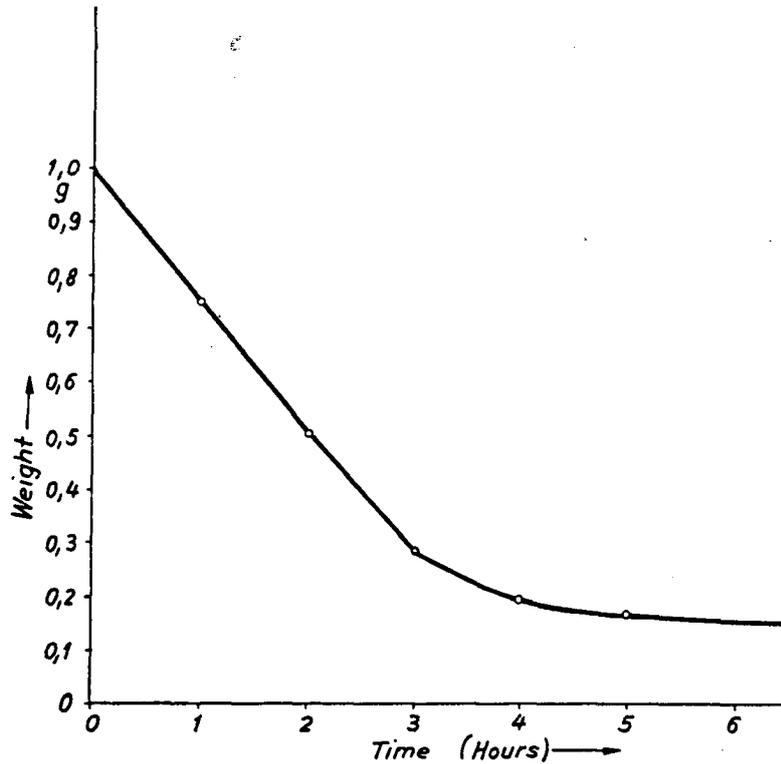


FIG. 13. Progress of the water elimination of a gelatin jelly in an air current of constant temperature.

Kroll⁽⁶⁴⁾ on soap gels, then we would expect that the drying time increases with the square of the thickness of the gel being dried.

2. Industrial drying of gelatins

For many years gelatin was dried in sheets. This form was copied from the glue boilers and was still used by some modern glue manufacturers until very recently. The gelatin solution was poured into a galvanized container. These containers were placed in cooling tanks where they were surrounded by water on all sides, to chill them, so that the gelatin solution set to a gel. The gelatin gels were cut into sheets that were laid on nets and dried in the open air or in drying tunnels. The cylindrical drying kiln appeared on the market, developed by Streidl, Munich, that was loaded with small gel particles, the gel crumbs. An agitator helps to keep the crumbs separate.

These drying systems were in use well into the 1950's and in some cases are

still in operation today, but they only have limited value now, in relation to modern manufacture.

Chilling gelatin solutions in galvanized containers and cutting sheets is not only a labour intensive operation, it is also unhygienic and takes a total of 16 to 24 hours. Even if the galvanized containers are replaced by stainless steel, the corners are still difficult to keep clean. The cutting and spreading of the gel sheets and stacking on drying nets by hand gives rise to many sources of infection (see "Hygiene in the gelatin factory" p. 361).

Control problems and the long period for the final stages of drying sheets of normal thickness make the tunnel drying process costly, steam wasting and time consuming. The cylindrical kiln process also involves heat wastage. In warm countries, severe difficulties with gel melting arises. While stainless steel band chillers reduced hand labour and were used extensively with tunnel driers the defects of the tunnel technique still applied.

Economical labour saving techniques are widely used for chilling, subdividing the gel and drying the particles produced in the modern industrial process.

3. *Chilling*

There are several systems for cooling or chilling the concentrated and prepared gelatin liquors in order to obtain rapidly a firm gel. Chilling rollers, chilling bands and, more recently, the Votator are widely used. All these systems are based on rapid chilling of the product.

One of the best known chilling devices is the patented Sandvik band cooler that can be applied both for the production of small gel particles as well as for thin slices. The gelling time depends on the difference in temperature between the chilling band and the gelatin concentrate as well as the thickness of the film of gelatin concentrate, factors that determine the length and the speed of the chilling band. The chilling of the seamless band is achieved with cooling water or cooling brine.

The gelatin liquor is fed onto the band by means of an overflow or weir tank onto the stainless band, which is fitted with rubber edges to prevent the gelatin liquor from flowing off the band while it is still liquid. If an 880 mm wide band is used, the resulting useful width becomes about 720 mm. Sometimes the gelatin liquor is precooled prior to pouring on the band. Thus the loading temperature can vary by 15–30°C, to a maximum of about 32°C. As the band moves along it passes over the water tanks constantly fed with cooling water with a slight excess pressure, so that the steel band actually flows or glides on the water surface. This improves the heat transfer between the band and the coolant. Figure 14 shows the mode of operation of the chilling band. The capacity of such a band is adjustable, from 1000 to 3000 litres per hour.

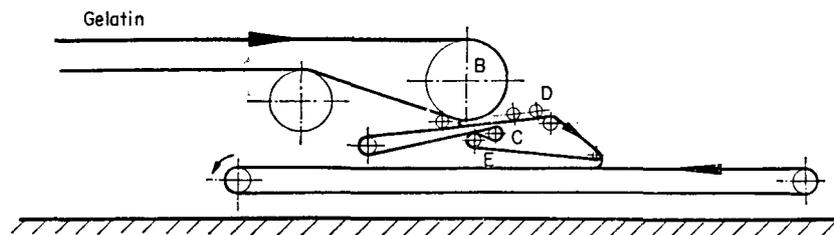


FIG. 14. Sandvik—chilling band.

A chilling drum operates on a similar principle. Here only cooling brine can be used because the chilling length—and thus the distance in which gelling must occur—is substantially shorter. The temperature of the chilling drum fluctuates between $+2^{\circ}\text{C}$ and $+4^{\circ}\text{C}$. The concentrated gelatin liquor flows on a slowly rotating chilling drum upon which a gel sheet of 2–4 mm thickness forms on the drum surface. The advantage of the chilling drum is that considerably less space is required for installation than for the chilling belt.

The gel band formed on the chilling band or chilling drum is then reduced in size. The gel band is peeled off the band or drum by a sharp-edged knife and is led directly to the cutting machine. The reduction is performed either by a mincing machine, which produces crumbs, or the sheet from the band or cylinder is cut down to mini sheets. An interesting device is the mini-sheet cutting machine made by Weiss-Trocknungs-Anlagen, Munich, in which the knife roller equipped with several circularly-shaped blades, intermeshes with a single roller blade that moves crosswise back and forth.

A different and very interesting processing method is applied by the Votator, because this simultaneously chills the liquor and extrudes the concentrated gel. During its passage through the Votator the fluid is progressively cooled and reaches a condition that may be described as supercooled, i.e. gel formation is immanent. In this supercooled state the product enters the gelling section attached to the outlet of the Votator cooling unit. In this section the material sets to a gel. The pressure generated by the feed pump extrudes the gel through a die plate in the form of noodles. During the cooling process build-up of gelatin on the heat exchanger wall is prevented by the action of the rotating scraper blades. The noodles are not perfect cylinders of gel but contain partial fractures creating internal surfaces during drying. With the Votator, the gelatin liquor enters the cooling unit at a temperature of about 45°C and emerges in a chilled condition at a temperature in the range of $15\text{--}20^{\circ}\text{C}$, depending on concentration and quality. To date it is possible to process highly concentrated gelatin solution (up to 40–50% for low grade gelatins) in the Votator plant; indeed this is its merit since a high degree

of water loss is achieved in the evaporation stage at less cost than in the drier. Glues of below 200 g double Bloom can be processed at even higher concentrations. The noodles are gravity fed by their own weight on to the drier feed conveyor. It is also possible to extrude the gelatin with a square cross-section. A cutting machine can be fitted to the end of the plate to produce small cubes. The chilling unit operates with an ammonia or Freon system, but brine is equally suitable.

Chilling bands or chilling drums are applied in the production of very thin gelatin sheets. A few firms have specialized in this form, considered economical such as Chemische Werke Stoess, Eberbach/Neckar, West Germany. Figure 14 is an illustration of thin sheet production. After discharge at the end of the band over the terminal drum (B) the gelatin is transferred to a small rubber band conveyor (C) on which it is cut to pieces (D). These are loaded on to wire mesh trays located on a slat conveyor (E), each tray containing 15 pieces of gelatin. At the end of the slat conveyor the trays are transported (or conveyed) into the drier.

4. *Drying plant*

A number of systems have been applied in drying gelatin, but two types have dominated the market. In recent times a third type has been added, which also has prospects of being successfully applied for drying gelatin.

These three types of driers differ in mode of operation and the manner in which they handle the gelatin. They are the

- batch drier
- band drier
- fluidized bed driers.

Batch driers

As already expressed in its name, the batch drier operates discontinuously. It is applied where the individual gelatin batches are to be dried separately and is best suited for small and medium production capacities. The standard line of batch driers Q 5 Ch built by Weiss-Trocknungs-Anlagen, Munich, has been developed along the lines of unit construction principles which is especially advantageous for an expanding gelatin production.

The capacity of the standard type is such that 1000 kg dry weight of gelatin can be dried in 24 hours. This is based on the following liquor concentrations: 1st extract: 18% solids, 2nd extract: 25% solids, 3rd extract: 35% solids.

The batch drier operates with hot air on the transverse flow principle, in a rotating drum with a cylindrical jacket that is permeable to air. The standard production line of the Weiss-Trocknungs-Anlagen is composed of three sections: the chilling drum (1), the patented square chip cutter (2), the drier (3).

With a single chilling and chip cutting setup several batch driers can be operated and the output enlarged stepwise by each drier capacity. The concentrated gelatin liquor's gel on the surface of the chilling drum to a gel film that is cut to gelatin chips, $6 \times 4 \times 2.5$ mm thick. A screw conveyor transports the chips to a container.

Each drier can be charged about every 7–8 hours. The drying process is automatically regulated over a total drying period of about 6–7 hours. The drying temperatures rise from the initial drying zone ($38\text{--}40^\circ\text{C}$) to 70°C in the last drying zone. This is followed by sterilizing the dried material at $80\text{--}90^\circ\text{C}$ and then cooled. The rotation of the drum keeps the material in constant motion. Discharge is by dropping the dry gelatin into a collection trough from which it is pneumatically transported to storage. After every drying process the interior of the drum is washed out with cold and hot water. Figure 15 illustrates the course of drying an 18% gelatin gel in the Weiss batch drier. The material being dried can be observed during the entire drying process through the transparent front of the drum. The batch drier needs only limited space. Further advantages are (i) automatic operation (ii) good steam consumption figures, i.e. 2–3 kg of steam per kg of evaporated water. In very hot countries the drying air must be conditioned to reduce its humidity. In countries such as Spain and Italy the batch drier can operate without air conditioning. The dried square chips are transparent and without flaws, in contrast to the products of other drying systems.

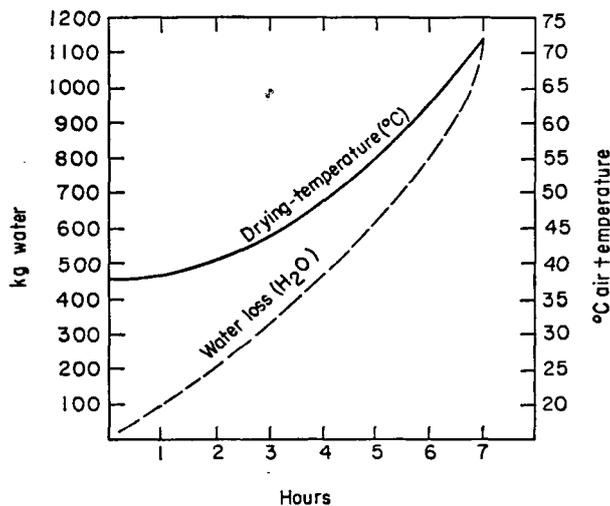


FIG. 15. Diagram of the batch dryer.

5. *Band drier*

Among the band driers, the one built by Gebr. Van Swaay, Den Haag Holland, under a U.S. licence, has given good service in industrial practice. The band itself is made up of several drying sections. The bottom of the drying sections is made from material through which air can freely pass. The band travels through a tunnel. It is arranged in such a manner that it passes through the initial drying zone at a slower speed than the later drying zones. The drying of gelatin gels in this drying system is best done using conditioned air, for which the Kathabar system has proved suitable. An interesting feature is that, in contrast to other systems, the air conditioning is achieved directly, so that no overcooling and reheating are required.

The conditioning of the drying air for the band drier is one of the prime requisites for drying gelatin gels. This also makes it possible to keep the drying time under 4 hours, depending on the quality and concentration of the material. Drying in the successive stages involves increases in air temperatures. In each stage the temperature and humidity of the drying air are fully regulated.

A Votator is preferably set up in front of the band drier to produce the gel. The noodles extruded by the Votator are brought onto the drying zones of the 1st drying band by means of a small reciprocating conveyor. The 1st drier band carries the gelatin gel through the initial drying zone and the noodles then drop onto the 2nd drying band. From this 2nd band the material is passed, fully dry, directly to a hammer mill where the gelatin noodles are broken down. This type of band drier is also used for the production of thin film gelatins.

The band drier of this type with the smallest capacity has an output of around 250 kg per hour of dry gelatin. This shows that such a drying plant is only suitable for large production capacities. As the separation of the various extracts may be difficult on a continuously operating drier, a throughput involving large quantities per extraction is essential. This band drier-system has prevailed on the world market, because gels of edible gelatin, pharmaceutical and photographic gelatins can be dehydrated in a germ-free milieu throughout the year under constant conditions.

6. *Fluidized bed driers*

A long period of experimenting with fluidized bed driers was necessary before useful results were obtained, enabling them to be used commercially. The latest machine of this type, the Haag fluidized bed drier (Fig. 16) built by Eugen Haag Apparatebau in Vaihingen/Enz, West Germany, has gained a foothold in the gelatin industry. Unfortunately no technical results are yet available to show the influence of the fluidized bed on the drying of gelatin gels. It is, however, known that the drying rate can be increased by fluidization,

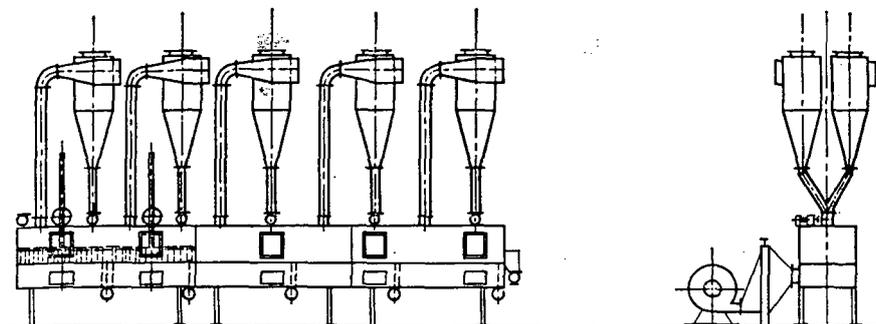


FIG. 16. Floating goods dryer.

because the air flow simultaneously agitates the bed and increases the heat and mass transfer coefficients. Air circulates from bottom to top. With this form of circulation, either the drying capacity can be increased or the drying rate. The drying time required in the Haag fluidized bed drier can be about 2 hours which is short compared with other systems. It is applicable to gel crumbs. The drier consists of 6 zones of which 3 are for initial drying and 3 for finish drying. The small gel chips are delivered to the 1st agitating zone by an impeller wheel. There they are distributed by an extra agitator and stimulated to fluidization. Over an overflow barrier the dry material is conveyed to two drying zones. The drying temperatures increase from zone to zone. Minute gelatin particles entrained by the air current are separated out by cyclones and returned to the products being dried as they pass over sluices at the beginning of the drying zone. The dried gelatin is delivered over an impeller wheel when finished.

The first three drying zones are arranged as agitating zones, in order to bring the still quite moist gel particles into turbulence. The three finish-drying zones are each equipped with an out of balance motor which creates a fluidization effect by vibrating the bottoms of the drier. This new type of drier has good prospects of being applied in the drying of gelatins because it makes possible continuous operation, even for small capacities. The short processing time for drying, the minimal space requirements and simplicity of operation are valuable features.

Any drier system for gelatins should be judged by the following:

- (a) no damage to the gelatins being dried
- (b) simplicity of operation
- (c) low operating and maintenance costs
- (d) magnitude of capital investments required
- (e) possibility to reduce the total operating cost.

The prime requisite for any gelatin drier is careful control. Today the drying processes can be programmed to simplify operation. For steam and power requirements a rule of thumb estimate is that about 3 kg of steam are required per kilogramme of water to be evaporated. Drying requires the 3- to 6-fold quantities of steam, to eliminate the same quantity of water, as an efficient evaporator. So highly concentrated gelatin liquors for drying are desirable. Practical limits arise in evaporation and in handling concentrated liquors. It is necessary to utilize to the full the heat in the drying air of a gelatin drier, so making recirculation desirable. By this means the steam required per kilo of evaporated water can be reduced to perhaps 2 kg. If conditioned air is used, then this figure is easier to attain than with outside air. The Weiss-Trocknungs-Anlagen plants can now reach these values even without air conditioning.

G. Grinding and Sifting of Gelatin

The value of gelatin in its various uses depends in part upon its particle size range. Gelatin dried as "minisheets" or in some other form has to be reduced to different degrees of particle size. The range of sizes extends from relatively coarse-grained particles through fine particles to powder. If the gelatin has to be transformed into sizeable granules or a finer grist, an additional requirement involved is the uniformity, with a minimal undersize or dust proportion. Different size reducing systems may be required, even in combination with screening or air classifying. (sifting).

The grindability of a material is, in the first line, decided by two factors, viz. the hardness or the toughness of the gelatin, and its thermal response. The brittle character of dry gelatin allows it to be subjected to mechanical impact grinding. For coarse breaking aiming at a large grain size with maximum uniformity, cutting mill type machines are used, characterized by rotation and stationary knives, positioned so as to be axially parallel. The grain size is decided by the screen sizes used.

Fine-ground end products are obtained by means of high-speed hammer mills, in which size reduction is obtained by a percussion system so that particles of the material strike fast-rotating impactors or upon suitably designed, stationary baffle plates. The velocities at the impactor tips range from about 80 to 100 m/sec. The grain size is determined by screens varying in hole size. Due to the poor conversion of the energy introduced, a considerable proportion is converted to heat. So effective cooling is needed while the heat-sensitive gelatin is being ground. Cooling is achieved by the air entrained by the impact rotor.

For ultrafine grinding with an end product smaller than 0.1 mm or still finer, size reduction alone will no longer be adequate. It will be necessary to

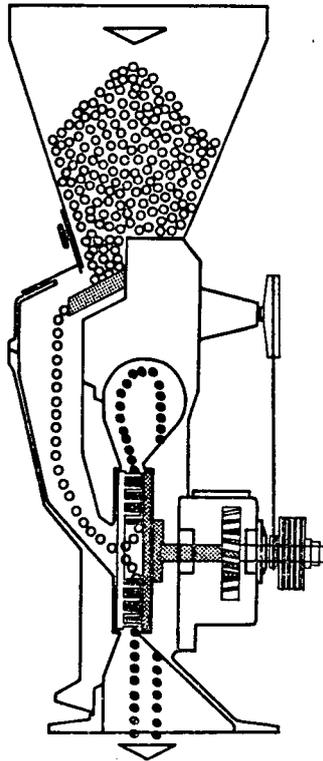


FIG. 17. ALPINE Kolloplex mill with a stationary and a rotating grinding disc. ⁽⁶⁾

combine fine-grinding with air classification. Size reduction takes place in fine-grinding impact mills, fitted with a peripheral grinding channel. Another system is the sieveless pin mill, fitted with one or two rotating discs that are featured by several rows of intermeshing pins. Figure 17 represents a Kolloplex mill with a stationary and a rotating pin disc.

There appears little difference in performance, product quality or cost between these latter impact mills and pin mills. There is some indication of the pin mill being better for low melting materials.

For the very finest grades, the mills are operated with spiral air classifiers, which remove the fine powder required from the ground product. The oversize separated in this way can be returned to the mill for further grinding. The Mikroplex spiral air classifier is capable of effecting separations within the range of from 10–16 microns diameter. It is essential to combine pulverizer and classifier to overcome the consequences of heat-sensitivity of the gelatin. This prohibits grinding alone if superfine particle size reduction is required. On its path between mill and classifier and back to the mill, the

unwanted oversize particles cool down so that it can be subjected to further grinding without damage.

H. Storage of Gelatin

Dried gelatins are first packed in bags of 100 lbs or other suitable containers and stored. The individual extracts or batches are kept separate until the physico-chemical properties have been determined in the lab. Standard ranges are produced by blending several types. Not until this stage are several batches, according to the required properties, ground together, screened and packed as specified by the customer.

Dry gelatin, stored under dry conditions, can be held in stock for many years.

I. Secondary Departments in Gelatin Production

1. *Steam*

The basis of power supply in the gelatin industry is the amount of steam required and the requisite operating steam pressure. The operating steam pressure is, as a rule, around 3–6 kg/cm². Steam quantities are per 1 kg of dry gelatin:

gelatin extraction	3–5 kg steam
evaporation	8–14 kg steam
hot water for cleaning, washing etc.	10–16 kg steam

This gives an average steam requirement of around 38–40 kg of steam per kg of dry gelatin. These values are based on statistics established in industrial gelatin production. They will of course fluctuate from factory to factory.

2. *Water supply*

An adequate supply of water for a gelatin factory is an essential requirement. Between 300 and 800 litres of fresh water are required per kg of dry gelatin. The water requirement fluctuates widely because of the various demands of raw material preparation.

These brief references to steam, electric current and water supply show that a thorough study of local conditions is imperative to ensure profitable gelatin production.

3. *Effluent*

The preparation and/or elimination of waste water has, in many countries, become a vital problem for the gelatin industry. The solution of this problem may be a matter of whether a gelatin factory can exist or not in a given area.

In order to tackle this effluent problem properly, the quantities of water from the main stages of production are required to be known, their solids

content, the organic content conveniently assessed as biological oxygen demand (B.O.D.) and the pH.

The waste water from a gelatin factory may be heavily contaminated, especially as a result of the liming process. A minimal requirement for all gelatin factories is that at least a mechanical purification of the waste water (settling, screening) ought to be provided. Waste waters containing lime can easily be clarified in a sediment tank in which the lime settles to the bottom. But this simple clarification is, today, no longer adequate in many industrial countries, as many countries require a more thorough mechanical and often also a biological purification of the water. A good form of mechanical purification of waste water is today provided by sludge centrifuges, also referred to as decanters. Such units are often applied to purify gelatin waste waters which can then go to the sewerage system. Many other methods—lagoons, activated sludge, etc. are available. The cost of a factory installation compared with the costs of discharging to sewers need careful comparison.

4. *Hygiene in the gelatin factory*

As referred to in detail in Chapter 13, gelatin is known to be a highly sensitive culture medium in bacteriology. For this reason hygiene and cleanliness in the production departments and in the personnel are of vital importance. All plants and equipment must be arranged in such a way that they can be rapidly, easily and thoroughly cleaned and disinfected. Above all, no plant should be so constructed that it has "dead corners" in pipe lines, pumps and elsewhere.

Washing and disinfecting tanks are necessary for cleaning and disinfecting the dismantled component parts and equipment. All entries to gelatin extraction, filter and evaporator stations should be provided with disinfection traps. In addition, blowing steam through the plant and pipeline does not give satisfactory cleaning and disinfection. Only more extensive and intricate methods are successful and efficient, when operated under a rigid schedule.

Rapid processing of gelatin liquors is of extreme importance. For, standing for too long, gives a noticeable increase in the total bacterial count. The gelatin liquor must be converted into its dry form as rapidly as possible. By strict adherence to cleaning and disinfecting programmes and by the sterilization of the gelatin it is possible to obtain a gelatin product that is low in bacteria so complying with all the demands of the foodstuff and pharmaceutical industries.

REFERENCES

1. Loeven, W. A. (1954). "Effect of alkali treatment of collagen on the pH-swelling curve of collagen and of gelatin products prepared from it", *J. Soc. Leather Trades' Chemists* **38**, 117-25.

2. Indian, P. 50806. "Dehairing Skins and Hides", Appl.: Council of Scientific and Industrial Research.
3. U.S. P. 2,453,630. "Glue or Gelatin", by Kenyon, J. and Silberstein, V.
4. Kowal, W. (1951). "Preparation of Bones", *Fleisch-Ind. USSR*, 22, No. 4, pp. 18-21.
5. Indian P. 45,583. "Gelatin", Appl.: Council of Scientific and Industrial Research.
6. Brit. P. 556,187. "Production or Extraction of Gelatin from Animal Skins", by Kernot, J. C.
7. Brit. P. 722,211. "Gelatin Extraction Process", Appl.: General Foods Corp.
8. U.S. P. 2,743,265. "Gelatin Extraction Process", Appl.: General Foods Corp.
9. U.S. P. 2,460,809. "Gelatin". Appl.: Eastman Kodak Comp.
10. Alexandrescu, G. and Suszer, A. (1966). "Die Verwendung hydrotropaktiver Stoffe in der Leimund Gelatinefabrikation", *Rev. Chem. (Bucaresti)* 7, 360-7.
11. Küntzel, A. and Heidemann, E. (1959). "Einstufige und zweistufige Kollagen-Gelatine-Umwandlung im alkalischen und sauren Medium", *Das Leder*, 10, 49-57.
12. Brit. P. 866,622. "Method of Recovering Gelatin", Appl.: General Foods Corp.
13. U.S. P. 2,517,487. "Improved Method for Extracting Glue and Gelatin from Hides and Bones", by Hill, J. E. and Hill, N. C.
14. Rutskiij, N., Epsktein, and Virnik, D. (1952). "Technological Improvements for Production of Gelatin", *Myasnaya Ind. UdSSR*, 23, 20-3.
15. U.S. P. 2,575,551. "Process for Extracting Gelatin and Glue", by Frechlin, R.E.A.
16. U.S. P. 2,577,429. "Apparatus for Counter-current Solvent Extraction of Glue and Gelatin from Animal Waste through a Plurality of Zones", by Mueller, C. J.
17. French P. 951,132. "Apparatus for Extracting Gelatin and Glue from Skins", by Frechlin, R. E. A.
18. U.S. P. 2,580,049. "Production from Gelatin", Appl.: Armour & Comp.
19. French P. 992,174. "Manufacture of Glue and Gelatin", by Coudun, C. and Coudun, R. G. H.
20. U.S. P. 2,676,168. "Food Products from Scrap Hides Particles", Appl.: Charles E. Ely.
21. U.S. P. 2,648,659. "Manufacture of Gelatin from Hide Glue", Appl.: Armour & Comp.
22. U.S. P. 2,557,871. "Comminuted Collagen Product", Appl.: Wilson & Comp.
23. U.S. P. 2,748,152. "Manufacture of Fat and Gelatin", Appl.: Armour & Comp.
24. U.S. P. 2,820,805. "Method of Defatting Bacon Skins", Appl.: Armour & Comp.
25. German P. 1,029,968, U.S. P. 2,908,615 and Brit. P. 817,367. "Method of Preparing Gelatin or Glue", Appl.: Armour & Comp.
26. U.S. P. 2,400,375. "Cleaning of Gelatin", Appl.: Eastman Kodak Comp.
27. Rousselot, S. (1943). "New Method of Demineralization of Gelatin", *C. R. hebdomadaire des Seances Acad. Sci.*, 216, 54-6.
28. U.S. P. 2,563,006. "Treatment of Liquids with Ion-Exchange Materials", Appl.: Sharples Corp.
29. German P. 1,024,655. "Verfahren zur Entfernung von öligen Bestandteilen aus Gelatine", Appl.: VEB Farbenfabrik Wolfen.

30. Lurgi, M. and Lykow, M. (1950). "Drying Bone Glue by the Spray-Drying Method", *Fleisch-Ind. UdSSR*, **21**, 31-6.
31. Kowal, W. "Preparation of Bones", *Fleisch-Ind. UdSSR* **22**, 18-21.
32. Edeling, C. (1947). "Spray-Drying", *Milchwissenschaft*, **1/2**, p. 227.
33. French P. 962,332. "Glue, Gelatin and Like Products", by Lemerte, J. L.
34. U.S. P. 2,824,807. "Spray-Drying Process", Appl.: General Foods Corp.
35. Hinterwaldner, R. (1957). "Herstellung von Gelatine, Haut- und Knochenleim", Moser-Verlag, Garmisch-Partenkirchen.
36. Ward, A. G. (1961, 1960). "Unsere gegenwärtige Kenntnis über Gelatine", *Das Leder*, **12**, 103-10 and *JSLTC* **44**, p. 505 ff.
37. Courts, A. (1960). *Biochem. J.* **74**, 238.
38. Courts, A. (1958). "Recent Advances in Gelatin and Glue Research" (Ed. Stainsby), p. 145, Pergamon Press, London.
39. Fysh, D. (1958) "Recent Advances in Gelatin and Glue Research" (Ed. Stainsby), p. 140, Pergamon Press, London.
40. Croome, R. J. and Clegg, F. G. (1965). "Photographic Gelatin", p. 21, Focal Press, London and New York.
41. Rousselot, A. (1943). "New Method of Demineralization of Gelatin", *C. R. hebdomadaire des Seances Acad. Sci.*, **216**, 54.
42. French Patent No. 899,036.
43. Janus, J. W., Kenchington, A. W., and Ward, A. G., (1951). *Research* **4**, p. 247.
44. Hinterwaldner, R. (1957). German Patent No. 964,200.
45. Hinterwaldner, R. Not yet published experiments (1967/1968).
46. Hinterwaldner, R. (1964). "Verfahrenstechnische Verbesserungen in der Gelatineindustrie", *Adhäsion*, p. 287.
47. Hinterwaldner, R. (1968). "Praktische Erfahrungen auf dem Gebiet der Gelatinefiltration", *Adhäsion*, p. 444.
48. U.S. Patent No. 3,073,380 (1963).
49. Gerngross, O. (1933). "Chemie und Technologie der Leim- und Gelatinefabrikation", Gerngross, O. u. Goebel, E., Sternkopff-Verlag, Dresden and Leipzig, p. 45.
50. Saunders and Ward, A. G. (1954). *The Journal of Photographic Science* **3**, p. 66.
51. Sauer, E. (1958). "Tierische Leime und Gelatine", Springer-Verlag, Berlin.
52. Veis, A. (1964). "The Macromolecular Chemistry of Gelatin", Academic Press, New York and London, p. 230.
53. Ames, W. M. (1947). *Journal Soc. Chem. Ind.* (London), **66**, 279.
54. German Patent No. 950,711.
55. Allé, Th. and Hinterwaldner, R. (1956). Unpublished experiments.
56. German Patent No. 1,047,970.
57. Hinterwaldner, R. and Elovsson, G. "Kontinuierliches, schonendes Sterilisieren von Gelatinebrühen", *Adhäsion*. **70**, (1966), S. 51-58.
58. Lindgren, B. and Swartling, P. (1963). "The Sterilizing Efficiency of the ALFA-LAVAL Vacu-Therm Instant Sterilizer", Milk and Dairy Research, Alnarp, Sweden, Report No. 69.
59. Thomé, K. E., Samuelson, E. G. and Holm, S. (1964). "Manufacture of sterile Milk Development Work with the ALFA-LAVAL VTIS", Milk and Dairy Research, Alnarp, Sweden, Report No. 70.
60. French Patent No. 1,469,354 (1967).

61. Gerke, K. (1915). *Kolloid-Z.*, **17**, 78.
62. Sauer, E. and Veitinger, J. (1958). "Tierische Leime und Gelatine", Springer-Verlag, Berlin, pp. 204-6.
63. Kamei, S. (1934). "Untersuchung über die Trocknung fester Stoffe", Vol. I-III, Kyoto Imperial University, pp. 35 and 37.
64. Krischer-Kroll. (1956). "Trocknungstechnik", Vol. I, Berlin, p. 264.

Chapter 11

Uses of Gelatin in Edible Products

N. R. JONES

*British Food Manufacturing Industries Research Association,
Leatherhead, Surrey, England*

I Quality Standards	366
II Properties Important in Choice of Gelatins	367
A. Presence of Various Impurities	367
B. Odour, Taste, Colour	368
C. Clarity	368
D. Origin	368
E. Bloom Strength	369
F. Viscosity	370
G. Surface Tension	370
H. Foaming Power	370
I. Iso-electric Point	370
J. pH Value	371
K. Melting and Setting Points	371
III Behaviour of Gelatin in Relation to Food Use	372
A. Breakdown by Heat and Acid	372
B. Breakdown by Enzymes	373
C. Compatibility with other Colloids	373
IV Functions of Gelatin in Food	373
V Handling of Gelatin	374
A. Hygiene	374
B. Dissolving Gelatin	374
C. Clarifying Gelatin	376
VI Use of Gelatin as a Jellying Agent	376
A. Comparison with Other Jellying Agents	376
B. Non-sugar Jellies	377
C. Gelatin-sugar Jellies	381
VII Use of Gelatin as a Whipping Agent	384
VIII Use of Gelatin as a Stabilizer	388
IX Use of Gelatin as an Emulsifier	390
X Use of Gelatin to Increase Viscosity	390
XI Use of Gelatin as an Adhesive	391
XII Use of Gelatin as a Binder	391
A. Sugar Pastes	391

B. Lozenges	391
C. Liquorice	391
XIII Use of Gelatin as a Fining Agent	391
References	392

Gelatin is used in foodstuffs as a processing aid because of its unique physical properties rather than for its nutritional value as a protein.

Gelatin jellies are unique in their easy reversibility, the closeness of the setting point to the melting point, and the ease with which they can be prepared. It is these properties which have led to the widespread use of gelatin in such a multitude of foodstuffs.

Much of the research work on gelatin has been carried out on highly purified gelatin and the results must be applied with some caution to the article of commerce. Furthermore much of the work has been carried out in very dilute solutions giving results that cannot be utilized under the conditions pertaining in many foodstuffs.

I. QUALITY STANDARDS

Various physical, chemical and bacteriological standards exist to govern the quality of gelatin for food use.

Chemical standards largely limit the content of poisonous metals such as arsenic, lead and zinc. Copper, which is limited to 30 p.p.m. in the United Kingdom and United States, can be important technically as can the ash content (3.25% in the U.K., 2.6% on a dry basis in Canada) and the sulphur dioxide content (1000 p.p.m. in the U.K., 500 p.p.m. in Canada). Gelatin is also required to be free from objectionable taste and offensive odour.

As far as jellying power is concerned, the British requirement is only that a 3% solution shall set at 16°C unless marked as "low setting strength". Generally setting power is of prime importance and gelatin is bought and sold by Bloom strength for all food purposes.

Although there is much variation in detail, bacteriological standards in the more advanced countries almost all require a total count not exceeding 10,000 per g and coliforms to be absent in 0.01 g (U.S.A., Canada, South African Bureau of Standards). There are no legal requirements in the U.K. Ward (1958) has reported total counts varying from about 6 to 6,000,000 per g on various samples examined. Coretti and Muggenberg (1968) found clostridia in 53 out of 73 samples of edible gelatins although in only one case was the count above 10 per g. They recommend that clostridia should be absent from 0.01 g for use in food for immediate consumption, absent from 0.1 g for use in fully preserved meat products and "complete absence" from gelatin for semi-preserves.

II. PROPERTIES IMPORTANT IN CHOICE OF GELATINS

A. Presence of Various Impurities

1. *Ash*

Although ash limits are frequently specified it is probably true to say that ash is not important except that a low ash content should mean a low calcium content which is of prime importance in many applications.

2. *Calcium*

A high calcium content must be avoided for many purposes where a clear bright jelly is required. In the presence of fruit acids, calcium citrate or tartrate may be precipitated (Selby, 1951) causing cloudy jellies and deposits on the surface of table jelly tablets (Taylor, 1933). For meat products, particularly such items as tongues or chicken in jelly, packed in glass jars, a low calcium content is vital to prevent white deposits, known as "white foots", produced by reaction with the phosphate in the meat extracts. Banfield (1927, 1930) showed that the maximum tolerable lime content was about 0.25% (as CaO) of the dry weight and suggested a working standard of 0.15%. Where the lime content was above this limit it could be removed by the methods dealt with later under "Clarifying" (p. 376).

When sulphur dioxide is present (as in most commercial gelatins) a calcium sulphite-phosphate complex may be produced (Edwards, 1931) causing opacity.

3. *Copper*

Gelatins containing 20–50 p.p.m. of copper can give purplish stains on the surface of tongues in jelly (Black, 1931) and copper from gelatin can cause similar stains on chicken (Edwards, 1931). Banfield (1931) found that while gelatin containing over 20 p.p.m. of copper gave grey discolourations, clarifying with egg albumen removed 40–80% of the copper.

4. *Iron*

It has also been stated that gelatin containing more than 2 p.p.m. of iron can cause grey stains on meat products (Anon, 1938a), but it must be remembered that this would produce a very low concentration of iron in the finished pack such as would readily be picked up from other sources.

5. *Sulphur dioxide*

The contribution of a high sulphur dioxide content to the production of an insoluble calcium sulphite-phosphate complex has already been mentioned. Sulphur dioxide can also bleach added colours although it may help to repress

the development of unwanted colour (Taylor, 1933). High sulphur dioxide contents may also cause corrosion of metal containers and lids (Edwards, 1931).

6. *Peroxide*

Leistner (1956) reported the presence of peroxides (50 mg of active oxygen per 100 g) in some samples of gelatin. This produced greenish discolourations in ham after 2 to 4 hours storage at room temperature in the dark. No reaction occurred with fresh meats.

B. Odour, Taste, Colour

Satisfactory gelatins should have little odour, taste or colour. Practically all official standards require it to be free of objectionable taste or offensive odour, but this is clearly a relative standard as samples are not normally free of a slight glue-like odour in warm solutions. Clyne (1958) points out that cheap gelatin needs more added flavour to cover its taste.

C. Clarity

Lack of clarity, unacceptable when a light jelly is required in some confectionery or meat products, can be inherent in the gelatin, because of the presence of foreign matter, in which case it can be removed by clarifying (p. 376). Filtration is seldom a practical proposition for treating the gelatin itself, although purification by centrifuge has been used (Idson and Braswell, 1957), but is sometimes used after clarification. Turbidity may also be produced from precipitates of insoluble calcium salts (p. 367) or be caused by changes of pH (see p. 371) or mutual precipitation with other colloids such as agar (p. 373). A gelatin of low pH can cause protein precipitation in some meat stocks if the system is only weakly buffered (Banfield, 1930).

D. Origin

Gelatins may vary considerably in their behaviour, depending on whether they are acid-processed or lime-processed. Many papers have been published which do not give an adequate description of the gelatin used and this could account for some of the discrepancies in the results found by different authors. Kramer (1965) states that pig-skin is the major source in the U.S.A. and is acid-processed, that some lime-processed calf-skin (tanner's stock) is also used, but that ossein (lime-processed) is little used except for photographic gelatin. The U.S. Pharmacopoeia classes acid-processed gelatins as type A and alkali-treated gelatins as type B. The type used can in some cases be critical as is shown later in the section on meat pies.

The most fundamental difference (from a user's viewpoint) between the two grades is the iso-electric point (q.v.) but other differences in chemical and physical properties of importance in food manufacture have been reported.

Oury stated in 1933 that ossein gelatin gave harder, more brittle jellies, whereas skin gelatin was more rubbery. Also it was easier to produce a low ash in ossein gelatin, 0.4% ash and 0.2% lime being reasonable limits for high-class products, but skin gelatin rarely had less than 1.0–1.5% ash and 2.0% was not uncommon.

Ossein gelatin is said to be more difficult to clarify than other gelatins (Kramer, 1965).

Downey (1950) makes the interesting suggestion that American users prefer type A gelatins because the jelly strength increases when the pH is raised above 4.0 (at which pH it is graded and its price fixed) whereas no such increase can be obtained with type B gelatins. With type A gelatins Downey states that Bloom strength and viscosity increase together but with type B gelatins the viscosity is not tied to the Bloom strength.

Ames (1947, 1952), using gelatins produced from ox hides and sinews, showed that the rate of breakdown under the action of acid or alkali and heat was different for the two types of gelatin. Alkali-prepared gelatin was stated to be more stable in alkaline solution, but acid-prepared gelatin was more stable in acid solution. Plotting the percentage loss in Bloom strength after 2 hours at 85°C, indicates that both types behaved similarly in neutral and alkaline solution, but that the lime-processed gelatin became progressively less stable at lower pH values. At pH 4.0 it had lost 60% of its Bloom strength compared with 30% lost by the acid-processed gelatin.

E. Bloom Strength

Commercial gelatins are generally obtainable from 90 to 300 g Bloom. When used in the manufacture of all types of jelly goods it is not always most economical to use high Bloom gelatins, because the price is largely tied to the Bloom strength and a cheaper gelatin of lower Bloom used in a higher concentration will often cost less. Hence the choice of gelatin needs to be made with regard to the other properties that accompany Bloom strength and to the market price.

To relate the cost of the product to that of the gelatin it is necessary to be able to compare jelly strengths at other concentrations than the standard 6 $\frac{2}{3}$ % concentration of the official Bloom test. Various conversion formulae have been used based on the approximate law that the rigidity varies as the square of the concentration. Ingleton (1965) for example gives theoretical tables of equivalent concentrations.

When the jelly strength is measured on a Bloom gelometer, jelly strength

has been found to vary approximately as the 1.7th power of the concentration (Rosenthal, 1963; Tiemstra, 1968). Both authors give conversion equations based on this power law. Such formulae do allow a rough calculation of equivalent costs to be made, but they make the assumption that rigidity, as measured by some simple specific test, is the important factor. Selby (1951) points out that the rigidity of two samples of gelatin may vary differently with temperature, and shows the rigidity/temperature curves of two samples that cross, so that it is not possible to even say which is the stronger without specifying the temperature.

Apart from economies, some control of texture may be achieved by selecting a suitable Bloom strength. The Bloom strengths recommended for different purposes are dealt with later in the commodity sections.

F. Viscosity

The viscosity of a sample of gelatin has only a small effect on the viscosity of the finished product, which depends primarily on gelatin concentration and total solids concentration (Taylor, 1941; Rosenthal 1963) and, at low temperatures, on any partial gelation which may occur (Rosenthal, 1963). Ossein gelatin has been said to give more "tailing" when pouring pastilles than a skin gelatin of the same Bloom strength and this was attributed to the higher viscosity of the gelatin (Anon, 1962b). Gelatins of high viscosity (and hence high molecular weight) give chewier jellies than gelatins of low viscosity which are more brittle (Ward, 1967).

G. Surface Tension

The depression of surface tension produced by gelatin in solution may be related to the beating properties of marshmallows (Taylor, 1946). The depression is much greater in strong sugar syrups than in water.

H. Foaming Power

Idson and Braswell (1957) say that the foaming power should be high for marshmallow production but Taylor (1933) states that the foaming power in water is no guide to that in marshmallow syrup.

I. Iso-Electric Point

The iso-electric point of acid-processed pigskin gelatin is usually in the region of pH 7.5-9 whereas that of lime-processed material is in the range 4.8-5.0. There may therefore seem to be reason to choose one particular type depending on the pH range of the finished product.

Thus at pH values in the neighbourhood of the iso-electric point the turbidity is higher, the swelling lower, the viscosity lower, the gel strength higher, and the syneresis higher (Clayton, 1932). In practice most of these effects can be neglected at the concentrations in which gelatin is used in foodstuffs, all of them being much more conspicuous in dilute solutions and in the absence of sugars. Nevertheless, where, for example, the utmost clarity is required at pH values in the range 5.0–6.0 (e.g. in many jellied meat products), this can be marginally important.

The iso-electric point is of vital importance when gelatin is mixed with certain other colloids because of the danger of mutual precipitation if the colloids are carrying opposite charges (see p. 373). Similar incompatibility is to be expected when the two types of gelatin are mixed together, and Tice and Blatt (1937) found that clear solutions could not be obtained at pH values between 4.8 and 7.5 (in which range the two types of gelatin molecule bear opposite charges).

J. pH Value

Various pH values are reported within the ranges 3.8 to 5.0 for acid-processed and 4.7 to 7.5 for lime-processed gelatin (Tice and Blatt, 1937; Ingleton, 1964; Kramer, 1965). The natural pH value is of importance only in lightly buffered systems. Banfield (1930) reported that protein deposits were thrown down from meat stocks by gelatin of low pH which reduced the pH of the stock from 5.7 to 4.7. Ingleton recommends that buffers should be added to cooked confectionery batches to give uniform products.

K. Melting and Setting Points

The melting points of gels from different gelatin samples vary considerably. Eldridge and Ferry (1954) showed that the melting point is a function of the molecular weight, and that, above 2 or 3% of gelatin, the variation between samples is much greater than that due to gelatin concentration. For example a gelatin of M.Wt. 72.1×10^3 had a melting point of 28°C at 3% and 30°C at 6%, whereas a sample of M.Wt. 48.0×10^3 had a melting point of 19°C at 3% and 21°C at 6%.

Selby (1955) found that the gelatin concentration (from 3–9%) in meat pie jellies did not measurably affect the melting point but that different commercial gelatin samples gave melting points varying from 24° to 33°C. This was not related to the origin of the gelatins. It is obvious that the melting point is important in choosing a gelatin for pies and jellied meat products to avoid liquefaction in hot weather. Even in the presence of sugar the choice of gelatin affects the melting point. Taylor (1946) found that the melting point

and setting temperature of a marshmallow mix was closely related to that of the gelatin used. Williams (1960a) states that gelatins of high melting point give jellies which have lower temperature coefficients of rigidity and hence give products less susceptible to changes of temperature.

III. BEHAVIOUR OF GELATIN IN RELATION TO FOOD USE

A. Breakdown by Heat and Acid

The susceptibility of gelatin to breakdown by heat and acid must be allowed for when gelatin is used in foodstuffs.

Many workers have published figures showing the rate of breakdown, both in fundamental studies and in application to food. The results are expressed in different ways and are difficult to compare, but when, by interpolation or calculation from formulae, the percentage loss of Bloom strength is derived from various papers the results are found to be extremely variable. Thus from four different sources (Croome, 1953; Ames 1947, 1952; Kramer, 1965; Tiemstra, 1968) figures for per cent loss of Bloom after 1 hour at pH 4 and 80°C were 8% and 46% loss for 2 different samples of alkali-processed material and 8% and 13% for a sample of acid-processed ox sinew and what were probably two samples of acid processed pig skin gelatin. Tiemstra derives a general formula and nomogram for calculating the gel strength after a given time at any pH value (from 3 to 9) and temperature:

$$\log D = 5 \log T + 0.08 (\text{pH} - 7)^2 - 11.771$$

where D is the degradation rate (from which the Bloom strength after a given time may be calculated) and T is the temperature in °C. He assumes that the breakdown rate varies symmetrically with pH about the value of 7, which is contrary to the findings of Ames, Croome and Kramer.

Values quoted for the pH at which breakdown is least also vary widely over the range from 5.5 to 8. Banfield (1930) states that at retorting temperatures (113°C) it varies with different samples from 5.5 to 6.2.

The loss of viscosity (as a percentage of the original) is more rapid than the loss of jellying power according to Ames's results.

Selby (1951), discussing problems in the use of gelatin in foods, comments that information is needed on whether different samples of gelatin vary appreciably in stability—certainly Tiemstra (1968) seems to assume that different samples behave similarly enough to make his nomogram useful.

For practical purposes, food manufacturers have contented themselves with trying to reduce breakdown to a minimum by taking precautions to avoid high temperatures in the presence of acid (breakdown under alkaline

conditions is not important in food manufacture). It is generally recommended to avoid temperatures above about 82°C by cooling batches to this temperature before adding the gelatin and by adding any acid at the last moment (Clyne, 1958).

To give consistent results buffers may be used to control the pH value. Thus Banfield (1930) suggests that for meat jellies, 4 to 8 pounds of sodium phosphates may be required per hundredweight of jelly. A patent for a gelatin coating buffered to pH 6.5-7 (Hall, 1949) claims that when used at 60-68°C the coating could be used over a period of 60 days.

B. Breakdown by Enzymes

Gelatin is also broken down by proteolytic enzymes. Probably the most important aspect of this is the warning (Lowe, 1955) against setting raw pineapple in a gelatin jelly, as the bromelin liquefies the jelly. They are, of course, also readily broken down by microbiological enzymes.

C. Compatibility with other Colloids

Gelatin is often used in conjunction with other colloids. At pH values below about 5 both types of gelatin carry a positive charge; above about pH 7.5 both types carry a negative charge. Between these pH values limed gelatin carries a negative charge and acid-processed gelatin a positive charge. Mutual precipitation or loss of jelly strength has been reported between gelatin and agar (Selby, 1955), Irish moss (Bronson, 1951), alginates (Selby, 1955; de Gloabec, 1947), gum arabic (de Jong and Bank, 1939) and pectin (Kramer, 1965). In all these cases the incompatible colloid carries a negative charge which neutralizes the positive charge on acid-processed gelatin, or on limed gelatin in acid products below pH 5. Only Selby and Bronson point out that both the type of gelatin and the pH of the product are critical. The other authors may have used only acid-processed pig skin gelatin.

In solutions containing high sugar concentrations precipitation does not seem to be a practical problem and gelatin is successfully combined with starch, milk protein, egg albumen, agar, gum arabic, sodium carboxymethyl cellulose, and gum tragacanth. Gelatin, both acid-processed and limed, is commonly used with milk in ice-cream.

IV. FUNCTIONS OF GELATIN IN FOOD

The uses of gelatin in foods are very extensive. Downey (1923) gave an extended list, but such lists tend to contain many products that are not

normally made with gelatin although it might be possible to use it. It is more useful to consider the common products classified according to the various functions of the gelatin.

Gelatin is used as a jellying agent, stabilizer, emulsifier, thickener, foaming agent, water binder, crystal growth modifier, glaze, adhesive, binder and fining agent.

There seems little doubt that these could easily be combined into a few basic functions, possibly gel formation, emulsifying, crystal growth control and fining. Nevertheless it is convenient to use more classes than this while remembering that the gelling properties, for example, are also fundamental to its uses as a glaze or a binder.

V. HANDLING OF GELATIN

A. Hygiene

Gelatin in solution or after soaking offers an excellent growth medium for bacteria and should be left in this state only if kept very cool or hot enough to destroy or inhibit bacteria. It is recommended that gelatin solutions for use in meat products should be brought to the boil, simmered for 5 minutes and then held at not less than 65°C until used. It is often cooled slightly immediately before adding to meat pies to prevent soaking into the crust or before use as a glaze so that the viscosity rises sufficiently to give a good coating. If this is done, it is important to cool only enough gelatin solution for immediate use. When jellying pies, the filling equipment must also be washed frequently with hot water to avoid a build up of micro-organisms.

Any jelly saved for the following day must be cooled rapidly before storing and reboiled before use as above, but it is far more satisfactory to avoid making more jelly than can be used in the day.

The danger is far less when the bacterial condition of the gelatin is satisfactory to start with, but even then the chance of contamination during use is quite high.

B. Dissolving Gelatin

Sheet gelatin, once very commonly used, must be soaked for at least an hour until quite flabby and until there is no sign of a whitish streak in the centre ("Mogul", 1963). With insufficient soaking, part of the gelatin will not dissolve. Mogul suggests wrapping the sheets in a wet cloth to allow equilibration without undue water absorption. The hygienic hazards of such a procedure are obvious, although in sugar confectionery production any bacterial

growth is unlikely to survive subsequent processing. The soaked sheets can then be melted in a water-jacketed bath or dissolved in hot sugar syrup.

Treated in this way the sheets absorb about their own weight of water; much less than is required by the use of powder. The entrapped air is also less. Apart from these advantages Mogul points out that sheet gelatin in bales is awkward to handle and occupies about six times the storage space of an equal weight of powder. Also the quantity of water absorbed is variable.

Powdered or flake gelatin can be soaked (30 minutes) and dissolved in a similar manner, but needs twice its own weight of water to avoid hard insoluble particles. Finely ground gelatin (30 mesh or finer) will clump if treated in this way and become difficult to dissolve.

Goodall (1959), investigating the formation of scum on table jellies, gives some interesting figures for the weight of scum formed and the volume of trapped air when using gelatins of different mesh.

TABLE I. Air content of soaked gelatin (%)

	Sheet	Powder	
		Coarser than 10 mesh	Finer than 20 mesh
Air in jelly ml/100 g	<0.05	0.13-0.17	0.17-0.28
Air trapped in soaked gelatin ml/100 g	Nil	1.06	2.87
Scum on jelly g/100 g	0.37-0.45	0.75-0.98	1.01-1.16

The larger bubbles formed by using the coarser grade rose more rapidly when the jelly syrup was kept hot. By melting the soaked gelatin and holding it for 30 minutes, the bubbles rose to the surface leaving a clear solution, but the jelly strength was reduced by some 25%.

The gelatin must be heated to at least 49°C to dissolve (except for low grade material) but 60°-70°C is more usual. When dissolved in sugar syrups it is usually recommended to have the syrup at about 82°C.

An alternative method for dissolving powdered gelatin (coarse or fine) is to add it in a fine stream to water at about 65°C while stirring at 40-50 r.p.m. (Mogul, 1963). Higher stirring speeds may result in air becoming trapped. Stirring must be continued until the gelatin is all dissolved (about 10 minutes).

It is possible to dissolve powdered gelatin directly in hot sugar syrups but solution is slow and it is difficult to see the undissolved grains because of the high refractive index of the syrup.

In the case of some meat products, such as canned ham and canned tongues, it is a common practice to add dry gelatin to the product in the can. The gelatin dissolves during processing and forms a jelly with the juices exuded from the meat.

Various cold water-soluble gelatins are described in the patent literature. These have been prepared by drying in the sol state (Mitchell and Seidel, 1958), dehydrating a sol with solvents (Corben and Steigmann, 1958), coating with emulsifiers or surface active agents (Steigmann, 1958; Gunthardt, 1958) or by chemical modification (Wingard, 1967).

Spray dried mixtures of gelatin and glucose solids (corn syrup solids) have recently become commercially available. These are soluble in cold water or milk and set to give firm jellies after standing for about 15 minutes at room temperature.

C. Clarifying Gelatin

For the utmost clarity in meat products, gelatin solutions can be clarified with egg white. About 3 pints of egg whites, or the equivalent in pre-dissolved dried egg albumen, are added to 10 gallons of jelly solution (Anon, 1938a,b), which is then heated to boiling point and held at this temperature until the albumen coagulates and carries down the impurities with it. Excessive copper can also be removed in this way. If excess lime (over 0.15%) is present, Banfield (1927, 1930) suggests that this is first precipitated by adding about $3\frac{1}{2}$ oz of sodium phosphate per 10 lb of dry gelatin and then an egg white clarification carried out. Clarification is seldom used in modern industrial practice.

VI. USE OF GELATIN AS A JELLYING AGENT

A. Comparison with other Jellying Agents

Gelatin forms thermally reversible gels with water and therefore resembles agar, carrageenan and furcellaran (all red seaweed extracts), and differs from pectin, low methoxy pectin, alginates, starch, egg albumen and milk protein which form irreversible gels. The main difference between gelatin and the red seaweed gels is the low melting point of gelatin gels, differing by only a few degrees from the setting point. It is for this reason that gelatin is universally used for table jellies which can be melted readily in hot water. The low melting

point gives the characteristic effect of dissolving in the mouth whereas an agar jelly, for example, needs biting because its melting point is much higher. On the other hand, gelatin jellies cannot be crystallized in warm syrups without melting. In addition a warm gelatin jelly can reform after the set has been broken by agitation, making it simple to use for flan jellies which must be poured when setting has commenced to give high viscosity and to avoid soaking into the sponge or pastry. Furthermore, although gelatin is susceptible to acid breakdown, it is much less susceptible than agar, carrageenan or furcellaran.

Jellies made with gelatin are much more elastic and rubbery than agar and pectin jellies and gelatin gives chewier nougats and marshmallows than egg albumen (Skuse, 1957). The texture of gelatin jellies can be modified by adding agar to give a shorter bite. For very hard jellies, gum arabic is mixed with the gelatin to avoid an impossibly tough product.

Skuse gives the strength of jelling solutions used in confectionery as: gelatin 4–10.5%; pectin (100 X) about 1.75%; agar 2–3%; starch 5–10%; gum arabic 50–65%.

The syneresis of gelatin is low, and this may be one reason why it is stated that its jellies cannot be removed from rubber moulds as can agar, pectin and alginates but must be cast in starch (Anon, 1958). In practice all these jellies can be very difficult to remove.

Zabik *et al.* (1962) compared the texture of egg custards containing gelatin, carrageenan or alginate and found that gelatin gave a smooth custard with a smooth cut surface but which clung to the mouth, carrageenan gave an even smoother product but alginate gave a coarse texture on eating and a rough cut surface.

B. Non-Sugar Jellies

1. *General considerations*

Gelatin jelly products fall conveniently into two classes, those containing sugar and those without, both because of the nature of the products and the different problems involved. Non-sugar jellies will be considered first.

(a) *Control of rigidity, melting point and setting point.* The rigidity and melting point of a gelatin gel depend on the sample used, the thermal history of the gel, the gelatin concentration and the pH values of the gel.

The first factor has already been considered on p. 371. Selby (1951, 1955) considers that for meat jellies the melting point is not itself as important as a low temperature coefficient of rigidity, so that the jelly is of an acceptable texture over a range of temperatures, provided the melting point is not too low. There is little scope for controlling the melting point by changes in

gelatin concentration in any case because this would give a disproportionate change in the rigidity.

The pH value tends to affect both rigidity and melting point in a similar manner. Various authors give data relating rigidity to pH value over various ranges (Briefer and Cohen, 1928; Clayton, 1932; Taylor, 1933; Downey, 1950; Selby, 1955; Ingleton, 1964). Various maxima and minima occur on published curves, the only common factors being a fall in rigidity at very low and very high pH values. It is difficult to compare these curves because the types of gelatin differ (and are not always specified), at least five different methods of measuring rigidity were used and it is not always clear whether part of the pH effect is an irreversible hydrolysis during preparation. Briefer and Cohen also showed that a minimum at low concentrations could occur at the pH value giving a maximum at high concentrations. There is only a limited scope for varying the pH value of the jelly because of considerations of flavour and stability. Near the iso-electric point, turbidity increases, but this is more apparent in very dilute solutions of little interest commercially.

The initial increase of rigidity on setting is much more rapid at low temperatures (Lampitt and Money 1936; Selby, 1955) but a slowly cooled jelly develops a higher rigidity after about a day. At the end of four days the rigidities become equal. This has little practical importance for jellied meat products where hygienic considerations demand a rapid cooling. Eldridge and Ferry (1954) show that the melting point was lowered by several degrees Centigrade for jellies chilled at 0°C as against 15°C. The rate of set is increased by higher gelatin concentrations (Ostwald, 1922).

The jelly strength (and also the whipping power) is affected not only by pH but also by the particular acid used. Of the acids commonly used in food-stuffs citric acid gives higher rigidities than tartaric acid at any given pH value (Lampitt and Money, 1936) the curves shown suggesting that the difference could be as much as 50%.

(b) *Syneresis*. Syneresis is also affected by pH, being greatest at the iso-electric point (Kuhn, 1928). Taylor (1933) showed an increase in syneresis from 2% to 14% on a 1% jelly for a change of pH from 4.9 to 6.8. Syneresis of stronger jellies is very slight.

(c) *Effects of additives*. In meat products the most important additive to gelatin jellies is probably salt. Salt increases the breakdown during heat processing and it is recommended (Anon, 1938b) that the overall salt content of glass-packed meat products should not exceed 3.5%. Banfield (1930) showed that the maximum rigidity occurred at higher pH values when salt was present in gelatin goods during retorting. Besides increasing hydrolysis salt is reported as reducing the rigidity and higher concentrations as precipitating the gelatin (Idson and Braswell, 1957; Kramer, 1965). Selby (1955) reports a 15-20% reduction in rigidity produced by 0.5% salt in a 5% gelatin jelly.

Sodium phosphate also lowered the rigidity, a point which should not be overlooked when using it as a buffer or to remove calcium. Both salts also lowered the melting point. Bello and Vinograd (1955) quote a fall in melting point from 31°C to 28°C for an addition of 3.7% of salt to a 5% gelatin gel and to 26°C for 7.3% of salt. Other salts, of little importance in foodstuffs, may raise the jelly strength (Selby, 1955; Kramer, 1965).

Milk raises the jelling power of gelatin (Carpenter *et al.*, 1928; Dahlberg *et al.*, 1928). This is dealt with further under ice-cream. (p. 389).

Selby (1955) reports at some length on the use of agar to raise the rigidity of meat pie jellies at temperatures just below the melting point, thus preventing collapse at high temperatures. This made the jellies cloudier and this treatment was possible only with lime-processed gelatins as acid-processed material precipitated the agar (see p. 373). Sodium alginate was found to reduce the rigidity.

(d) *Effects of freezing.* Moran (1926) and Hardy (1962) showed that at temperatures above -3°C gelatin gels did not freeze as a whole, but that ice crystals gradually penetrated from the exterior. Above concentrations of 2% of gelatin the separating crystals contained a solid solution. On thawing, the gelatin reabsorbed the water or solution. On rapid cooling below -3°C crystallization occurred throughout the jelly and the structure was retained on thawing. This resistance to breakdown of the structure on rapid cooling makes gelatin valuable in ice-cream manufacture.

2. Meat products

The normal use of gelatin in meat products is as a jelling agent although it has also been used to thicken canned or bottled soups and pie gravies. It is used to provide a jelly for brawns, aspics and similar products, canned hams, picnics, tongues, and other meats and fish, chicken or tongues in glass, and for binding and absorbing juices from chicken or turkey rolls, meat loaf etc. A very important use is for the jelly in cold pies.

Gelatin glazes and coatings are only stronger jellies and can be considered here.

Gelatin jellies are normally flavoured with stock and salt or a natural jelly can be extracted from pork rinds. Selby (1947). The jelly solution can be clarified with egg albumen when great clarity is required (see p. 376) any excess lime being removed beforehand, but for quality products a gelatin of good clarity and low lime content should be selected.

Gerrard (1968) recommends the following grades of gelatin for jellied meat products:

90–180 g Bloom (powdered)—pork and veal and ham pies, brawns, presses, potted meat etc.

160–240 g Bloom (cubed, kibbled or sheet)—glazes, canned hams, chicken, tongues, meat loaf etc.

(a) *Pie jelly*. The jelly stock is prepared by the procedure described under Hygiene (p. 374) and is added to the cooked pie through a hole in the lid. It is good practice to cool the pie before jellying so that the jelly sets rapidly without soaking into the pastry. The use of agar to raise the effective melting point in summer conditions has already been mentioned (p. 379). It should be noted that rind jellies extracted by an acid process will cause a precipitate in the same way as acid-processed commercial gelatins (Selby, 1955).

Jelly stocks for meat pies require gelatin concentrations in the range 5–9.5% according to the strength of the gelatin and the texture required.

(b) *Jellied meats etc.* The concentration of gelatin required for these products depends on the heat process to be applied. If the product is to be fully processed then extra gelatin is required to allow for breakdown in retorting. Thus Selby (1951, 1955) recommends 8–10% to allow for 1 hour's processing at 113°C. The jelly strength can be halved by such a process.

In canned products the gelatin is often added as powder but can conveniently be added in the form of a strong jelly (1 part gelatin, 2 parts water). This material must be stored in a cool place. For open pack brawns etc, a jelly stock is more convenient and may contain 10–15% of gelatin in the stock.

Various precautions in making jellied products have been dealt with already in connection with "white foots" (p. 367) caused by lime, discolourations caused by iron and copper (p. 367), peroxides (p. 368) and the effects of sulphur dioxide in excess (p. 367).

Gelatin has been used in Russia to make an artificial caviar by cooling drops of the mixture (flavoured with cod liver oil and herring extract) in cold oil (Anon, 1971).

(c) *Edible glazes*. Glazes need a high gelatin concentration to give a high viscosity so that the solution does not run off too quickly and give too thin a coating. Gerrard (1968) suggests 10–15% of gelatin. Edwards (1931) warns against the temptation to allow the temperature to fall in order to thicken the glaze. Only small quantities that can be used up rapidly must be cooled because of the hygiene risk.

(d) *Non-edible coatings*. Protective coatings can readily be made with a gelatin basis and can carry preservatives, antioxidants, etc. Many such mixtures have been patented.

Two patents (Hall, 1949; Lavrova *et al.*, 1953) describe coatings containing such ingredients as glycerin, propylene glycol and benzoic acid as preservatives, various buffers to prevent breakdown in use and reducing sugars to prevent oxidation of the gelatin. The gelatin contents vary from about 20–40%.

These coatings are suitable for such products as ham, bacon, sausages, fats and cheese.

A gelatin coating (3% gelatin) has been used for applying antioxidants to frozen turkeys (Klose *et al.*, 1952).

(e) *Other products.* The use of gelatin to prevent the seepage of juice from cooked beetroots has been patented (Cahn, 1971).

C. Gelatin-Sugar Jellies

1. *Effects of sugars*

It is surprising how little work has been published showing the quantitative effects of sugars on the properties of gelatin jellies. Sheppard and Sweet (1921) show curves for the effects of glycerin and alcohol on rigidity but simply state that sugar increases the jelly strength. Zabik *et al.* (1952) reported an opposite effect in custards, but as the sugar was added to the gelatin solution, the gelatin concentration was thereby reduced and this could have been the cause of the loss in rigidity. Some of the custards were compared by a sag test, and in this case the increased density would act to give a greater sag.

Unpublished work at the British Food Manufacturing Industries Research Association has shown a considerable increase in rigidity when sucrose is present provided the gelatin concentration was kept constant. The rigidity increased by 50% to 100% for different gelatin samples when the sugar content was increased from 0% to 60%. Invert sugar and glucose syrup gave somewhat lower strengths. The melting points also increased by about 9°C. The author plotted stress-strain curves for sugar concentrations (sucrose-glucose solids mixtures) up to 78% and found that the rigidity fell again beyond 60% but the texture became more viscous (unpublished work). This effect is similar to that found by the author for agar jellies (Jones, 1968).

Kramer (1965) states that sugars may undergo a browning reaction and also that aldehydes tend to crosslink gelatins (causing insolubility). Duck (1960) produced marshmallows from sucrose, glucose syrup or dextrose and studied the changes in toughness on storage. All three types of marshmallow toughened rapidly for the first 24 hours. After that time the sucrose marshmallow continued to toughen, the glucose syrup one did so at a slower rate, but the dextrose marshmallow actually began to decrease in toughness after 72 hours. Duck ascribed the toughening to cross-bonding between gelatin molecules and attributed the effect of the reducing sugars to an interference with this process as the aldehyde groups combined with the side groups of the gelatin molecule.

The viscosities of gelatin solutions containing 75% of sugars varied with pH in a similar manner to aqueous gelatin (Taylor, 1941). He also showed (as

would be expected) that the viscosity of a gelatin sample in water was a good guide to its viscosity in a strong sugar syrup.

2. *Some general effects in sugar jelly products*

(a) *Hardening by citrus oils and aldehydes.* Reference has already been made to the combination of gelatin with aldehydes (p. 381). Stainsby (1937) found that terpeneless lemon oil which had become oxidized could render pastilles insoluble in boiling water after a month's storage, whereas fresh oils had no effect. The activity was found in the aldehyde fraction. Russoff in 1953 found that butylated hydroxy-anisole (BHA), an antioxidant added to stabilize citrus oils, also prevented the development of insolubility in citrus oil-gelatin flavour compositions. The action of some flavours may render gelatin capsules containing them insoluble (Williams, 1953). A patent proposes the use of a dried granulated flavour mixture to avoid "tanning" in gelatin desserts (Monsanto, 1971).

(b) *Shrinking of fruit in jelly.* An interesting effect has been found when cherries have been set in gelatin jelly. After a few weeks the cherries shrink. Anthistle (1961) investigated this phenomenon and found that it was pH dependent. Cherries which shrunk 20–30% at pH 3.5 in a lime-processed gelatin underwent a slight swelling at pH 4.5. This effect could be explained as due to the Donnan effect, the cherry skin acting as a semi-permeable membrane. A pH value of 4.0 was suggested as a good compromise between shrinkage and acid flavour. With an acid-processed gelatin a higher pH is required which is incompatible with an adequate acid flavour.

3. *Sugar jelly products*

(a) *Table jellies and gelatin desserts.* American gelatin desserts or British jelly crystals consist of mixtures of gelatin powder, sugar, acid and other flavour and colour. The gelatin content varies from about 7% to 14% to give about 1.7% to 3% in the prepared jelly, the British product usually containing the higher proportions, probably because American practice assumes that the jelly will be set in a refrigerator. Bronson (1951), Williams (1953) and Merory (1968) all state that the gelatin should be at least 220 or 225 g Bloom. It is better to assess the gelatin by practical use tests. Bronson points out that the use of a low Bloom gelatin would not give the necessary quick setting and high melting point. Merory recommends the addition of a buffer to give a quick set and maintain gel strength. Although citric acid gives higher gel strengths it is more prone to caking than tartaric acid. Fumaric acid may now be used.

In Britain table jellies are more often made from jelly tablets in which the gelatin is predissolved with the other components. Tablets contain about 9–12% of gelatin. The main problems in table jelly manufacture are dissolving

the gelatin without adding excess water or introducing excess air, and avoiding gelatin losses through acid hydrolysis by adding the gelatin or acid at too high a temperature. The hot gelatin syrup needs to stand for some time to clear and must be skimmed or strained to remove the scum before casting. A full description of the process is given in Williams' book (Williams, 1953).

Jelly compounds for making up with milk must contain no acid or the milk will curdle. Suitable flavours are maple coffee, caramel etc. (Williams, 1953). The old U.K. standards (now revoked) allowed up to 20% of starch (usually cornflour or farina) in compounds. This allows a lower gelatin content. Jelly compounds merge into blancmange powders containing only 2-3% of gelatin as a stabilizer.

Flan jellies are commonly made from special modified starches, but some gelatin may be added to give a firmer set. Bennion (1966) gives a recipe containing 4.8% of arrowroot and 2.4% of gelatin.

(b) *Confectionery jellies*. The manufacture of confectionery jellies is similar to that of jelly tablets except that the jellies are cast in depressions in moulding starch. Soft jellies may contain from about 7% to 9% of gelatin of various grades from 100 to 200 Bloom or above. Soft jellies may be ready to remove from the starch after 2-3 hours and are then treated by various methods to prevent them from sticking together (e.g. sanding with granular sugar, dusting with icing sugar and cornflour, or wet crystallizing in sugar syrup). It must be remembered that soft gelatin jellies will not stand much heat treatment, such as steaming to make sugar adhere, or they will melt.

The gelatin, besides producing the gel, also delays crystallization of the sugar in the jellies (graining). Jelly babies are traditionally allowed to develop a grained surface to prevent them from sticking together without the expense of further treatment, and hence the gelatin content is reduced to about 5%. To give the necessary strength a high Bloom strength is desirable.

Although the texture of gelatin jelly sweets is partially controlled by gelatin content, it is also largely controlled by the water content. Williams (1960a) quotes boiling points equivalent to the following syrup concentrations as suitable for jellies of increasing toughness.

Tender jellies	—	85%
Firmer jellies	—	86%
Pastilles	—	87%
Gums	—	89%

The tougher jellies were at one time usually made with mixtures of gelatin and gum arabic, and gums (as the name implies) of gum arabic alone. Nowadays much more gelatin is used, often with no addition of gum at all. In order to reduce the moisture content sufficiently the jellies are stored in

stoves while still in the starch impressions until sufficient water has evaporated. This may take several days.

Mixtures for pastilles or jujubes (usually rather softer) may contain from 20% to 35% of gum arabic and 2% to 7% of gelatin (before stoving) according to the texture required. All-gelatin goods may contain from 10% to 15% of gelatin. When the higher concentrations of gelatin are used the goods may be unstoved.

(c) *Glazes for confectionery and baked goods.* Sweetened or unsweetened glazes may be applied to gums (see above), liquorice, buns etc. A "cake shine" recipe (Blumenthal, 1947) contains 2.7% of gelatin in sugar and water and a typical liquorice glaze about 10% of gelatin in sugar, glucose syrup and water.

(d) *Sugarless preserves.* Gelatin may be used to make sugarless jam or marmalade, e.g. for diabetic diets.

VII. USE OF GELATIN AS A WHIPPING AGENT

Besides its use to form jellies, gelatin is used extensively as a whipping agent. The most important foodstuff in which it is so employed is marshmallow. Although other whipping agents have been used for this purpose, principally egg albumen which gives a more tender product (and also mixtures of gelatin with egg albumin or lactalbumin), the simplicity of using gelatin alone has often made it the choice for mass production, and most of the scientific work (of which a great deal has been published) has been confined to gelatin marshmallows.

1. *Gelatin marshmallows*

Marshmallows are made by beating air into a warm strong sugar syrup containing from about 2% to 3% of gelatin and 18-25% of water. The finished foam can be poured on a slab and subsequently cut, poured into starch impressions, or extruded to form "drops", "snowballs" etc.

There have been many different opinions as to whether low Bloom or high Bloom gelatins should be used; all values up to 300 g have been recommended. Bronson (1951) recommends choosing the Bloom strength according to the texture required. Thus 75 to 125 g Bloom for a light fluffy marshmallow for novelties, up to 225 g Bloom for pieces sold in bags which must be sufficiently resilient to keep their shape. Downey (1950) gives figures to show that the cost is reduced appreciably when a higher Bloom strength gelatin is used, and for this reason the modern tendency seems to be to prefer a high Bloom gelatin.

Some properties important in a marshmallow are: over-run, texture, viscosity, setting time, beating time and setting temperature.

(a) *Over-run.* Taylor (1937, 1946) found that an increasing gelatin concentration gave a better over-run, provided the syrup concentration was high and also found that the expansion increased with increasing syrup concentration, e.g. an over-run (ratio of foam volume to syrup volume) of 2.7 at 70% and 3.5 at 78% under the same conditions. Tiemstra (1964) found that the over-run decreased above a certain gelatin concentration, e.g. one experiment showed an optimum concentration of 1.85% in a 72% syrup. However, he stresses that the gelatin concentration must be chosen for the texture required and that extra volume must be obtained by other means, such as the use of additional whipping agents or additives or, in continuous processes, the use of higher air pressures. He shows a graph relating air pressure and dwell time required in a continuous pressure heater to give an over-run of 3.25. The use of special marshmallow gelatins containing phosphates enables shorter dwell times to be used, thus increasing production.

Taylor found higher over-runs with greater beater speed but that high beating temperatures reduced the expansion. Tiemstra states that the optimum beating temperature depends on the solids content. Both Taylor and Tiemstra found that a low Bloom gelatin gave a greater volume than a stronger one, although more gelatin was required to give the optimum expansion. Briefer and Cohen (1928) found that for gelatins of the same Bloom strength the more viscous samples gave the greater volume.

Various additions have been suggested for improving the whipping power of gelatins. Downey (1950) showed that citrates improved the whipping power independently of the increase of pH value which also improved the volume slightly when raised from the normal 4.2 to 4.7 or 5.2. The use of phosphates (e.g. 0.5–5.0% of sodium hexametaphosphate) was patented by Grettie (1940) and the U.S. Federal Regulations (U.S.F.D.A., 1964) allow the addition of 0.5% of sodium lauryl sulphate to gelatin for marshmallow manufacture. Other substances suggested are phytic acid and various esters (see Idson and Braswell, 1957).

(b) *Texture.* The toughness or resilience of a marshmallow is dependent principally on the gelatin concentration and Bloom strength but high syrup strengths also increase the resilience (Taylor, 1937, 1946). Lees (1960a) recommends the use of 250 g Bloom acid-processed porkskin gelatin, stating that low Bloom gelatins give a rubbery texture.

(c) *Viscosity.* Viscosity is increased by high over-runs and hence all the factors which give a high over-run give an increased viscosity. The viscosity can be reduced by using very high Bloom strength gelatins when making extruded marshmallows.

(d) *Rate of set.* As the rate of set determines how long the marshmallow must stand before cutting, removing from starch or packing, it is of great economic importance.

It is generally agreed that a higher Bloom gelatin gives a faster set (Taylor, 1946; Gorfinkle, 1953; Tiemstra, 1964). Tiemstra also examined the various other factors involved in setting time. He found that marshmallow achieved a maximum set in 24 hours (unlike gelatin jellies). The set could be speeded up by cooling in an air blast, by using special phosphate gelatins or by raising the pH value, although the last had little effect on the special gelatins. pH values above 6.0 could lead to browning.

(e) *Beating time.* Small changes in the gelatin concentration can have very marked effects on the time taken to reach a maximum over-run. Carlin, (1938) quotes a 30% reduction in beating time for a 0.2% increase in gelatin concentration. According to Rosenthal (1963) acid-processed gelatins need less beating time than limed ones.

(f) *Melting point and setting point.* Tiemstra (1964) and Gorfinkle (1959) state that the use of higher Bloom gelatins at reduced concentrations (to give a standard rigidity) gives lower melting points whereas Bronson (1951) stated that some manufacturers used a low Bloom in winter and then changed to a high Bloom in summer to compensate for a greater melting tendency at higher temperatures.

Taylor (1946) found the melting and setting points of the marshmallow to be linearly related to those of the gelatin (in 6.7% aqueous solution). The melting and setting points of marshmallow differed by about 4°–9°C compared with about 2°–4°C for the gelatins. Using four different gelatin samples, the setting point of the marshmallows varied from 19.4° to 33.7°C.

(g) *Extruded marshmallows.* When marshmallow pieces are extruded instead of being deposited in starch the pieces must be fit to handle within a very short time (e.g. 1 hour). Cast marshmallow pieces form a dry crust on the outside which enables them to be handled easily, whereas an extruded piece, with its moisture evenly distributed, is much too tender to handle unless its gelatin content is increased. A typical increase would be from 2.25% to 2.8% of 250 g Bloom gelatin. This results in a higher viscosity which in turn necessitates a higher extrusion temperature and consequently gives slower setting. Gorfinkle (1959) recommends overcoming these problems by using an extra strong gelatin of 300–325 g Bloom, requiring only 2% with 22% of moisture.

Boettger (1965) proposes the addition of 2.7% of waxy maize starch to an extruded marshmallow, containing about 2.7% of gelatin, for a more rapid set.

(h) *Bakers' marshmallows.* Bakers' marshmallows are provided whipped or in jelly form ready to whip in the cold or to add hot water. Recipes may

contain other colloids such as egg albumen and boiling starch (Anon, 1957) or be pure gelatin marshmallows (Bennion *et al.*, 1966). The incorporation of hot water is obviously easier with the all-gelatin product because the gel is thermally reversible.

2. *Nougat*

Nougat, like marshmallow, can be made with gelatin or egg albumin or a mixture, and can exist in grained and ungrained forms giving a variety of textures, some products being extremely tough and chewy. In the case of nougat the hardness is controlled by the water content, which is not possible with marshmallow if good beating properties are to be obtained.

Gelatin concentrations can vary considerably (figures quoted being from about 0.6% to 3% or more), generally using Bloom strengths from 110 g to 180 g (Williams, 1955, 1960b; Anon, 1962a; King, 1952).

Extra ingredients, fat, and fine coconut, are added to improve the bite and ease of cutting, with cornflour, icing sugar, nuts and fruits.

3. *Fruit chews and chewing toffee*

Gelatin can be incorporated in fruit flavoured chewing sweets, containing acid and usually some fat. 0.4 to 1.5% of a low Bloom gelatin is added in a beaten form.

Gelatin can also be added to toffees to make them extra chewy. About 0.4-0.6% is required (Skuse, 1957). It can be boiled with the batch or the soaked material dissolved in the boiled syrup.

4. *Frappés*

As an intermediate product in confectionery manufacture, a beaten syrup containing egg white and gelatin, called a frappé, is often manufactured. The whipping properties are largely due to the albumin, the gelatin acting as a stabilizer. Frappé is added to many products to lighten their texture.

5. *Whipped desserts*

Soufflés, mousses, and chiffon pies, are all whipped products. Williams (1953) gives recipes for soufflés containing 2-3% of gelatin and various other ingredients such as fruit juice, cocoa, dried egg and milk powder. In chiffon pies a meringue portion is blended with a custard. Glabau (1955) showed that the addition of gelatin of 75 g Bloom (2½%) reduced syneresis and equivalent amounts of higher Bloom strength gelatins (125-250 g Bloom) prevented it. The higher Bloom gelatins gave the greater foam volumes. Mousse is a whipped frozen gelatin dessert containing milk components but not the egg content of a soufflé.

VIII. USE OF GELATIN AS A STABILIZER

The function of a "stabilizer" is very ill-defined. It can be considered for the present purposes that stabilization involves the control of crystallization and the prevention of separation of phases. This latter function would seem to be partly bound up with the formation of a weak gel or simply an increase of viscosity. It is also connected with electric charges and adsorption and so is not truly distinguishable from the function of an emulsifier, but it is convenient to consider emulsifying action as applying to fairly liquid products.

Gelatin in solution inhibits the crystallization of sugar or reduces the size of crystals produced. Similarly it controls the production of ice crystals when the solutions are frozen. Duecker (1926) showed that 1.5% of gelatin almost completely prevented crystallization in 70% sugar syrups. Gelatin also affected the forms of the crystals, single crystals being produced when up to 0.45% of gelatin was present and cloudy agglomerates at 0.6% or higher concentrations. Gelatin contents of over 1.5% also (using his recipe) prevented crystallization in marshmallows. Theories as to the action of gelatin in restraining sucrose crystallization are given by Karacsony and Pentz (1955) and by Susich *et al.* (1959).

Callow (1952) measured the rate of growth of ice at -3°C . In water the rate was 1800 cm per hour and this was reduced to 710 cm/hr in 2% gelatin and 7 cm/hr in $2\frac{1}{2}\%$ gelatin. This effect on the formation of ice crystals is one of the main functions of gelatin and other stabilizers in ice-cream and related products.

1. Ice-cream

(a) *Functions of gelatin in ice-cream.* Stabilizers are used in ice-cream to prevent the formation of coarse ice crystals and gelatin was the earliest stabilizer used. It also decreases the rate of melting, gives body and a firm smooth texture. It is not used to increase over-run and Dahlberg *et al.*, (1928) state that the over-run is not affected by the sample used or the concentration within a reasonable range (0.2–0.6%). Over-run is more conveniently controlled by the addition of emulsifiers.

(b) *Bloom strength and concentration.* It is possible to produce good ice-cream with gelatin of any Bloom strength. Tobias and Edman (1951) found that 1.25% of a 62 g Bloom gelatin gave a similar ice-cream to 1.0% of a 263 g Bloom gelatin. These concentrations seem rather high. Frandsen and Arbuckle (1961) give equivalent figures for ice-cream as 0.50% of 150 g Bloom, 0.42% of 200 g Bloom, and 0.35% of 250 g Bloom. They suggest that the normal range would be 0.25–0.50% of 250 g Bloom and that the choice of

gelatin should be based on cost per unit of gel strength. Rothwell and Palmer (1965) quote 0.4% of 250 g Bloom as equivalent to 0.6% of 60 g Bloom.

An alternative test, known as the Dahlberg test, has been developed for assessing gelatins for use in ice-cream (Carpenter *et al.*, 1928; Dahlberg *et al.*, 1928). It measures the concentration which will just set in skimmed milk cooled in ice water. Skimmed milk gives stronger jellies than water, even when allowance is made for the reduced water content. With acid porkskin gelatins and limed calf-skin gelatins up to 50% more gelatin was required to give a set in water than in skimmed milk. The effect was much less with bone gelatins. They found that low Bloom gelatin hardened more on cooling than high Bloom gelatin. A formula is given to determine the concentration required for an ice-cream from the Dahlberg factor (Frandsen and Arbuckle, 1961).

(c) *Body and texture.* Too little gelatin gives a coarse, crumbly, weak texture whereas too much gives a gummy, rubbery body (Dahlberg *et al.*, 1928). The range of concentration for satisfactory results can vary over about 0.1% for a low grade gelatin or 0.05% for a high grade one. It would appear that an actual gel forms in ice-cream, jellying agents giving better shape retention than non-gelling thickeners (Moncrieff, 1954). Gelatin reforms its gel after whipping without remelting and also recovers its original viscosity after 48 hours (Dahlberg *et al.*, 1928).

(d) *Melt down.* Gelatin decreases the rate of melting, but the correct quantity is essential to avoid a thin melt with too little or a gelatinous melt with too much (Dahlberg *et al.*, 1928). Melt down is controlled by gelatin only if the gelatin is present at the surface of the oil droplets. If the non-fat milk solids are all homogenized with the fat, the milk proteins exclude the gelatin, but by using only part of the milk solids in this way, the gelatin is able to reach the fat surface and impart the necessary structure (Musselwhite and Walker, 1971). The eating properties depend on the melt, as a slow melt down with a sufficient viscosity are essential to give a creamy texture.

(e) *Comparison with other ice-cream stabilizers.* Gelatin has the useful property of giving a low viscosity in the mix and although it needs time to age, this is not a real disadvantage for many production processes. Stistrup and Andreasen (1962) compared its behaviour under modern high temperature heat treatments, followed by hardening at -35° to -40°C with other colloids. Gelatin was found to stand up to this treatment very well, as did alginate, carboxymethyl cellulose and carrageenan.

Against this Nash (1960) states that it melts too rapidly and is not sufficiently resistant to heat shock (ice crystal growth due to temperature fluctuations). Rothwell and Palmer (1965) state that it gives insufficient resistance to melt down and produces ice-cream lacking body. There is a tendency now to use it in conjunction with other stabilizers.

2. *Yoghurt*

Gelatin can be added to yoghurt and fruit yoghurt to reduce syneresis. It masks the flavour less than some other gums (Volker, 1970a,b).

3. *Icings*

Gelatin serves various purposes in icings for cakes etc. Mickevic *et al.*, (1956) say that gelatin (and other stabilizers) prevents liquid from soaking into the cake when the amount of liquid phase increases in hot weather, improves the gloss, and stabilizes aerated icings made with albumen. The quantities used are of the order of 1–2%. Idson and Braswell (1957) say that besides acting as a setting agent in boiled icings, gelatin also controls the sugar crystal size.

It can be used in fondant icing to reduce the brittleness of coatings of *fourrés* (sweets dipped in fondant). Skuse (1957) suggests 1% for this purpose.

IX. USE OF GELATIN AS AN EMULSIFIER

Gelatin promotes oil-in-water type emulsions (Clayton, 1932), but in practice does not appear to be used extensively to produce food emulsions. Blumenthal (1947) gives a recipe for a flavouring emulsion of lemon oil in syrup, emulsified with about 0.25% of gelatin.

Where regulations permit, it can be added to whipped cream, Blumenthal suggesting that 0.35% of gelatin used in this way will not only stabilize the whip but will also reduce fat losses. The U.K. Cream Regulations 1970 permit up to 0.3% of gelatin in whipped cream. It can also be used to make a mock cream from fat, milk and sugar, Williams (1953) giving a recipe requiring 5% of gelatin. Idson and Braswell (1957) also suggest its use in butter icing.

It has also been used to emulsify the fat in some meat products, such as pastes and cream soups, to supplement that naturally present.

Downey (1923) stated that it could be used in mayonnaise, but there do not appear to be any recipes in the literature.

X. USE OF GELATIN TO INCREASE VISCOSITY

Nash (1960) mentions the use of 0.1% of gelatin to thicken flavouring syrups for ice-cream and Gerrard (1968) states that 30–80 g Bloom gelatin can be used to thicken canned soups and as a gravy thickener in steak and kidney pies. For most thickening purposes, however, a non-gelling colloid would require less precise control.

XI. USE OF GELATIN AS AN ADHESIVE

Gelatin is used in several sugar confectionery products as an adhesive. For this purpose it is dissolved in strong sugar syrups, recommended gelatin concentrations varying from 1.5% to 9% for this purpose. Typical examples are a 4% gelatin syrup for sticking the different layers in liquorice allsorts (Anon, 1960a); 1.5% of 110 g Bloom gelatin in syrup for sticking nonpareils ("hundreds and thousands") to liquorice allsorts (Anon, 1966a); 9% in syrup to stick coconut to confectionery centres (Skuse, 1957).

XII. USE OF GELATIN AS A BINDER

A. Sugar Pastes

In sugar pastes, icing sugar is dispersed through a syrup and so bears a resemblance to fondant except that the particle size is much coarser. To maintain body it is necessary to add gelatin. Bloom strengths suggested vary from 75–150 g and concentrations from 1% to 9% in the syrup phase (Skuse, 1957; Williams and Reece, 1961; Anon, 1960b). The gelatin, besides acting as a binder, gives a less crumbly texture and so facilitates clean cutting.

B. Lozenges

Lozenges are much harder than cream paste goods, and gum arabic or gum tragacanth are generally used as binders. Mixtures with gelatin may be used and Skuse gives one recipe for a mucilage of 3.2% of gelatin with 2.1% of gum tragacanth.

C. Liquorice

The main bodying agent in liquorice goods is wheat flour which constitutes 30% to 50% of the finished goods. Gelatin is commonly added towards the end of the batch, suggested proportions varying from 1½% to 12½% of 160–180 g Bloom. Lees (1960b) states that the gelatin prevents cracking of sheet assists binding, adds body and prevents drying out.

XIII. USE OF GELATIN AS A FINING AGENT

Gelatin may be used as a fining agent in such products as fruit juices, wines and vinegar. The procedure is to produce a flocculant precipitate with tannin,

either natural to the product or added separately. On standing the precipitate carries down with it the materials causing a cloud in the product. This precipitate is then removed by filtration or other means. Quantities of gelatin are likely to be of the order of $1\frac{1}{2}$ to 5 oz per 100 gallons with about $1\frac{1}{2}$ oz of added tannin (Fiene and Blumenthal, 1939). This is dealt with more fully in Chapter 12.

REFERENCES

- Ames, W. M. (1947). *J. Soc. Chem. Ind.* **66**, 279-84.
 Ames, W. M. (1952). *J. Sci. Fd Agric.* **3**, 454-63.
 Anon. (1938a). *Food* **7**, 336.
 Anon. (1938b). *Food* **7**, 227-9.
 Anon. (1957). *Confect. Prod.* **23**, 274.
 Anon. (1958). *Confect. Prod.* **24**, 688, 692.
 Anon. (1960a). *Confect. Prod.* **26**, 146.
 Anon. (1960b). *Confect. Prod.* **26**, 840-2.
 Anon. (1962a). *Confect. Prod.* **28**, 268.
 Anon. (1962b). *Confect. Prod.* **28**, 462-4.
 Anon. (1966a). *Confect. Prod.* **32**, 313.
 Anon. (1969a). *Fd. Trade Rev.* **39**, (8), 61.
 Anon. (1969b). *Fd. Manuf.* **44**, facing pp. 64, 65.
 Anon. (1971). *Fd. & Drink Wkly*, 13th Aug. p. 6.
 Anthistle, M. J. (1961). *J. Sci. Fd. Agric.* **12**, 208-11.
 Banfield, F. H. (1927). *Brit. Fd. Mfg. Inds Res. Ass., Fd. Res. Rpts*, No. 2.
 Banfield, F. H. (1930). *Brit. Fd. Mfg. Inds Res. Ass., Fd. Res. Rpts*, No. 9.
 Banfield, F. H. (1931). *Brit. Fd. Mfg. Inds Res. Ass., Fd. Res. Rpts*, No. 12.
 Bello, J. and Vinograd, J. R. (1955). U.S. Army Contract No. DA-49-007-MD-298.
 Bennon, E. B., Stewart, J. and Bamford, G. S. T. (1966). "Cake Making." Leonard Hill, London.
 Black, J. W. (1931). *Fd. Technol.*, London **1**, 162.
 Blumenthal, S. (1947). "Food Products." Chemical Publishing Co., Brooklyn.
 Boettger, R. M. (1965). *Mfg. Confect.* **45**, (9), 47.
 Briefer, M. and Cohen, J. H. (1928). *Indust. Engng. Chem.* **20**, 408-13.
 Bronson, W. F. (1951). *Fd. Technol.*, Champaign, **5**, (2), 55-8.
 Cahn, R. I. (1971). BP. 1,229,325.
 Callow, E. H. (1952). *J. Sci. Fd. Agric.* **3**, 145-50.
 Carlin, G. T. (1938). *Pennsylvania Mfg. Confects' Ass., 2nd Prod. Conf.* 104-13.
 Carpenter, D. C., Dahlberg, A. C. and Hening, J. C. (1928). *Indust. Engng. Chem.* **20**, 397-406.
 Clayton, W. (1932). "Colloid Aspects of Food Chemistry and Technology". Churchill, London.
 Clyne, E. J. (1958). *Confect. Prod.* **24**, 507, 509-10.
 Corben, L. D. and Steigmann, A. E. (1958). U.S. P. 2,834,683.
 Coretti, K. and Muggenberg, H. (1968). *Fleischwirtsch* **48**, 623-9.
 Croome, R. J. (1953). *J. appl. Chem.* **3**, 280-6.
 Dahlberg, A. C., Carpenter, D. C. and Hening, J. C. (1928). *Indust. Engng. Chem.* **20**, 516-26.

- Downey, T. B. (1923). *Indust. Engng. Chem.* **15**, 602.
- Downey, T. B. (1950). *Pennsylvania Mfg. Confects' Ass., 4th Prod. Conf.*, 31-7.
- Duck, W. N. (1960). *Pennsylvania Mfg. Confects' Ass., 14th Prod. Conf.*, Section III, 1-9.
- Duecker, W. W. (1926). *Mfg. Confect.* **6**, (11), 28-30.
- Edwards, P. R. (1931). *Fd. Technol., London*, **1**, 100-2.
- Eldridge, J. E. and Ferry, J. D. (1954). *J. phys. Chem.* **58**, 992-6.
- Fiene, F. and Blumenthal, S. (1939). "Handbook of Food Manufacture". p. 246, Chapman and Hall, London.
- Frandsen, J. H. and Arbuckle, W. S. (1961). "Ice Cream and Related Products". Avi Publishing Co., Westport.
- Gerrard, F. (1968). *Meat Trades J.* No. 4195, 15.
- Glabau, C. A. (1955). *Bakers' Weekly*, **166**, 82.
- de Gloabec, V. C. E. (1947). U.S. P. 2,430,180.
- Goodall, H. (1959). *Brit. Fd. Mfg. Inds. Res. Ass., Technical Circular* No. 154.
- Gorfinkle, W. I. (1953). *Pennsylvania Mfg. Confects' Ass., 7th Prod. Conf.*, 54-8.
- Gorfinkle, W. I. (1959). *Pennsylvania Mfg. Confects' Ass., 13th Prod. Conf.* Section X, 1-4.
- Grettie, D. B. (1940). U.S. P. 2,196,300.
- Gunthardt, H. (1958). U.S. P. 2,819,971.
- Hall, L. A. (1949). U.S. P. 2,477,742.
- Hardy, W. B. (1926). *Proc. roy. Soc.*, Series A, **112**, No. A760, 47-61.
- Idson, B. and Braswell, E. (1957). In "Advances in Food Research", (Ed. Mrak, E. M. and Stewart, G. F.) Vol. 7, pp. 235-8.
- Ingleton, J. F. (1964). *Confect. Prod.* **30**, 365-69, 372.
- Ingleton, J. F. (1965). *Confect. Prod.* **31**, 296, 298-300, 320.
- de Jong, H. G. B. and Bank, O. (1939). *Protoplasma*, **33**, 321-40.
- Jones, N. R. (1968). In Soc. Chem. Ind. Monograph No. 27 "Rheology and Texture of Foodstuffs", p. 96.
- Karacsony, D. and Pentz, L. (1955). *Elelmezesi Ipar*, **9**, 45-52.
- King, J. A. (1952). *Pennsylvania Mfg. Confects' Ass., 6th Prod. Conf.*, 208-30.
- Klose, A. A., Mecchi, E. P. and Hanson, H. L. (1952). *Fd. Technol.*, Champaign, **6** (8), 308-11.
- Kramer, F. (1965). *Mfg. Confect.* **45** (9), 35-40.
- Kuhn, A. (1928). *Kolloid Z.* **46**, 299-314.
- Lampitt, L. H. and Money, R. W. (1936). *J. Soc. chem. Ind.* **55**, 88T.
- Lavrova, L., Lyaskovskaya, Y. and Ivanova, A. (1953) *Myasnaya Ind. S.S.S.R.* **24**, (4), 29-34.
- Leistner, L. (1956). *Fleischwirtsch* **8**, 118.
- Lees, R. (1960a). *Confect. Prod.* **26**, 458-60.
- Lees, R. (1960b). *Confect. Prod.* **26**, 922, 924-5.
- Lowe, B. (1955). "Experimental Cookery", pp. 178-91. Wiley, New York.
- Merory, J. (1968). "Food Flavours, Composition, Manufacture and Use". Avi Publishing Co. Westport.
- Mickevic, M., Alikonis, J. and Glabe, E. (1956). *Bakers' Dig.* **30**, 34-6, 49.
- Mitchell, W. A. and Seidel, W. C. (1958). U.S. P. 2,634,771.
- "Mogul", (1963). *Confect. Prod.* **29**, 315-16.
- Moncrieff, R. W. (1954). *Fd. Manuf.* **29**, 314-15.
- Monsanto Co. (1971). B.P. 1,249,250.
- Moran, T. (1926). *Proc. roy. Soc. Series A*, **112**, No. A760, 30-46.

- Muir, R. J. (1947). *Confect. Prod.* **13**, 20, 23.
- Musselwhite, P. R. and Walker, D. A. (1971). *J. Texture Studies* **2**, 110-16.
- Nash, N. H. (1960). *Am. chem. Soc.*, Advances in Chem Series No. 25, 45-58.
- Ostwald, W. (1922). "An Introduction to Theoretical and Applied Chemistry". Wiley, New York.
- Oury, S. J. (1933). *Fd Manuf.* **8**, 133-5.
- Rosenthal, H. (1963). *Boston Section, Am. Ass. Candy Technologists*, May 14th.
- Rothwell, J. and Palmer, M. M. (1965). *Dairy Inds.* **30**, 107-8, 118.
- Russoff, I. J. (1953). U.S. P. 2,657,997.
- Selby, J. W. (1947). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Rpts*, No. 1.
- Selby, J. W. (1951). *Food.* **20**, 284-6.
- Selby, J. W. (1955). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Rpts*, No. 65.
- Sheppard, S. E. and Sweet, S. S. (1921). *J. Am. chem. Soc.* **43**, 539.
- Skuse (1957). "Skuse's Complete Confectioner", Bush, London.
- Stainsby, W. J. (1937). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Records* No. 61.
- Steigmann, A. E. (1958). U.S. P. 2,819,970.
- Stistrup, K. and Andreassen, J. (1962). *XIVth Int. Dairy Cong. Copenhagen. Section VI: 1*, 19-28.
- Susich, G., King, A. O. and Doghotti, L. M. (1959). *Science*, **130**, 567-8.
- Taylor, A. M. (1933). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Records* No. 37.
- Taylor, A. M. (1937). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Records* No. 57.
- Taylor, A. M. (1941). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Records* No. 82.
- Taylor, A. M. (1946). *Brit. Fd. Mfg. Inds. Res. Ass., Res. Records* No. 106.
- Tice, L. F. and Blatt, W. G. (1937). *Am. J. Pharm.* **109**, 29-35.
- Tiemstra, P. J. (1964). *Fd. Technol., Champaign*, **18**, 915-27, 1084-96.
- Tiemstra, P. J. (1968). *Fd. Technol., Champaign*, **22**, 1151-2.
- Tobias, J. and Edman, G. (1951). *Dairy Inds.* **16**, 663-4.
- Volker, H. H. (1970a) *Ernährungswirts.* **17**, 320, 322, 324.
- Volker, H. H. (1970b) *Süsswaren* **14**, 510, 512, 514.
- Ward, A. G. (1958). *Brit. Fd. Mfg. Inds. Res. Ass., Scientific and Technical Surveys* No. 31.
- Ward, A. G. (1967). *Home Economics Quarterly, Rev. of Nutrition*, No. 9, 13-16.
- Williams, A. E. (1953). "Gelatine Desserts and Table Jellies". Food Trade Press, London.
- Williams, C. T. (1955). *Confect. Manuf.* **1**, 235-7.
- Williams, C. T. (1960a). *Confect. Manuf.* **6**, 155-6, 167.
- Williams, C. T. (1960b). *Confect. Manuf.* **6**, 253-4.
- Williams, C. T. and Reece, M. C. (1961). *Confect. Prod.* **27**, 731, 733, 735, 749.
- Wingard, W. H. (1967). U.S. P. 3,332,782.
- Zabik, M. E., Miller, G. A. and Aldrick, P. J. (1962). *Fd. Technol., Champaign*, **16**, 87-91.

Chapter 12

Uses of Collagen in Edible Products

A. COURTS

*British Food Manufacturing Industries Research Association, Leatherhead, Surrey, England**

I Sausage Skins	396
A. Natural Casings	396
B. Artificial Casings from Collagen	401
II Clarifying Reagent for Beer	405
A. Gelatin as a Finings Reagent	405
B. Isinglass Finings	406
C. Eucollagen Finings	407
D. The Finings Reaction	407
E. Summary on Beer Finings	409
F. Fundamental Properties of Isinglass	409
References	412

Other chapters in this book will make it abundantly clear that collagen is a structure protein and is a major component of mammalian tissue. Animal husbandry leading to meat processing leaves a number of valuable collagenous materials at the end of the line, but the chapter of uses will inform as to the great versatility to which the solubilized forms can be put.

It is not always appreciated that the precursor protein, collagen occurs natively in other forms and plays an important part certainly in three commercial areas, two of which come under the heading of edible products. The third form, the surgical suture is still utilized by the body and can be coped with, even in the crosslinked state, by phagocytosis. It might be added that substantial quantities of collagen protein are consumed unobtrusively in association with the muscle protein in meat and meat products.

Two forms of collagen are processed for association with food products. These are (I) intestine from cattle, sheep and pigs, which are utilized as casings or skins for sausage and hence may be defined as a packaging material

* Present address: Imperial College of Science and Technology, London, England.

and (II) the outer tunic of the swimbladder of the sturgeon and other large fishes which in its acid dispersed state is the isinglass preparation used in the clarification of beer and wines. In this sense, it is behaving as a flocculant.

I. SAUSAGE SKINS

A. Natural Casings

While the sausage as we know it to-day is a relatively recent food commodity, the form of casing used derived from the viscera of domesticated animals have been utilized as packaging materials for meat products for many centuries.

The consumption of sausages in modern times accounts for about 600 million pounds of products each year in the U.K. alone. There is a similar per capita consumption in the U.S.A. taking in about 200 million pounds per annum. If one adds to this the heavy production in Germany, Hungary and Poland, the utilization of animal intestine for casings is likely to exceed one million miles each year.

These intestinal tissues are rich in collagen, as is shown in Table I. The values of Melnick and Courts (1957) were calculated from hydroxyproline determination on bovine tissues.

TABLE I. The collagen content of intestine

Tissue	Collagen (%)	Hypro N
		Total N
Large intestine	37.6	0.44
Small intestine	28.4	0.39
Stomach muscle	28.4	0.39
Stomach mucosa	71.0	0.72
Purified collagen	100	0.82

The values for collagen content quoted in Table I are based on dry, fat free and ash free values. They can only represent a guide in so far as these other components are both large and very variable between samples. The mineral component for example may be as high as one quarter of the dry weight, consisting of inorganic cell inclusions. The fat content of the large intestine and stomach muscle may account for almost half of the dry weight. This is present in two forms. It will be laid down as adipose tissue, such as the mesenteries about the gut wall. The mesenteries are the connective tissue membranes which tie the intestine, holding it in loops. Fat will also appear in the cellular structure as globules in the cells.

The average weight of the washed intestines taken from cattle between 2½

and 3½ years of age is 5.4 kgs for the large intestine and 1.4 kgs for the small intestine. About 85% of these weights are water. Bowes (1959) points out that the ratio of hydroxyproline nitrogen to total nitrogen of a proteinaceous tissue is a valuable indication of its collagen content relative to other associated protein. A value of 0.82 represents pure collagen so that the value of 0.72 for stomach mucosa suggests a relatively high collagen content for this tissue. The small and large intestines, on the other hand, may well have the collagenous fibres associated with a connective tissue protein of another type. Elastin is a possibility here. Certainly this associated material is physically restraining in its influence. The collagen in the gut is relatively unaffected by the acid swelling which is normally a strong characteristic of collagen taken from other tissues. Swelling factors for skin and tendon, for example, are demonstrated in Courts (1963).

1. Parts of the bovine tract

The division of the intestinal tract for the various packaging purposes has been described by Wang (1959). This is shown diagrammatically in Fig. 1.

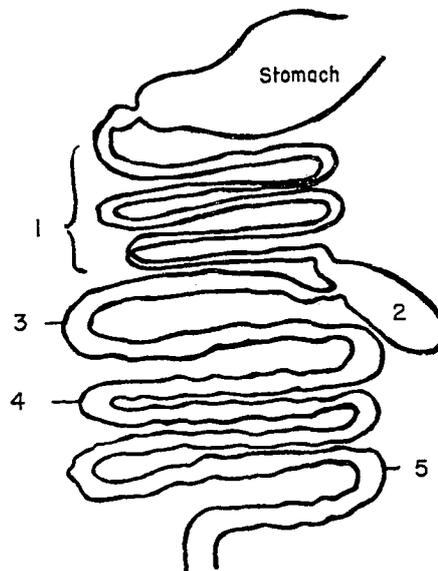


FIG. 1. Diagram of generalized beef intestinal tract showing its divisions and casings derived therefrom. The stomach shown is the last of four compartments characteristic of the stomach of ruminants. Division No. 1, small intestine, makes the "Rounds"; Division No. 2, *Caecum*, produces the "Bung"; Divisions Nos. 3, 4, and 5 are consecutive portions of the large intestine which make, respectively, "Narrow-end Middle," "Wide-end Middle", and "Fat-end Middle" (Wang, 1954). Reproduced by kind permission of the Director of the American Meat Institute Foundation.

This is a bovine intestinal tract including the fourth stomach characteristic of ruminants.

Section 1: the small intestine, giving the "rounds".

Section 2: the caecum, giving the "bung" (beef) or "cap" (pork).

Sections 3, 4, 5: the large intestine which is divided to give respectively "narrow-end-middle", "wide-end-middle" and "fat-end-middle".

Wang (1954) reports that along the complete length of the intestine the tissue architecture is unchanged, even though the wall thickness and the tube diameter show substantial variations along the length. The wall of the intestine comprises five distinct layers, as shown in Fig. 2.

- (a) The mucosa
- (b) The submucosa.
- (c) Circular smooth muscle.
- (d) Longitudinal smooth muscle.
- (e) The serosa, forming the outer lining.

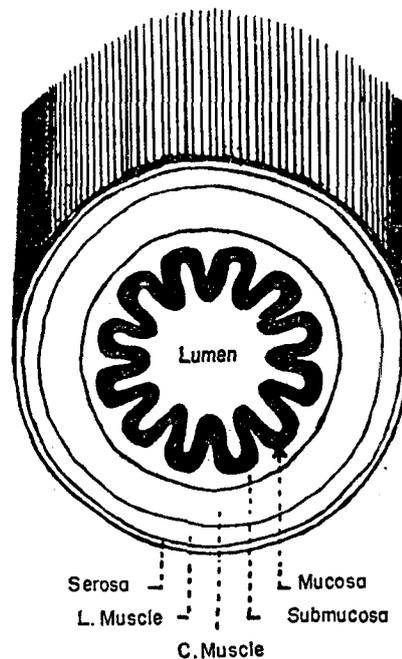


FIG. 2. Diagram of a sector of intestine with one end cut open for identification of the various structures of its wall. (Wang, 1954). Reproduced by kind permission of the Director of the American Meat Institute Foundation.

(a) *The mucosa.* The membrane of the mucosa is composed of epithelial cells some of which have gland like functions of secretion. They perform the digestion and absorption functions. Strands of muscle cells are present with some fibre attachments. Near the basal region is the lymphatic tissue, the centre of lymphocyte organization.

(b) *The submucosa.* The bulk of the submucosa contains connective tissue of collagenous and elastin forms. Other tissues, with blood vessels and portions of the digestive glands may also be associated.

(c) *The circular muscles.* These smooth muscle cells form a layer which is supported by collagenous fibres. These also act as a link with the submucosa layer and the longitudinal muscle layer. The circular muscle cells are arranged with the fibre axis around the tract. The reticular collagen fibres frequently branch from the main organization parallel to the muscle cells leading to a three dimensional network.

(d) *The longitudinal muscles.* These cells are orientated in the longitudinal direction of the tube and are somewhat patchy in grouping. This layer has a high content of collagenous fibres. This muscle layer together with the circular muscle layer alternately contract and relax in unison giving rise to progress of food matter along the digestive tract.

(e) *The serosa.* The serosa is mainly collagenous in structure, lined with a layer of mesothelia cells.

(f) *Discussion.* While the intestinal tract is represented as five layers, each layer also appears to have a mingling of collagenous fibres interconnecting with layers on each side.

There exists, therefore, a continuous network of connective tissue across the entire wall culminating in the main density of the serosa. It is this special structure which allows the intestine to play such an important part as a casing for sausage meat.

It is interesting that in some ways intestinal collagen exhibits the same type of property as other connective tissue collagens. For example, a freely suspended strip has a shrinkage temperature of about 70°C compared with about 67°C for bovine dermis. Yet when acting as a casing, that is in a condition when shrinkage is restrained, the same material can withstand temperatures of 150°C and above before shrinking or splitting.

In instances where the skins shrink or split at lower temperatures this is often due to some softening treatment carried out on the skins to improve texture.

In at least one respect, a typical collagen property is not found in intestinal collagen. This is the swelling feature, which is so prominent with skin and tendon collagens in particular, is comparatively negligible for intestinal collagen at acid pH values. Similarly bone collagen has only a limited swelling factor as shown by Courts (1963) and McKernan and Dailly (1966). These

authors have suggested the possibility of limited amounts of phosphate bridging as being responsible for this. The extensive cellular activity in the intestine compared with, say, skin dermis would suggest some possibility of an unusual crosslink in intestinal collagen.

2. *Pork casings*

The porcine intestine wall is considerably different in structure from the bovine material in that it appears to be more simply constructed. This becomes most apparent when comparisons are made between the processed (cleaned) and unprocessed gut.

With both the small intestine and large intestine the structure pattern of tissue from pig follows closely on that for beef. However, with the small intestine from pig the circular muscle layer is reported by Wang (1955) as being noticeably thicker than the layer of longitudinal muscle. Of like importance from the sausage casing aspect is the characteristic difference noted in the serosa structure. After processing (sliming) the tissue consists entirely of the connective tissue fibres of the submucosa. Thus in this process on hog intestine the serosa and the two layers of muscle cells have been stripped off. Since the submucosa from the pig small intestine has a very low fat content, the casings processed from this tissue are almost pure collagen. Similarly, the large intestine sections process to leave a relatively pure collagen network but with more fat than both the pig small intestine and the related tissue from beef. The terminal portion of the large intestine has a poor collagen structure with relatively high fat content and has little value as a casings material.

It therefore becomes evident that the continuous network of collagen fibres which permeate throughout the various layers of bovine intestine wall to give a firmly bonded structure are not similarly present in the pig intestine. Casings prepared from the latter tissues are free from the serosa layer and therefore less likely to be troubled by fat contamination. The absence of muscle cells from porcine casings represents an additional advantage over the bovine tissue. These may well be the properties which lead to the preferred performance of casings prepared from pork intestine.

Processing. The processing of intestine into natural casings is labour intensive and requires considerable hand cleaning and washing. The casings are measured off and strung into bundles. These bundles are treated with salt, which helps to preserve and partially de-water the collagen. After draining, the bundles are packed into barrels or, increasingly, plastic boxes. If kept in cool storage sites, the salted materials show no microbiological deterioration over very long periods.

A recent modification of this stage takes the casings without rubbing in salt, but packs them into brine and then freezes them (Froda, 1972). It is claimed that the process reduces the number of handling stages for both the

casings producer and the sausage manufacturer. Instead of storing casings into hanks they can be processed in a ready to use form by threading into a cylinder made from an elastic composition and then stored under a liquid preservative (Ekhammer, 1969). A variety of mechanical devices for handling casings are described in the patent literature. In particular a single support for transferring the casing length from stage to stage without handling is described by Wildenauer (1970). Reduction in bulk to ease transportation costs is possible by freeze-drying natural casings (Klimas, 1970). The moisture content can be reduced to 8–10%. A process which combines natural casing material with the artificial collagen substance is described by Jacob (1969). Natural casing was cut into strips longitudinally and a tube formed by winding around a mandrel with edges overlapping. A coating of collagen slurry can then be applied and the length dried.

B. Artificial Casings from Collagen

There are four major incentives for the production of a manufactured casing for sausages. Firstly, the growth of the sausage component of the meat processing industry could be severely restricted by the amount of suitable gut available. It could only expand if (i) carcass production increased substantially, which is probably unlikely, (ii) if the consumer could be induced to take skinless sausages, and it is not an easy task to persuade her, (iii) if an artificial edible skin could be manufactured.

Secondly, the cleaning and preparation of gut for natural casings is an unpleasant job and requires considerable manhandling and wastage. The third aspect is based on price, since the cost of the natural gut is extremely high, amounting in its prepared state, to about 15% of the retail price of the sausage pack. Finally, the uniformity of the artificial casing makes it ideal for automated sausage production.

A number of processes have been described for the preparation of an artificial edible skin, most of them based on extrusion processes, for example, the extrusion of an alginate solution into calcium chloride, or the extrusion of solubilized ground nut protein into acids. However, since the natural gut is mainly collagen, it seems reasonable that other forms of this protein should be examined for the ability to extrude into a composition sausage casing.

Two processes are successful and clearly distinguishable. The first of these has been operating over thirty years in Germany. This is a most remarkable procedure, relying very strongly on clever engineering. Hide splits are minced, washed and finely disintegrated in a mill resembling a colloid mill. The uniformity of the disintegrated fibre bundles is a critical part of the operation. This collagen suspension at about 20% solids level is then treated with an

edible organic acid—lactic acid or tartatic acid are suitable. Because of the enormous surface area presented, the swelling is very fast and gives rise to a tough collagen dough, which is extremely elastic and to the untrained eye is barely distinguishable from rubber. This dough is then subjected to a hydraulic filtering mechanism which pushes aside the collagen lumps which have not been sufficiently broken down. The dough is then fed into a ram extruder producing a continuous tube, which is inflated, air-dried and reeled. The pressures required for this extrusion are of the order of 500 atmospheres and because of the great cohesion of this collagen film, the tube is self-supporting. This collagen system is, of course, viscoelastic and therefore shows the Wiessenberg effect of flowing away from the orifice of the die. Clearly this calls for great ingenuity of die design. This product is the "Naturin" sausage casing which is made in a number of European centres. The product has never met with great success for enrobing the sausage which is to be subjected to hot oil frying conditions. The denaturation temperature of these skins is well below that for the natural skin so that in the worst instances the synthetic skin might shrink and convert into gelatin well below 150°C.

Modern processes aimed mainly at the American and U.K. markets have very little in common with this high pressure technique. Although the first stage is still the fine comminution of the hide material, very much lower concentrations of swollen acid collagen are used (for example between 3 and 6%) and corresponding lower pressures are required to extrude; perhaps only 2 to 5 p.s.i. However, tubes of collagen extruded under these conditions lack sufficient cohesion to be self-supporting. A precipitating stage is therefore required. A number of salts will do this: strong sodium chloride, sodium or ammonium sulphate, alums and so on. Some methods also claim that the presence of ammonia in the precipitating bath aids the cohesion of the film. The great bulk of this work has been carried out in the U.S. by the Johnson and Johnson Corporation, who manufacture the "Devro" casing. The research and development work emanating from this organization is built up on experience gained from the utilization of collagen systems for surgical sutures. Their publications form a highly significant contribution to collagen fibre technology.

A number of important principles are involved in the development of a successful collagen tube.

1. It is critical that the collagen fibre suspension should be homogenous and a fibre length of only a few millimetres is aimed at. Any large fibre bundle which appears in the casing is likely to be the centre of a tearing operation in the final tube, particularly while still in the wet state.

2. The addition of carboxylic acid has to be controlled to ensure uniform adsorption and swelling to avoid lump formation.

A number of acids have been described for the swelling and dispersion stage, Hochstadt *et al.* (1960) have examined cyanoacetic acid. The smaller perfluoro-carboxylics such as perfluorobutyric at pH 2 to 3 were examined by Klevens and Nichols (1960) who have shown that strands prepared from perfluorobutyric acid have greater dry strength than those prepared from malonic acid.

3. The solution mass is visco-elastic and it also shows thixotropic properties. It is clearly an advantage before it reaches the extrusion nozzle to exert work on the system in order to reduce the viscosity, and this may be achieved by introducing a shearing disc, just before the extrusion stage.

4. Even the slightest pressures exerted on to a flowing suspension of fibres tends to orientate them in a single direction, along the lines of flow. A film produced in this way would have maximum strength in the direction of flow and minimum strength at right angles to this line. Since the film needs to be uniformly strong in all directions of strain, it is an advantage to extrude through a contra-rotating nozzle which disorientates the fibres in a suitable random fashion.

5. Critical to the success of the operation is the washing process. Clearly the precipitating salts must be eliminated but in such a way as to avoid undue swelling of the film taking place. The Devro casing goes through a very extensive washing system.

6. The selection of hide plays a critical part in the operation and it is an advantage from the homogeneity point of view to utilize hide from young animals. It has also been found a strong disadvantage to use collagen from the grain layer since the swelling property of collagen from this side is inferior to the swelling characteristics of collagen from the flesh side.

It is interesting that hide is the only successful form of collagen for this process. The swelling powers of comminuted ossein collagen are very limited, (Courts, 1963), and the solution mass shows no cohesion.

7. *Introduction of cellulose fibres.* It is relevant to consider the part that meat plays in the overall system. When the sausage is cooked, it first expands then it contracts by about 20% of its original volume. While the origins of casings are lost in history, it might well go on record that the man who first used animal gut for sausage casing was something of a genius, because in most cases this material follows the meat in these exertions extremely well.

The pure artificial collagen casing is, however, not successful. As previously mentioned, they shrink to a very much greater extent than the meat and invariably split. Two distinct developments have managed to overcome this.

The Devro group of scientists (Johnson and Johnson, 1965) examined the action of filling the collagen dough with inert materials and finally alighted with tremendous success on cellulose fibres.

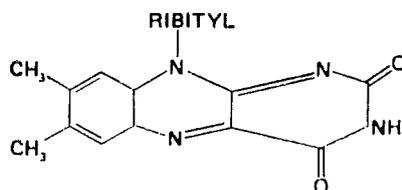
These fibres are quite short and, for example, breaking down wet Kleenex

tissue gives a suitable filling material. About 10–15% of cellulose on a dry weight basis makes a remarkable difference to the shrinkage properties of the skin and enables it to match the sausage meat more closely.

The second approach has been to reconsider the alternative raw material for sausage casings: that is the alginate casing. The major objection to alginate as a sausage skin was its complete lack of shrinkage when cooked with the meat so that in the final product the meat components had free movement inside a hard skin.

If alginate and collagen could be merged so that they were mutually compatible then the over-shrinkage of one could be counterbalanced by the under-shrinkage of the other. This has, in fact, been found to be the case by Bradshaw *et al.* (1966). A close ionic protein-polysaccharide linkage does take place and the mixture will extrude under the lightest pressures. The final skin is formed by precipitation into calcium salts. The interaction of collagen with polysaccharides is referred to later on, but certainly in the present context it is the polyuronic acid which is important. The methylated forms, as with pectin are not effective either for strong ionic linkage or for forming insoluble calcium salts.

8. Chemical crosslinking is not normally considered advisable for this product and careful attention to modulated drying can give the required final strength. However an interesting development from this work by the Devro group has been the use of light sensitive hardening agents described by Kunz (1964). This utilizes the principle that collagen can, in fact, crosslink as adjacent chains through local reactive groups and without a chemical bridge. The reaction can be photocatalysed by certain dyes, for example the isoalloxazines, or the closely related structure riboflavin 5-phosphate.



The dye needs to be of such a structure as will form only a weak complex with collagen and then be readily removable. It is claimed to be effective with gelatin so that a 2% gelatin sol treated in a similar manner produces a gel which does not melt at 70°C. The native occurrence of riboflavin with connective tissue has been reported on at least two occasions. Blair and Graham (1954) have found it in snake skin, while in a paper on sturgeon skin it was suggested by Courts (1960) that riboflavin might behave as an orientation factor in the laying down of young collagen.

Eucollagen casings

In reducing skin collagen, which is a tough structure protein, into a plastic mass suitable for extrusion, areas of resistance are invariably encountered. Lumps of collagen and strongly encased fibre bundles have to be filtered out prior to extrusion. This is minimized by using specially selected calfskin splits.

A considerable advance in the technology of producing a homogeneous plastic dough, suitable for extrusion, has arisen from the use of eucollagen for artificial casings (Courts, 1967). The process can utilize cattle splits without selection and the alkali modification generally reduces the resistance of protective fibres and membranes to give a favourable mechanical breakdown. The extruded tubes can then be treated in the same way as collagen extrusions. The ease of handling makes it a relatively cheap source of structurable material. The sausage casing is one of the most expensive packaging materials used in the food industry, representing about 15% of the cost of the finished article. Thus, any reduction of cost arising from that component could represent a useful economy.

Collagen edible film. The disintegration principles used for converting collagen into artificial sausage casings can also be used to produce a packaging film. A number of patents (e.g. Tsuzuki, 1971; U.S.M. Corporation, 1969) describe certain details for the process. The packaging of snack products, particularly dried meats and foods are described by Autry (1972). The foods are emulsified and extruded onto collagen film and covered with a second film.

II. CLARIFYING REAGENT FOR BEER

The fermentation processes which are part of the production of alcoholic drinks always give rise to colloidal suspensions which have to be removed in order to give bright and palatable liquors.

The various industries processing these beverages, whether from the grape or from apples and pears or from malt, hops and yeast all rely on reagents which are themselves natural. The two materials in common usage are both based on collagen. One of these is gelatin, the other is isinglass. They may be added to the liquors after primary fermentation to decrease the load on filtration and also to improve the colloidal stability of the beverage, particularly when filtration is not employed.

A. Gelatin as a Finings Reagent

Gelatin appears to be an admirable colloid for clarifying cider and perry. The presence of tannins in these liquors, and this can be very high in perry, aids

the flocculation but two reservations must be made. Firstly that excess gelatin will remove too much tannin and so impair the flavour and the desirable amber colour. Secondly the adoption of a good grade of gelatin may be advisable, since low viscosity gelatins can act as resuspending agents.

Both gelatin and isinglass are used in the wine trade, but for beer, at least in the U.K., only isinglass is acceptable to the trade.

However, in the United States, gelatin is very acceptable as a flocculant for beer, where most of the product is bottled. The draught beer is not as extensive as in Europe. There is no doubt that gelatin is not as efficient a finings reagent as isinglass in its reaction with beer, and it would appear that the molecular shape has some effect on this reaction. The rigid form which collagen molecules take up are certainly more effective in flocculating the suspended matter in beer, than the more random molecular form which gelatin takes up. In fact, when isinglass solutions have been exposed to long periods of standing and become, what is technically called "overcut", then they are inefficient as finings reagents. In some instances they have been known to take up the opposite effect and they behave as re-suspending agents.

Rudin (1956) believes that there should be some degree of fibrous structure in a finings for optimum effect. In order to lead to the best effects in the clarification of beer it is also likely that a wide variety of molecular weight species in the finings reagent would be valuable to the reaction. The use of gelatin in the American cellaring operation is probably two-fold; firstly it decreases the load on the filters during the primary filtration operation, and secondly it is said to improve the colloidal stability of the final product. Thus, Moeller (1964) suggests that the more sedimentation which can be brought about before the filtration stage then the less risk there is of further hazes developing during the storage of the final product. The gelatin preparation which is used for clarifying beer is prepared as a $\frac{1}{2}\%$ solution in water. This concentration is roughly the same as the protein level used in isinglass finings, but now with gelatin, no acid is employed. It is regarded as beneficial to inject the warm gelatin solution at about 60–65°C into the beer preferably by proportional pumping, since it is considered that the sudden charging of a warm solution into a cold beer stream would result in clogging of the transfer lines. By this method 70–80% of the clarity of the product can be achieved in about 5 days. Again Moeller (1964) stresses that a sedimentation period of 5 days or more is valuable in reducing the diacetyl level of beer. It is sound brewing practice not to accelerate the clarification stages unduly.

B. Isinglass Finings

It is not always appreciated that isinglass is a relatively pure form of collagen. It is derived from the tunic of the swim bladder of certain large

fishes. At one time only the Beluga sturgeon was thought of sufficient status for this important job, but after the restriction of exports of isinglass from the U.S.S.R. in 1939 a number of other fish materials have been utilized. The swim bladders are classified by names such as Penang Pipe, Saigon Leaf, Bombay Purse and Brazilian Lump.

Isinglass as imported is a dry, hard lumpy material which has to be processed for use as a flocculating agent, or as it is called a fining agent. It is allowed to swell and soften in dilute tartaric acid, or some similar edible carboxylic acid, in the presence of SO_2 . It is then broken down by beating until it disperses evenly throughout the liquor and appears to be a solution. The process is called "cutting" and may take from 2-3 days to several weeks. For use with the beer, the solution is adjusted to give 0.5% protein concentration and is used, depending on the brewery, at about 3 pints per barrel of 36 gallons or roughly 1 part per 100 of beer.

The colloiddally suspended material in beer is heterogenous in composition. It is mainly yeast, say up to 95%, occurring as $1\frac{1}{2}$ million cells per millilitre. The other components are likely to be dextrans, hop resins, polyphenols and the particle size will range from 0.1 micron to 0.001 micron. It is the variety of these components and those in solution which affect clarification. Certain agents tend to resuspend flocs, agents such as glutamine and asparagine, calcium ions and some sugars.

C. Eucollagen Finings

The eucollagen doughs referred to on p. 405 can be dispersed in citric or tartaric acids as 0.5% protein solutions. In this form they have been described by Courts and Johns (1970) as excellent finings for beer and may be regarded as a substitute for isinglass. Whereas eucollagen from ossein is unlikely to be of value for sausage casings, this material is of similar value for fining alcoholic beverages as the product from hides. Eucollagen of both types can be used to clarify wine, cider and perry as well as beer. Some of the advantages are described in Courts (1971).

D. The Finings Reaction

The action of clarifying beer by a finings reagent is one of some complexity. It will depend not only on certain factors of the beer itself, such as the strain of the yeast employed and the pH of the beer together with proportions of certain salts that are likely to be present, but these have to be viewed in association with certain of the properties of the finings reagent itself. The physical properties of the finings solution for example, particularly the

viscosity have been shown by Leach, and Barrett (1967) to be important. However in studying the colloidal nature of the reagent, it is necessary to take into account the level of small non-soluble fibres which are dispersed evenly throughout the solution. In recent years particular attention has been placed on the electrical properties of the finings and of the beer itself. For example, the question of the iso-electric point of the finings has been introduced by some authors.

Leach (1967) suggests that the isoionic point of isinglass protein may well be at around pH 9, but it is likely that the iso-electric point of finings as prepared and as affected by the buffer acids is in the region of pH 5. Thus at values below pH 5, the protein molecules will be positively charged. This is the state at which they function when introduced to beer which will be in the range pH 4-4.6. The yeast colloidal material on the other hand, the main constituent of beer which has to be flocculated, will be about pH 4. It has been suggested therefore, that it is the electrical potential between these two charges, that is to say, their zeta potential which actually gives rise to the flocculation of the yeast, induced by the finings reagent. Rudin (1958) has showed that this potential is diminished by the addition of simple salts although the intensity of this effect depends upon the strain of yeast which has been employed. Different yeasts, for example, will have different zeta potentials. The present writer takes the view that the flocculation reaction is not simply one by which the yeast cells are bound to the colloid by electrostatic forces, but rather that this interaction has the more important effect of interfering with the hydration shell of the yeast particles in colloidal form, causing dehydration of the system.

In a different context a similar type of complexing and precipitation in the collagen-alginate and gelatin-polysaccharide reactions at pH 4 was reported by Courts and Giles (1965). This paper showed for example, the way in which the orientating ability of a range of gelatins affected the formation of the flocs when reacted with chondroitin sulphate. The stoichiometric ratio of the two components was always constant but the level of hydration was proportional to the modulus of rigidity and hence the ability of the gelatin to take up the collagen fold on maturing. Similarly with the heparin complex with gelatin, the hydration effect was most pronounced when the gelatin, derived from acid pigskin, had matured for several hours.

The effect of pH was studied with the same matured pigskin gelatin using sodium alginate. These developed a complex quite rapidly in the range pH 3.5 to 6.5 but the formation of flocs was considerably delayed above this value, although sedimentation work showed a heavy molecule. Pectin, which is a non-ionic polysaccharide, did not complex with gelatin in the same way. Veis and Bodor (1965) have reached similar conclusions of the hydration of complexes between gelatin and yeast D.N.A.

E. Summary on Beer Finings

It is likely that all beer produced in the U.K. is now subjected to finings. For the draught beer trade, the finings are added at the brewery and the barrels are subjected to agitation in transit to the public house. Alternatively finings may be added to storage tanks by proportional pumping along with the beer. The flocs therefore must have the property of packing down well as a sediment after agitation. Some brewers, anticipating a rush, expect this to happen after a few hours—others will allow several days.

Bottled beers and keg beers are fined as an aid to filtration giving much longer life to the filter beds. Attention may be paid to this after primary and secondary fermentation. For this purpose the efficiency of sedimentation probably is not quite as important.

The relative usage of gelatin compared with isinglass in the U.S.A. is not openly disclosed, although with a beer consumption around one quarter of the world production, each finings reagent is probably well employed.

On considering the quantity of beer in current production in the U.K. at about 1000 million gallons per annum, this may well rely on over 200 tons of isinglass as a necessary adjunct.

F. Fundamental Properties of Isinglass

It is reasonably clear from the amino acid composition of Beluga Sturgeon (*A. huso huso*) isinglass that this material is a typical fish collagen. Chemical analysis (Eastoe, 1957) demonstrating this is given in Table II. Compared with a mammalian collagen the total for imino acid residues, proline and hydroxyproline, are typically low at 184 compared with 232 residues, while the total hydroxyl groupings were high at 173 compared with 157 or thereabouts expected for mammalian collagen.

The shrinkage temperature of sturgeon isinglass tissue in free suspensions was 50°C, again in the range of fish collagen values rather than the mammalian region of 60–70°C. A range of T_s values for commercial isinglass is given between 41–57°C. This isinglass is readily convertible into gelatin by warm water extraction particularly at mildly acid pH values. An isinglass gelatin prepared by Courts (1966) at 60°C and pH 4.5 had a modulus of rigidity at 60,000 dynes/cm² at 10°C, which is on par with a bovine gelatin extracted in the same way.

Leach and Barrett (1967) have carried out a detailed evaluation on the intrinsic viscosity of various finings preparations in order to assess the relationship between this property and finings efficiency. Each reagent was divided into 2 parts for these evaluations, with the aliquot intended for viscosity work subjected to centrifugation. In these latter preparations the

TABLE II. Amino acid composition of ox skin and Beluga Sturgeon swimbladder

	Ox skin collagen	Sturgeon swimbladder collagen
Alanine	99.6	118.9
Glycine	338	337
Valine	27.1	18.0
Leucine	39.9	17.7
Isoleucine		11.4
Proline	122.3	102.2
Phenylalanine	14.1	14.1
Tyrosine	5.1	2.4
Serine	29.9	50.5
Threonine	17.9	29.2
Methionine	5.0	8.8
Arginine	46.0	52.4
Histidine	4.5	4.8
Lysine	28.6	21.8
Aspartic Acid	44.0	47.5
Glutamic Acid	71.7	70.5
Hydroxyproline	99.6	82.0
Hydroxylysine	6.3	10.7
(Amide Groups)	43.9	41.0

TABLE III. Intrinsic viscosity of finings

Isinglass source	(dl/g)	
	Solution as prepared in tartrate	Reconstituted fibres in citrate
Long Saigon Leaf	24.5 (88%)*	22.4
Round Saigon Leaf	25.7	
A	38.5 (91%)	25.6
	37.9	
B	35.7 (93%)	21.4
	36.1	
East Indian Leaf	24.7 (90%)	19.7
Brazil Lump	29.9 (88%)	
Purse	23.0	
Karachi Leaf	27.8	
Penang Leaf	19.5	

* The percentages in parentheses give the proportion of reprecipitable material.

soluble collagen represented 88–95% of the soluble protein present in the clarified solution.

The intrinsic viscosity values are given in Table III. They are certainly as high as values normally given for soluble collagen from a variety of sources and for some samples the values must be amongst the highest recorded. A measure of aggregation seems likely in these samples since the reconstituted fibres obtained from solution by dialysis which were then redissolved in citric acid gave viscosities at about two thirds of the value shown by the original extracts.

The viscosity stability of the three samples so investigated was very good, being unchanged at 40°C after 6 weeks. Thermal denaturation of these collagens gave the typical severe reduction in viscosity indicating transformation to gelatin, although perhaps surprisingly, to high molecular weight forms of gelatin.

The relationship of intrinsic viscosity to finings efficiency is not clear cut. There appears to be a smooth relationship between the viscosity of finings, as represented by the citrate soluble fractions, and the residual haze in the beer and this is demonstrated in Fig. 3.

However, there is a lack of relationship in the finings as prepared between the viscosity and the residual haze value it imparts. Where the relationship,

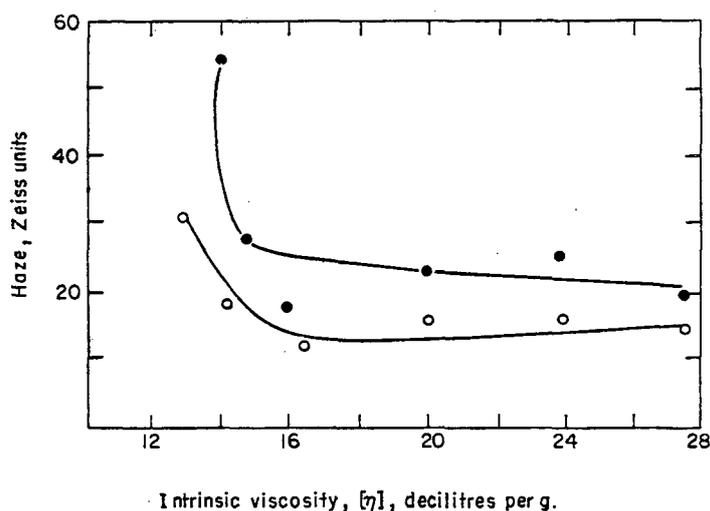


FIG. 3. The relationship between the intrinsic viscosity of finings from blends and single types of isinglass and the residual haze in a pale ale.

- 1 Pint of findings per barrel.
 - 2 Pints of findings per barrel.
- (Leach and Barrett, 1967)

such as is shown in Fig. 3 exists then it applies mainly to samples where the viscosity is smaller than 16 dl/g, but levels off above this. Where the quality of the beer is such that haze readings after finings are high, then the significant level of viscosity is about 20 dl/g before the series plateaus.

Leach and Barrett (1967) place particular importance on the true viscosity of the fibre-free solution, rather than the finings as prepared, in showing up the true potential of the type of isinglass as an efficient finings reagent.

REFERENCES

- Autry, R. F. (1972). U.S.P. 3,664,849.
- Blair, J. A. and Graham, J. J. (1954). *J. Biol. Chem.*, **56**, 286.
- Bowes, J. (1959). *J. Soc. Leather Trades Chem.* **43**, 203.
- Bradshaw, N., Schram, C. J. and Courts, A. (1966). B.P. 1,040,770.
- Courts, A. (1960). *Nature* **185**, 463.
- Courts, A. (1963). *J. Soc. Leather Trades Chem.* **47**, 213.
- Courts, A. and Giles, B. (1965). In "Structure and Function of Connective and Skeletal Tissue" (S. Fitton Jackson and R. D. Harkness, Eds.), p. 235.
- Courts, A. (1971). B.P. 1,082,852.
- Courts, A. and Johns, P. (1970). B.P. 1,184,502.
- Eastoe, J. E. (1957). *Biochem. J.* **65**, 363.
- Ekhammar, J. A. (1969). German P. 1,492,550.
- Froda, R. H. (1972). B.P. 1,273,313.
- Hochstadt, H. R., Park, F. and Lieberman, E. (1960). U.S.P. 2,920,000.
- Jacob, H. (1969). German P. 1,492,717.
- Johnson and Johnson, Inc. (1965). B.P. 991,183.
- Klevens, H. B. and Nichols, J. (1960). U.S.P. 2,919,998.
- Klimas, G. (1970). German P. 1,812,370.
- Kunz, E. (1964). U.S.P. 3,152,976.
- Leach, A. A. (1967). *J. Inst. Brewing* **8**, 73.
- Leach, A. A. and Barrett, J. (1967). *J. Inst. Brewing* **73**, 246.
- McKernan, W. M. and Dailly, S. D. (1966). In "Third European Symposium on Calcified Tissues" (H. Fleisch, H. J. Blackwood and M. Owen, Eds.), p. 171.
- Melnick, S. and Courts, A. (1957). Unpublished Information.
- Moeller, W. M. (1964). *Brewers Digest* p. 66.
- Rudin, A. D. (1956). *J. Inst. Brewing* **62**, 4141.
- Rudin, A. D. (1958). *J. Inst. Brewing* **64**, 392.
- Tsuzuki, T. (1971). B.P. 1,219,463.
- U.S.M. Corporation, (1969). B.P. 1,175,622.
- Veis, A. and Bodor. (1965). In "Structure and Function of Connective and Skeletal Tissue" (S. Fitton Jackson and R. D. Harkness, Eds.), p. 228.
- Wang, H. (1954). *Am. Meat Foundation, Bulletin No. 17*.
- Wang, H. (1965). *Am. Meat Foundation, Bulletin No. 25*.
- Wildenauer, A. (1970). German P. 1,807,015.

Chapter 13

Technical and Pharmaceutical uses of Gelatin

MRS. P. D. WOOD

Gelatin and Glue Research Association, Birmingham, England

I Introduction	414
II Pharmaceutical and Medical Uses	415
A. Capsules	415
B. Tablets and Pastilles	416
C. Gelatin Dressings	416
D. Gelatin Sponge	417
E. Surgical Powder	417
F. Suppositories	417
G. Medical Research	417
H. Plasma Expanders	418
III Microencapsulation	419
IV Sizing of Paper	422
V Emulsification	424
VI Flocculation	425
VII Printing Applications	426
A. Carbon Printing	426
B. Collotype	427
C. Silk Screen Printing	427
D. Photogravure	427
E. Wash-Off Process	428
F. Printers Rollers	428
VIII Coated Abrasives	429
IX Adhesive Uses	430
A. Gummed Paper Tape	431
B. Carton and Box Manufacture	431
C. Furniture Industry	431
D. Bookbinding	432
E. Modified Adhesives	432
X Miscellaneous Uses	433
A. Gelatin as a Sizing Agent	433
B. Manufacture of Golf Balls	434
C. Diffraction Gratings and Holographic Plates	434

D. Cork Compositions	435
E. Paper Gaskets	435
F. Bacterial Culture Media	436
G. Electrolytic Metal Refining	436
References	436

I. INTRODUCTION

Gelatin is one of the most versatile natural products known. It is a material with a wide range of physical properties and it is these properties which are responsible for the numerous and varied applications in which gelatin plays an important part. The type of gelatin for a particular application must be chosen with care as in some cases inferior products may result due to a gelatin with the wrong characteristics or wrong grade being used.

Possibly the most important single property is the strong reversible gel-forming characteristic which is exceptional among polymers. This property is important in film formation in, e.g. the sizing of paper and textiles where uniform films are produced by drying the gelatin gel., The sol-gel transition is an important feature in the use of medicated pastilles, capsules and suppositories.

Flexible films are formed by the addition of plasticizers such as glycerol and sorbitol, the flexibility being controlled by the ratio of plasticizer to gelatin. Elasticity imparted to gelatin by plasticizing is utilized in printers rollers.

Gelatin is unusual in that it is capable of acting both as a protective colloid and as a flocculating agent depending upon the conditions employed. Thus, it acts as an emulsifying agent in many cosmetics, pharmaceuticals, water paints and disinfectants while small concentrations of gelatin are utilized as a flocculating agent in the extraction of uranium ore. Viscosity is another important property in many of these uses. In many forms gelatin acts as the stabilizing agent. In the electroplating and electrolyte metal refining industry gelatin is used to control the crystal growth.

Low molecular forms of gelatin in particular have the special property of tackiness and adhesion—characteristics that are of primary importance in gummed paper, packaging, abrasive cloths and paper, and many wood-working applications.

Varied chemical activity makes gelatin easy to cross-link and this further extends the number and type of uses. In addition gelatin may be chemically modified, e.g. to introduce the property of coacervation which is made use of in microencapsulation—a technique which is being increasingly used for a wide variety of applications.

The protein nature of gelatin is important in its use as a plasma substitute or as a bacteriological medium.

Lists of the known uses of gelatin appear to be never-ending and yet new uses are constantly being found. Some of the uses of technical and pharmaceutical gelatins are described in detail below but no claim is made that the coverage is in any way complete.

II. PHARMACEUTICAL AND MEDICAL USES

A. Capsules

The pharmaceutical industry uses approximately 6.5% of the total production of gelatin, of which capsules account for the largest proportion. Since the invention of capsules by the French pharmacist Mothes over one hundred and thirty years ago an expanding industry has developed. Mothes dipped a small leather bag filled with mercury into a solution. On removal of this bag, he allowed the gelatin film to dry, removed the mercury and subsequently the leather bag leaving the first crude capsule. The dipping method of capsule manufacture has evolved directly from his discovery. This method, now utilizing steel pins is the basis of today's large-scale production of hard pharmaceutical capsules, although it still has limited use for the manufacture of soft capsules for such purposes as lighter fuel.

In 1932 R. P. Scherer developed the first continuous method of encapsulation—the Rotary Die Method—which at a later date was refined to make it a completely automatic process. In this method the material to be encapsulated is injected between two sheets of plasticized gelatin at the exact instant when the capsule is formed between two revolving dies or rollers. Much of the moisture is removed from the capsules by a series of five heating stages followed by further drying at ambient temperature. This process produces many of the so-called "soft" or elastic capsules which are widely used for a variety of medicinal products. The Rotary Die Method has the advantage of producing a capsule which has an accurate dosage (approximately $\pm 1\%$) and involves no wastage of either encapsulated material or plasticized gelatin which can be melted and re-used. These capsules are hygienically filled and there are no air inclusions making oxidation impossible. This is of particular importance in the encapsulation of vitamins which can remain stable for several years when packaged in this form.

Photochemical deterioration of light sensitive substances may be prevented by incorporating suitable dyes in the gelatin composition. Capsules may be manufactured in many shapes and sizes and possess the further advantage of being easy to swallow since on contact with the saliva in the mouth the outer-most layer dissolves coating the capsules with a layer of gelatin solution which acts as a lubricant.

Hard gelatin capsules consist of pure gelatin and small quantities of edible dyes in two interlocking parts. They are manufactured on a large scale to very close tolerances. Hard capsules possess the following advantages over tablets:

- (i) A capsule dissolves in the stomach within a few minutes whereas tablets often remain whole until reaching the small intestine.
- (ii) Gelatin capsules remain stable on storage whereas tablets may deteriorate on long storage.
- (iii) Most solid substances can be contained in them.
- (iv) They are tasteless.

Gelatin capsules on treatment with formaldehyde become insolubilized rendering them enteric, i.e. resistant to disintegration by acids and enzymes in the stomach. Disintegration then occurs in the small intestine where the encapsulated medication is absorbed.

A medium grade gelatin is normally used for soft capsules whereas a gelatin having a high gel strength is most suitable for the manufacture of hard capsules.

Microencapsulation is also used to control and prolong the release of drugs into the gastrointestinal tract (Paradissis and Parrott, 1968) over a period of time. Accurately controlled microcapsules can be manufactured which may be filled into hard capsules or alternatively form one of the components of tablets.

B. Tablets, Pastilles etc.

Gelatin acts as a binding and compounding agent in the manufacture of many tablets. The medicated ingredients are usually dispersed in a gelatin solution to obtain complete mixing and then dried. The dried compound is then ground to a powder which is subsequently compressed into tablets. A gelatin finishing coat may also be applied to tablets, the advantages being the protection of the medication and the masking of unpleasant tastes.

Glycerinated gelatin is extensively used as a base for medicated pastilles where it serves as a binder of the ingredients, a soothing agent for the throat and supplies the medicant over a period of time. Gelatin and gum arabic is another pastille base.

C. Gelatin Dressings

Many specialized protective dressings such as zinc gelatin (British Pharmacopoeia, 1963a) or Unnas paste which is used in the treatment of ulcerated varicose veins, contain gelatin as an important ingredient. The medication which consists of finely sifted zinc oxide, gelatin, glycerol and

water is applied to the wound in liquid form at body temperature. Pastes containing various medications are used to hold dressings in many cases where there is sensitivity to ordinary adhesive tapes and bandages. Gelatin thus functions as an adhesive in this application.

D. Gelatin Sponge

A haemostatic sponge is formed when a sterile solution of gelatin is whipped into a foam, rendered insoluble by treatment with formaldehyde and then dried. After cutting into various sizes it is further sterilized by dry heat (British Pharmacopoeia, 1963b). Increasing use is being made of this sponge in surgery as an absorbable local haemostatic sponge which may be produced in various degrees of flexibility. Proteolytic enzymes completely digest the sponge which can therefore be left in position as the tissue heals. The sponges may also be used to implant a drug or antibiotic directly into a specific area. Blain (1951) has shown that the action of thrombin and penicillin is not inhibited by gelatin sponge and it does not cause tissue irritation or foreign body reactions.

E. Surgical Powder

After treatment at 142°C for approximately one day gelatin loses its tackiness in cold water and in this form it may be used as a sterile dusting powder for surgical gloves. On contact with open wounds it improves healing as it possesses properties similar to gelatin haemostatic sponge.

F. Suppositories

Glycerinated gelatin finds another use as a base for suppositories where it is superior to other materials. The nature of the base greatly influences the effectiveness of the drug and it has been shown (Tice and Abrams, 1953) that certain antiseptics are effective against *S. Aureus* and *S. Typhosa* when glycerinated gelatin is the vehicle but ineffective with other bases. Although in most cases either a limed hide or an acid-pigskin gelatin is satisfactory, compatibility of the gelatin with the added medications must be considered as in a few cases, one type of gelatin is superior to the other.

G. Medical Research

Recently, use has been made of gelatin in medical research to separate viable lymphocytes from human blood. Coulson and Chalmers (1964) used gelatin as a sedimentation agent for defibrinated blood and subsequently

recovered the lymphocytes from the supernatant liquor. This method, which is now widely used, was found to be quicker than other methods with little risk of cell trauma.

H. Plasma Expanders

The protein character of gelatin is important in its use as a plasma expander. Although there is no substrate for blood in cases of very severe shock and injury, less severe cases can be treated with a plasma substitute which restores circulatory blood volume in emergencies without the danger of contamination with the virus *S. Hepatitis*.

Dilute solutions of specially selected gelatins have been successfully used as plasma expanders. The selected gelatin must be an absolutely sterile uniform product which is non-antigenic, pyrogen-free and capable of maintaining osmotic pressure. It has been shown (Cambell, 1956) that the osmotic pressure of gelatin, and other macromolecules, is dependent on the number average molecular weight. Thus the effectiveness, and retention time, of the plasma expanders is dependent on the size of the molecule—high molecular weight preparations showing greater retention than the low molecular weight materials which pass readily through the capillary walls. Once an effective material has been prepared, subsequent batches should have the same molecular weight distribution.

Gelatin as a plasma substitute is effective for 24–48 hours and in general no adverse effects on the body tissues are recorded, although transient swelling of the kidneys has been reported by Hartman and Behrmann, (1953). Some of the gelatin is metabolized in the body although different workers report varying percentages. Macromolecules such as gelatin interact with erythrocytes giving rise to an increased sedimentation rate and interfering with blood typing. This however causes no ill effects.

Gelatin is readily available at a low cost—an important consideration particularly in times of national emergency. Solutions which are normally 3–6% can be made to vary widely in their physical properties. The viscosity of the solution is important for parenteral use. The main limitation of gelatin as a plasma substitute is its low setting or gelling temperature. Thus at a concentration of 3% it exists as a gel at room temperature. Before administration the gel must therefore be warmed to body temperature. This limiting feature of gelatin as a plasma substitute has led to extensive research to modify the gel point while at the same time preserving its advantageous properties. Degradation of the molecule by physical, chemical or enzymic means has been unsuccessful for although the gel point may be depressed below normal room temperature, the retention time of the product is also reduced.

Numerous chemically modified forms of gelatin have been described for this use. Oxypolygelatin which was developed about 1945 is formed by a condensation reaction of gelatin with glyoxal until maximum viscosity is reached, followed by oxidation with hydrogen peroxide. Some molecular degradation also occurs. This product overcame the gelation problem and has been successfully used as a plasma expander. The reaction is described in Chapter 7.

The ability to modify the isoelectric point of gelatin thereby increasing its effective osmotic pressure by changing the relative proportions of free amino and free carboxyl groups is used in the preparation of a group of gelatin derivatives suitable for plasma expanders (Charles B. Knox Gelatin Company, Inc., 1955). These derivatives are produced from reactions of gelatin with poly-aliphatic carboxylic acid anhydrides or chlorides, e.g. succinic anhydride or succinyl chloride. After fractionation, all the above products were found to possess properties suitable for plasma expanders but the succinyl product was evaluated in detail (Tourtellotte and Williams, 1958). Another plasma expander—Haemacol—has been prepared by cross-linking degraded gelatin with urea bridges (Moeller and Skyudes, 1962). Reports of two years' experience with Haemacol (Froeschlin, 1962) show no adverse effects. A more recent modified gelatin consists of inter- and intra-molecular cross-linking peptide bonds between the free carboxyl and amino groups (Biotest Serum Institute, 1963). Preferential decomposition by splitting some of the original peptide bonds results in a product with spherical molecules of suitable molecular weight, the solution of which is liquid at normal temperatures.

III. MICROENCAPSULATION

Although pharmaceutical and other materials have been packaged by encapsulation for over 150 years, it was not until the 1950's that the technique of microencapsulation was first introduced. Microencapsulation may be defined as the process of enveloping a microparticle or nucleus in a continuous polymeric phase. Capsules containing liquids are usually spherical whilst those containing solids take the form of the solid particle. The size range for these microcapsules is somewhat arbitrary the lower limit being of the order of a few microns.

Microencapsulation was first introduced commercially as the now well-known carbonless "carbon" paper (Green, 1955) in which a suitable dye material enclosed in microcapsules is coated onto a sheet of paper. When the capsules are ruptured by stylus, pen or typewriter key blue marks are formed on contact with the surface of a special backing paper sensitized

with an acid-clay material such as attapulgite. The printing substance encapsulated was 3,3 bis (p-dimethylamin-phenyl)-6-dimethylamino phthalide dissolved in the oily trichlorodiphenyl. The immediate blue colour tended to fade with time. A secondary colour reactant benzoyl leuco methylene blue developed a coloured form after a few hours making the record permanent.

Coating for pressure-sensitive recording paper consisting of a rupturable continuous gelatin film containing droplets of the oily printing fluid had previously been described (Green, 1945) but the introduction of microcapsules considerably improved the durability and storage characteristics. The microcapsules adhere to the paper and each other in such a way that cracks formed in the coating by folding the paper run between the capsules and not through them. Thus the oily fluid is not released by random cracks in the film but only on intended rupture.

A method of making microscopic oil-containing capsules for this and other uses has been described (National Cash Register Co., 1956). The capsules are formed by preparing aqueous sols of acid pigskin gelatin (iso-electric point of pH 8) and gum arabic and emulsifying the oil to be encapsulated in one of them. After mixing the resulting emulsion and sol, coacervation is brought about by dilution or adjustment of the pH of the mixture. The complex colloid material is thus deposited around the oil droplets and subsequently gelled to form the microcapsules. Necessary conditions are that two colloid ions in the mixture before coacervation possess different electric charges and one or both of the colloids must be capable of forming a gel. Oil-containing microcapsules may also be produced by a salt coacervation process using gelatin as a single hydrophilic colloid sol and causing coacervation by adding a salt solution to the emulsion.

The gelatin coat may be hardened with formaldehyde or glutaraldehyde to increase the resistance to heat and moisture. When the capsules are dispersed in a large volume of water they may exist individually but on drying they form an agglomerate which may be ground and washed to remove any oil that has escaped from accidentally ruptured capsules. The finished product before drying may however be used for coating, the microcapsules adhering to the substrate on drying.

A method of encapsulation has been recently reported (Veis *et al.*, 1967) using an acid-precursor gelatin (having an isoionic pH of 9.0) and an alkali precursor gelatin (having an isoionic pH of 5.0). The potential for this technique lies in replacing gum arabic which is more expensive than gelatin.

Chemically modified gelatin has also been used to produce microcapsules (Clark *et al.*, 1967). The chemical modification renders the gelatin sufficiently hydrophobic to coacervate from an aqueous sol without the aid of a

second colloid simply on adjustment of the pH to around 4.3 (see also Chapter 7).

Any water-immiscible oils which are inert to the encapsulating medium and also capable of forming emulsions may be encapsulated by the processes outlined above. Although water-miscible liquids can not be encapsulated by these methods, it is claimed that they can replace encapsulated oily nucleus materials by molecular diffusion through the capsule walls (N.C.R., 1964). In this process the capsules are stirred in an excess of the liquid polar vehicle which must also be a solvent for the originally encapsulated oil.

Since the introduction of microencapsulation the tremendous potential for this technique has been realized in many industries where it is desirable to maintain a material in an inert state until required. Numerous patents utilizing and improving the technique demonstrate its value in consumer products and the research field. Some advantages of microencapsulation using gelatin are:

- (i) Unpleasant tastes may be masked.
- (ii) The capsule wall protects many substances from oxidation.
- (iii) Liquids may be converted to a free flowing powder containing a high percentage of liquid. In this way liquids may be handled as solids.
- (iv) Release of the encapsulated material may be controlled.
- (v) Physical separation of reactive materials may be achieved until mixing is desired.

Materials encapsulated by gelatin may be released in the ways listed below:

- (a) Increase in pressure.
- (b) Increase in temperature.
- (c) Generation of gas from inside the capsule.
- (d) Dissolving the capsule wall.
- (e) Diffusion of the internal phase through the capsule wall.

It is not possible to list here the many uses claimed for microcapsules but a few of the applications may be considered in brief. Heat-rupturable core containing capsules have been used in fire-extinguishers (N.C.R., 1961). Carbon tetrachloride or perchloroethylene is encapsulated and automatically released by the gas pressure which develops within the capsule walls by the heat of the fire to be extinguished.

Solvents may be encapsulated which on the application of heat are released to activate a potentially adhesive material. For example an "adhesive" tape coated with a suitable capsule may be activated by means of a hot iron.

Gelatin microcapsules find a use in the laundry industry where they are used to delay the introduction of bleach-sensitive brighteners into a laundry

bath containing a bleaching material (N.C.R., 1963). The brighteners are protected by the gelatin until the deleterious effect of the bleach is reduced. This application makes use of the fact that gelatin may be case-hardened by subjecting the cross-linked capsules to a prolonged dry heat-treatment. The outer skin of the capsule is thus chemically modified to a depth proportional to the heating time.

A zinc chromate corrosion-inhibitive primer has been encapsulated using a gelatin-carrageenan system for use in the aircraft industry (Dalton, 1965). A serious exfoliation corrosion problem has been solved by bonding the microcapsules (using an alkyd resin) to places which are highly prone to corrosion.

Transport problems have been solved by microcapsules. Briquettes containing approximately 95% petrol have been formed. In this form petrol can be transported without containers and recovered later by compression in a screw press.

IV. SIZING OF PAPER

Tub-sizing or surface sizing with gelatin has been used in paper making for a great many years—indeed paper makers formerly processed their own gelatin for this purpose. With the demand for accurately defined technical paper, manufacturers began to buy their gelatin stating the desired properties for their sizing agents. Davidson and Bodenhausen (1959) investigated gelatin characteristics and testing methods necessary for paper sizing.

Gelatin is used today for hand-made and almost all high quality paper, in particular all-rag-content papers. Either alone or as a starch/gelatin mixture it is also used as a sizing agent for all-wood or rag/wood papers. Hand-made papers are usually sized with a 7–10% solution of gelatin containing 3–5% alum. For high quality rag, wood and esparto papers a 4–8% solution is used containing 3–5% alum and 0.1–0.75% formaldehyde to harden the gelatin after drying. The average paper takes up approximately 5% of its weight of tub-size when dried. Most grades of gelatin may be successfully used for tub-sizing but economic considerations often dictate a low to medium grade. Even so one paper mill uses a bone gelatin (Barker, 1962) which is only just below edible quality.

The functions of a good gelatin size for papers is to impart the following properties:

- (i) Increased wet and dry strength and resistance to surface abrasion. The dry strength reflects the folding properties.
- (ii) Reduction in surface fluff.
- (iii) It ensures the desired degree of resistance to ink and oil.

- (iv) Surface quality.
- (v) Rattle, i.e. the crackling noise when paper is handled.

Paper makers are able to control the surface and bulk properties of their paper by accurate control of such factors as the viscosity of the sizing bath and the time of application. Thus a relatively high viscosity and a short time of application gives rise to a product with a high concentration of gelatin at the paper surface. On the other hand by using a low viscosity material and a longer time of application it is possible to control the bulk characteristics of the paper. Drying must also be carefully controlled as this affects the resistance of the surface to liquids.

Characteristics such as high resistance to liquid penetration and surface abrasion are important in photosensitive and technical paper, e.g. blueprint, chart and currency papers. In these cases the ability of gelatin to become insolubilized by treatment with cross-linking agents is utilized. Paper suitable for blueprinting must be free from chemical impurities and contains 25–100% rag-stock. Care should be taken that the gelatin size-coat imparts the correct degree of hardness and absorbency to the paper. The degree of penetration of the sensitizing solution is important and may be controlled (Hanson, 1958) by using a gelatin size containing a cross-linking reagent together with magnesium hydroxide.

A water resistant gelatin size-coat in chart paper permits slow drying inks to be used without feathering occurring.

Gelatin may be used either alone or in combination with other adhesive materials, e.g. casein, starch and soya protein, in clay and pigment coatings for paper. The purpose of this coating is to ensure a level surface by filling up the small surface indentations of the paper with mineral to ensure good printing reproduction. Applications for coated papers include playing cards, posters, wallpapers—both washable and non-washable—paper used in glossy magazines and many cartons used in packaging.

All grades and types of gelatin are suitable for use in the coated paper industry although for most uses a low grade is usually chosen. For special coatings however a higher grade is used whilst for photographic (baryta) coated papers only a photographic grade is suitable to avoid fogging. When calcium carbonate is used as the pigment it is claimed that hide materials are superior to bone gelatins giving foam-free coatings (Tutt and Lane, 1961).

Gelatin is compatible with pigments and, due to its amphoteric colloidal properties, is capable of combining with anions or cations depending on the pH of the system. It has a high adhesive strength and the coating imparts a smooth glossy surface to the paper which remains flexible and does not crack upon drying. Clay is usually used either alone or in combination with other pigments such as titanium dioxide, calcium carbonate and satin white

(a mixture of alumina and calcium sulphate). The rheological properties of the coating solution are extremely important (Tutt and Lane, 1961) different properties being required for machine coating than for off-machine coating. A thixotropic material with a high solids content (65–70%) is required for on-machine applications. The coating is applied to the paper by means of rollers and breaks down to a fluid consistency at the high rates of sheer encountered. At lower machine speeds a lower solids content is required.

In off-machine coating the solids content may be as low as 30%, the percentage varying with different coating machines. In this process the coating mixture normally possesses near Newtonian properties.

Preparation of the coating material entails dispersing the clay in water in the presence of a de-flocculating agent such as sodium phosphate or sodium silicate, the percentage of de-flocculant required depending on the type of glue or gelatin. The gelatin solution is then added to the suspension. When gelatin is used in combination with other proteins it is important to add these materials after the gelatin has been incorporated into the slurry to ensure that the conditions are correct for optimum fluidity (U.S.P., 2,513,121). This patent describes the preparation of coatings in detail, the final pH of the suspension being in the range of 8–9.5.

The moisture resistance of gelatin coatings may be improved by washing in a hardening solution, a property which is utilized in the production of washable wallpaper. Gelatin may also be used with synthetic latex emulsions in pigment coatings.

V. EMULSIFICATION

Gelatin is an excellent emulsifier and stabilizing agent for many emulsions and foams. This property is due to the ability of gelatin, as a protective colloid, to form a film around oil droplets. Only small amounts of gelatin are required for emulsification but careful selection of type of gelatin and rigid control of pH conditions are vital for optimum results. The anionic or cationic behaviour of gelatin is important when used in conjunction with other ionic materials. For instance when gelatin is used in emulsions containing vegetable gums the charges on the two materials must be considered to ensure compatibility ("Gelatin", 1954) because precipitation takes place if the materials possess opposite charges.

Many pharmaceutical and cosmetic emulsions utilize this stabilizing property of gelatin particularly where a low viscosity product is required. More viscous emulsions are obtained by adding a thickening agent such as vegetable gum because increasing the proportion of gelatin leads to gelling at room temperatures.

The emulsifying ability of gelatin is sensitive to impurities either in the gelatin itself or in the oil phase. Kragh (1958) has examined the stability of a wide variety of oil-in-water emulsions utilizing gelatin as the emulsifying agent. Stability was examined as a function of gelatin concentration. He found that conditions unsuitable for emulsification were obtained using unrefined fat and limed hide gelatin. Acid pigskin gelatin gave better emulsions with the crude materials. Limed hide gelatin however was found to produce good emulsions with the refined oils. Poor emulsification with unrefined materials was due to the combination of the phosphatides in these materials with calcium from the limed gelatins to form a complex which inhibited emulsion. Better emulsions were thus formed with acid pigskin gelatin which is virtually free from calcium.

In a slip-coating process, used in the manufacture of refractory articles, gelatin finds another interesting use as a stabilizing agent (Smith, 1960). Without the addition of an organic stabilizing agent such as gelatin the aqueous slurry (or slip) of finely divided refractory material and colloidal silica would settle out on standing. Such suspensions containing gelatin are more viscous at low pH values and the viscosity may be controlled by pH adjustment.

VI. FLOCCULATION

With increasing controls on river pollution throughout the world, recent attention has turned to efficient and low cost flocculation agents. Although gelatin is a powerful protective colloid, under certain conditions e.g. in very low concentrations it exhibits the opposite effect and functions as a flocculating agent. It is extensively used in South Africa as a filtration aid during the extraction of uranium ore. Quartz, kaolin, montmorillonite, silicon carbide and colliery washwater suspensions are other examples of systems which may be readily flocculated by technical gelatin.

During the extraction of uranium ore, a slurry of uranyl sulphate is formed, the solid content being of the order of 65-70%. When a gelatin solution is added to this (approximately 1 lb. dry gelatin per ton of ore), the slurry at once increases in viscosity due to the formation of large aggregates which rapidly settle in a porous mass. The amount of flocculating agent required depends on the surface area of the solid, and the optimum concentration is dependent on the nature of the slurry (Kragh, 1959). With an excess of gelatin however flocculation does not take place. Kragh and Langston (1962) have investigated the flocculating power of gelatin with a number of mineral suspensions from the viewpoint of the amount of gelatin absorbed and the electrophoretic mobility of the particles. They show that the optimum

coverage of the mineral particles by the gelatin is about 33% thus supporting the theory that gelatin forms bridges between the particles to bring about flocculation. More than 99.9% of the gelatin added is shown to be absorbed under optimum conditions. Flocculation is also shown to be pH dependent. The settling rate is dependent on the log viscosity number (L.V.N.), the gelatin with the highest L.V.N. settling faster than those with lower L.V.N. Charge effects are also significant, although it appears that flocculation is not now regarded as a charge effect.

Gelatin and Sveen glue solutions are widely used as retention aids during paper manufacture. Sveen glue consists of 1% gelatin glue and 0.1% rosin size, adjusted to pH 4.5 by the addition of alum. A preservative such as santobrite (sodium-pentochlorophenol) or formic acid is also usually added. Fullerton (1961) comparing gelatin, Sveen glue and a number of synthetic polyacrylamides as retention aids shows gelatin extremely favourably on a cost-effective basis. Although a high quality polyacrylamide was more efficient its cost was almost three times that of the gelatin (gel strength 180 g Bloom).

Conditions for optimum flocculation depend on the particular material in suspension, processing conditions, pH, type of gelatin, concentration of gelatin and presence of impurities in both the suspension and flocculating agent.

VII. PRINTING APPLICATIONS

Many photo-printing methods depend on the effect of light on a gelatin film which has been sensitized by immersion in a solution of potassium or ammonium dichromate. On exposure to light the sensitized gelatin film hardens in direct proportion to the intensity of light received. This renders the exposed area insoluble while unexposed portions of the film remain soluble and may be removed by washing in warm water. In this way a film of varying thickness is produced. The chemical action of active light on bichromated gelatin is extremely complex and is not fully understood (Kosar, 1965).

A. CARBON PRINTING

Carbon printing consists of a paper tissue coated with a layer of pigmented (i.e. containing powdered carbon) gelatin which is sensitized before use by immersion in a solution of potassium dichromate. After exposure and subsequent washing the particles remain embedded in the tanned gelatin. Collotype printing which gave poor reproduction was evolved from this process.

B. Collotype

This is a direct lithographic process which is particularly noted for its high quality reproduction without distortion. It is ideal for producing art prints up to a maximum of about 5,000 copies. A collotype printing plate is prepared by coating a ground glass, zinc or aluminium plate with dichromate/gelatin to produce a uniform thin film. The drying process which is of the utmost importance results in a fine grain reticulated surface. Carefully controlled drying is carried out in an oven, the temperature being raised stepwise from 20–40°C. If the oven temperature is too high, the resulting film has a grain which is too coarse and conversely a lower temperature results in a grain which is too fine. After exposure to light through a continuous tone negative, the plate is developed to remove all traces of unreacted dichromate. During printing there is preferential acceptance of printing ink, by the exposed gelatin areas and almost total rejection by the moist unexposed highlight areas. The ink film on the finished print varies in thickness giving first class depth of colour and fine detail with complete absence of any screen pattern as in photogravure and screen-process printing.

C. Silk Screen Printing

The silk screen process consists essentially of forcing ink through a stretched piece of silk in which some areas are blocked to form a stencil. Since the advent of synthetic materials, nylon and terylene are frequently used in preference to silk. Stainless steel meshes are also used. Photostencils may be produced either directly or by an indirect method. The production of indirect photostencils is based on the carbon tissue process, the paper forming a support for the stencil until when completed it is pressed on to the screen and the temporary support removed. The main use for indirect screen printing is in publicity work. Improvements reducing the processing steps use products of a presensitized type containing ruby or magenta dyed gelatin (Kyle, 1967).

Direct photostencils may be produced photographically by impregnation of the screen mesh with dichromate gelatin which, when dry, is exposed by contact printing with a positive transparency and developed by washing. This process was developed for printing materials other than paper and is an important method in the textile and ceramic industries.

D. Photogravure Printing

This method also uses a pigmented gelatin coating on a paper base sensitized by potassium dichromate. After careful drying, the gelatin side of

the plate (paper, metal, plastic or glass) is exposed to light through a photogravure screen consisting of a matrix of equi-spaced opaque dots. The tissue is thus tanned in a grid pattern. To produce the photogravure this is exposed through a photographic positive of the desired subject. The gelatin which was at first exposed between the grid lines now becomes exposed by an amount depending on the density of the positive. The exposed pigmented tissue is then applied to a copper-printing surface and developed in warm water. The backing paper and the unexposed gelatin are removed before drying. The gelatin thus forms an etching resist controlling the rate at which an etching solution, e.g. ferric chloride, reaches the metal surface. During printing the etched intaglio surface is filled with a low viscosity ink and varying tones are produced on the printed surface by the variation of film thickness.

E. Wash-off Process

A further use of light sensitive dichromate gelatin is in the reproduction of engineering drawings. Photosensitive tracing paper is prepared by coating with a composition consisting of gelatin, potassium dichromate and a silver halide. The sensitized paper is exposed to carbon arc light under a negative which is in extremely close contact with it. After development the silver image is intensified by ordinary photographic means.

F. Printers Rollers

The printing industry has used gelatin composition rollers for about 100 years. The speed of many printing presses has increased from about 1400 to 4000 impressions per hour. This has limited the use of gelatin rollers, although they are still used today for high quality work. Gelatin rollers must be run slowly to avoid excessive heat buildup which may cause the rollers to melt. Composition rollers consist basically of a tough, elastic composition of gelatin, water and a plasticizer such as glycerol, sorbitol (which has a narrower humectant range), or a mixture of the two. Compositions vary according to the manufacturers, grade of gelatin and the flexibility required (Griffin and Almy, 1945; Griffin, 1945) but a typical formulation might contain equal parts of gelatin and plasticizer with approximately 10–20% water. A notable advantage of these rollers is that the composition may be melted and re-used—an important economical consideration. Many manufacturers incorporate a small percentage of previously used material in their rollers.

Other advantages of composition rollers are:

- (i) They are much cheaper than other rollers.
- (ii) High quality results are obtained.

- (iii) They may be tailor-made for different purposes and conditions. The shore hardness is normally in the range 14–30 although in special cases this may be much higher.
- (iv) The roller surface is unaffected by oils and oil-soluble or water-insoluble materials. Rollers may thus be easily cleaned.

Two of the disadvantages of composition rollers, viz. the susceptibility to changes in relative humidity and the tendency to melt at high speeds have been overcome to some extent by incorporating a small percentage of a cross-linking agent such as formaldehyde, hexamine or chrome alum in the mix. The melting point is thus increased whilst the composition retains its elasticity. The gelatin in this composition cannot however be reclaimed and used again although it is possible to reclaim the glycerol.

The composition is prepared by first soaking the gelatin, adding the glycerol and dissolving in a steam jacketed mixer under vacuum. The composition is run off, allowed to set in trays and then stored for several weeks to mature. The prepared metal roller stocks are held centrally in a "gatling" gun which is a water-jacketed cylinder containing about 25 accurate moulds. After moving the gun to a vertical position, a suitable equilibrium temperature is reached. The composition is remelted in a pressure kettle and then injected into the gun from the bottom to avoid trapping air. In the case of non-meltable rollers the hardening agent is added before injection. Rollers are stored for a few days to mature before use.

Another type of roller is known as the "Davis Process" roller which is a combination of soft rubber with a coating of gelatin composition about 1/10" thick. This is more stable to changes in humidity and the coating completely covers the rubber base making it impossible to absorb materials from the ink unless the gelatin surface becomes cut or damaged. The Davis roller runs at a lower temperature than normal composition rollers and can therefore be used on machines operating at higher speeds.

Soft gelatin rollers are used in roller coating particularly in the United States (Letsky, 1958). It is claimed that this process gives a good reproduction of wood grain in the finish of such items as radio cabinets, car facia panels etc.

VIII. COATED ABRASIVES

Large quantities of low and medium grade technical gelatin and gelatin glues are used in the coated abrasive industry which may be broadly split into two sections, viz.

- (i) Sandpapers and sandcloths of various shapes, sizes and types.

- (ii) Set-up abrasive polishing wheels, bobs, belts, and discs used in workshops and factories.

Gelatin adhesives are selected for their great strength, flexibility, gelling properties and also for easy machinability. The large number of widely different uses of these products in the metal and wood finishing industries has given rise to many types of coated abrasive products, the grade of gelatin being determined by the specific use of the product. For heavy work employing silicon carbide or aluminium oxide grains a high grade technical gelatin is used whereas for light polishing work (using emery, garnet or flint), low to medium grades are selected.

In the manufacture of sandpaper and sandcloths the cloth backings are frequently sized with gelatin or a starch/gelatin mixture to give increased body and strength. A gelatin film (making coat) is next applied to the paper and the abrasive grains are deposited upon it. The viscosity and gelling properties of the glue are extremely important in this step. After partial drying a dilute glue film (sizing coat) is applied to hold the grain in position. Controlled drying then yields the finished product.

The concentration of the gelatin solutions used is dependent on the quality of the gelatin and the type of abrasive grain. In general however concentrations lie between 25% and 40% for the making coat which is applied at a temperature in the region of 60°C. Fillers and extenders may be added. The size coat is at a lower concentration, approximately 10–15% of the same gelatin.

Set-up polishing wheels have a thicker coating of abrasive than sandpapers. The manufacture is basically similar however although more than one coat of abrasive may be applied and the final sizing coat is often unnecessary.

Correct drying is an important factor in the working life of set-up wheels (National Association of Glue Manufacturers, 1951). Ideally the wheels should be dried at a temperature of approximately 35°C for four to eight hours at a relative humidity of 40%. Oven dried wheels are liable to slip due to the coating being brittle while under-drying gives a short life. Modern abrasive coatings fix a layer of urea formaldehyde resin on top of the glue coat. This marries the toughness of the resin with the flexibility of the glue.

IX. ADHESIVE USES

Low molecular weight or low grade gelatins find numerous uses due to their well known properties of adhesion and rapid tack. Gelatin adhesives or animal glue are widely used in the manufacture of gummed paper tape, in the furniture industry and in the manufacture of boxes and leather goods.

Gelatin glue is used where a quick tack and strong bond are required and where at least one of the surfaces to be joined is porous. It has the advantage over most synthetic adhesives of an indefinite pot-life.

A. Gummed Paper Tape

One of the most important uses of gelatin adhesives is the production of gummed paper tape, which accounts for approximately 40% of this grade of gelatin. The tape consists of a kraft paper-backing coated with a layer of gelatin glue. Where extra strength is required a cloth-backed kraft may be used. Modern reinforced tapes include a nylon or fibreglass webbing set into the paper. The paper backing varies in quality with the desired use and the weight of adhesive applied is usually about 25% of the weight of the paper. The adhesive is coated onto the paper by means of rollers as a 50–60% aqueous solution at a temperature of at least 50°C. The subsequent drying in hot air of the tape is critical to the process, great care being taken to ensure that the glue does not gel before drying.

The re-wetting properties of the glue film determine the performance of the tape in use. This film redissolves on wetting to produce a viscous solution which immediately starts to re-set to a gel producing an extremely tacky film. By varying the formulation, grade and type of gelatin a wide range of properties may be obtained. It is common practice to extend coating glues with up to 20% by weight of starch adhesive.

B. Carton and Box Manufacture

Rapid development of tack is also important in the manufacture of cardboard cartons. Thus, the flaps of the cartons remain in position without the necessity of pressure being applied for any length of time. High speed machines can therefore be used giving a higher production rate. Gelatin glues are extensively used when a strong bond is needed making it a useful adhesive in many packaging applications.

C. Furniture Industry

Although gelatin glues have been replaced to a large extent in veneering and assembly work, their special properties ensure a continued use in processes such as chair-frame making. As synthetic adhesives have proved to be superior for high-speed production methods, gelatin adhesives are today largely used only for hand assembly processes. It is widely used in the reproduction furniture industry for high quality veneering where the use of

synthetic adhesives has resulted in cracking of veneers. In addition to its adhesive use it also acts as a filler for fine cracks and gaps giving further stability to the veneer itself. The quick tack of gelatin glue make it useful for the assembly of sub-joints. Paraformaldehyde may be used as a hardener for gelatin glue giving an adhesive having similar properties to urea-formaldehyde resins but which hardens slowly thus reducing the cracking of veneers but giving a bond which is relatively water-resistant (Leheimann, 1968).

In chair-making gelatin glues are generally considered to be vastly superior to synthetic materials of similar price. On drying the cohesion of the glue film remains showing no tendency to creep under load and is resistant to sudden shocks. The thickness of the glue-line is not critical when gelatin glues are used as in the case of many synthetic adhesives. Any bond formed with gelatin adhesives is usually stronger than the wood itself.

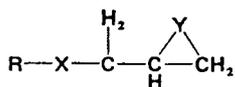
The viscosity of the glue solution is an important property in the glueing of wood as it controls the thickness of the spread layer. If the viscosity is too low, the glue will penetrate too far into the wood leaving an insufficient layer to provide a continuous film. Gel depressants, such as urea, may be added to slow down the development of tack when it is important for all joints to be glued and the furniture to be cramped before setting.

D. Bookbinding

A plasticizer is added to gelatin adhesives used in bookbinding to give a permanent tough, flexible glue line with adequate holding of the pages of the book. This flexible glue film is necessary to prevent cracking on opening and closing the book. Glycerine or sorbitol or a mixture of the two are the plasticizers usually recommended. Sorbitol has a narrower humectant range thus giving a better bond at high humidities. It is also claimed that sorbitol compositions have a higher tensile strength.

E. Modified Adhesives

Gelatin glues may be modified to give them additional properties, e.g. increased water resistance or to render them thermoplastic for use as a hotmelt adhesive. Modifying reactants having the general formula



may be used where R is an organic radical controlling the physical properties

of the adhesive, X is selected from the group consisting of $-O-$ and $-CH_2-$ and Y is selected from the group consisting of $-O-$, $-NH-$ and $-S-$ (Merrill, 1965).

Where the use of heat is impracticable gelatin glues may be formulated to remain liquid at room temperature. Gel depressants such as urea or thiourea achieve this effect, the exact formulation depending on the grade of gelatin, and the specific use (National Association of Glue Manufacturers, 1951).

X. MISCELLANEOUS USES

Gelatin is unaffected by most organic solvents. Wooden paint brush handles may be coated with gelatin by dipping into a gelatin solution. Drying and hardening this coating by dipping into a formaldehyde solution produces a hard polished surface unaffected by the organic solvents normally used for cleaning paint brushes. Rubber based adhesives may be applied using gelatin rollers again utilizing this ability to withstand organic solvents.

A gelatin composition finds an unusual use as a ballistic recovery medium bullet trap or for tracing the path of a fired projectile. The composition contains large amounts of glycerol (70–97% by weight) and little if any water. It is claimed that stable gels resistant to bacterial decay are formed.

Gelatin is used as a binding agent in many industries. In the production of matches it plays an important part in achieving a match-head of uniform composition and correct density. In the sanitary fireclay industry gelatin is used as a binder in the engobe composition used to mask coat a pure colour over the fireclay ware before glazing. Blocks of sodium chloride crystals may also utilize about 0.1% w/w gelatin as a binder (I.C.I. Ltd., 1960).

A. Gelatin as a Sizing Agent

Although the main use of gelatin as a size is in the paper industry there are many other sizing applications. It may be used in the textile industry for sizing threads and fibres to prevent breakage during weaving processes. Ends of fibres projecting from the yarn frequently become caught in the machinery thus impeding the free running of the yarn. Sizing provides a protective surface coating thus imparting strength and improving surface characteristics. A low or medium grade gelatin with a low viscosity is used to facilitate penetration into the thread. Gelatin is readily removed by washing after the weaving process.

Pebbled effects in fabrics are obtained by using a gelatin size to hold a high degree of twist in the weft yarn. On removal of the size after weaving this twist is released causing the crepe effect.

A process for stiffening and proofing natural and synthetic fabrics for hat making utilizes a gelatin solution containing chrome alum (Herman, 1965).

Low and medium grade gelatins and gelatin glues find a use in undercoat paint compositions for sealing porous surfaces (Harborne, 1961). Low grade technical gelatins are also used in dilute solution as decorators size. The main function of the size is to provide an even surface before hanging wallpaper or applying water-based distempers. It also facilitates the work of paper-hanging as the presence of the size coat allows a small degree of slip thus enabling the wallpaper to be properly positioned.

B. Manufacture of Golf Balls

The inner core of games balls (e.g. golf balls) consist of an elastic envelope filled with a viscous medium containing a suspension of finely divided dense particles to give the desired weight (Twiss and Neale, 1949). The core material must be solid during manufacture to facilitate winding the rubber thread under tension on to the core. A gelatin gel may be used for this core material and liquefied at a later stage to give a viscous solution of predetermined viscosity. Liquefaction of the gel may be accomplished by the high temperature utilized in vulcanizing the outer cover or by incorporating proteolytic enzymes. Alternatively the gelatin may be chemically hydrolysed by the presence of substances such as potassium persulphate or maleic anhydride with generation of free acid.

C. Diffraction Gratings and Holographic Plates

Dichromated gelatin is used in the production of diffraction gratings (Merton, 1950; Dew and Sayce, 1951) where an accuracy of the order of a micro inch is required. A cast is taken of a plane or helical grating by means of a plastic pellicle and transferred to an optically flat gelatin surface.

Dichromated gelatin has recently been used as a new holographic plate material (*Science Journal*, 1968) where the efficiency is claimed as 96%. Only 1/20 of the laser beam power required by conventional plates is necessary for retrieval of information from the high density optical memory systems produced from these holograms. Spectroscopic diffraction gratings can also be economically, accurately and quickly produced by this new technique which basically involves exposing a dichromated gelatin film to an argon laser beam and then developing it in water before drying. Gratings have been produced with 2000 lines per mm.

D. Cork Composition

Cork compositions of the types used in gaskets, cricket balls etc., frequently include gelatin as the binding agent. A typical formulation for this application is:

Cork	100 parts
Glycerol	20 parts
Gelatin	6-7 parts
Water approx.	3 parts

Paraformaldehyde or formaldehyde is added to give the correct degree of hardness and flexibility.

The cork in the form of granules is coated with gelatin solution containing the other ingredients before filling a mould. This composition is then compressed by an amount depending on the density required in the finished product. The mould is heated, either di-electrically or by placing it in an oven, until the granules are set. After leaving the mould the cork composition mat or sheet is cut into the desired articles. It is vital in many products, in particular gaskets, that the density is uniform throughout. If the cork composition is removed from the mould too soon, the cork granules relax or re-expand after removal of the compressive force giving a product of varying density. Although only a few minutes at about 90-95°C is necessary to cross-link the gelatin with the paraformaldehyde, the cork granules, which are extremely resilient and tend to return to their original unrestrained condition, do not acquire a permanent set until some time later. After di-electric heating the composition is therefore left in the mould for a further 5 hours to allow the internal stresses developed to relax. The rate of stress relaxation varies with the size of the granules, being faster with small particles. The gasket or other article is then cut from the cork composition sheet.

E. Paper Gaskets

Plasticized gelatin also acts as a composition agent in the manufacture of paper and fibre gaskets of the type used in industrial machinery and internal combustion engines. The gaskets are manufactured by impregnating a paper base with the gelatin solution containing a suitable plasticizer, and then passing it through a hardening bath of formaldehyde. Extreme care must be taken to ensure the finished material is of the correct thickness, bursting strength etc. (National Association of Glue Manufacturers, 1951). The finished dried weight of the paper should be at least 50% greater than the starting material. Gaskets of the desired dimensions are then cut from the paper sheet. A wide range of gelatin grades are used depending on the use of

the finished gasket. The viscosity of the gelatin solution is however, important as this controls the coating weight and degree of penetration into the paper base.

F. Bacterial Culture Media

Special grades of gelatin may be used as bacterial culture media. Nutrient gelatin is useful for the differentiation of micro-organisms by their proteolytic effects and gelatinase activity is determined by gelatin charcoal discs.

Nutrient gelatin consists of approximately 3 grams of beef, 5 grams of peptone and approximately 100 grams of gelatin per litre of distilled water. This is filtered and sterilized in an autoclave at 120°C, 15 lb./sq. in. for 15 minutes.

Gelatin charcoal discs consisting of finely powdered charcoal and formalin denatured gelatin are completely sterile (Kohn, 1953) and may be added to liquid or solid media. The appearance of charcoal particles preceding complete disintegration denotes the presence of gelatinase. Results by this method are extremely rapid and may be obtained in a few hours.

G. Electrolytic Metal Refining

The electrolytic refining process for many metals is improved, by the addition of small amounts usually less than 0.1 % of gelatin to the electrolyte. For example the cathode deposit of copper in the presence of gelatin is smooth and dense, while without gelatin the formation is so irregular that short circuiting with an adjacent anode can take place. A rough deposit traps pockets of electrolyte which cannot be washed away. Current efficiency is also improved by the addition of gelatin. The function of the gelatin, which may also be used in conjunction with other addition agents e.g. casein and polyvinyl alcohol, is not understood. The grade of gelatin varies with different metals but it should have a low grease content and should not cause excessive foaming.

REFERENCES

- Barker, E. F. (1962). *Paper Trade Journal*, 20-22.
Biotest Serum Institute (1963). British Patent 1,013,577.
Blain, G. (1951). *The Lancet*, 427-9.
British Pharmacopoeia (1963a) p. 886, The Pharmaceutical Press, London.
British Pharmacopoeia (1963b) p. 339, The Pharmaceutical Press, London.
Cambell, H. (1956). *J. Pharm. Pharmacol.*, 8, 93-109.
Charles B. Knox Gelatin Co. Inc. (1955). British Patent 742,594.
Clark, R. C., Sutton, D. A., Leach, A. A., Wootton, J., Gibbs, J. A. and Pratt, A. N. (1967). British Patent 1,075,952.

- Coulson, A. S. and Chalmers, D. G. (1964). *The Lancet*, 468-79.
- Dalton, A. S. (1965). *Official Digest*, 1593-1622.
- Davidson, P. B. and Bodénhagen, H. B. (1959). *Tech. Assoc. Pulp Paper Industry*, **42**, 720-33.
- Dew, G. D. and Sayce, L. A. (1951). *Proc. Roy. Soc., a*, **207**, 278-84.
- Froeschlin, W. (1962). *Deut. Med. Wschr.*, **87**, 811-19.
- Fullerton, H. (1961). *The Paper Maker (International No.)* 93-101.
- "Gelatin" (1954). Gelatin Research Society of America, Inc.
- Green, B. K. (1945). U.S. Patent 2,374,862.
- Green, B. K. (1955). U.S. Patent 2,712,507.
- Griffin, W. C. (1945). *Ind. Eng. Chem.*, **37**, 1126-30.
- Griffin, W. C. and Almy, E. G. (1945). *Ind. Eng. Chem.* **37**, 948-52.
- Hanson, H. H. (1958). U.S. Patent 2,866,707.
- Harborne, M. R. (1961). British Patent 871,200.
- Hartman, F. W. and Behrmann, V. G. (1953). *J. Amer. Med. Assoc.*, **152**, 1116-20.
- Herman, L. (1965). Australian Patent 260,211.
- I.C.I. Ltd. (1960). British Patent 853,316.
- Kohn, J. (1953). *J. Clin. Path.* **6**, 249.
- Kosar, J. (1965). "Light Sensitive Systems—Chemistry and Applications of Nonsilver Halide Photographic Processes," John Wiley and Sons, Inc.
- Kragh, A. M. (1958). In "Recent Advances in Gelatine and Glue Research", pp. 231-5 (G. Stainsby, ed.), Pergamon Press, London.
- Kragh, A. M. (1959). *Adhesives and Resins*, **7**, 103.
- Kragh, A. M. and Langston, W. B. (1962). *J. Coll. Sci.* **17**, 101-23.
- Kyle, E. J. (1967). *Printing Technology*, 5-8.
- Leheimann, H. L. (1968). *Cabinet Maker and Retail Furnisher*, 759.
- Letsky, B. H. (1958). *Wood*, 216-217.
- Merrill, R. E. (1965). U.S. Patent 2,958,605.
- Merton, T. (1950). *Proc. Roy. Soc., A*, **201**, 187-91.
- Moeller, J. and Skyudes, A. (1962). *Deut. Med. Wschr.* **87**, 726-9.
- National Association of Glue Manufacturers (1951). "Animal Glue in Industry".
- National Cash Register Co. (N.C.R.) (1956). British Patent 751,600.
- National Cash Register Co. (N.C.R.) (1961). British Patent 872,438.
- National Cash Register Co. (N.C.R.) (1963). British Patent 927,157.
- National Cash Register Co. (N.C.R.) (1964). British Patent 949,910.
- Paradissis, G. N. and Parrott, E. L. (1968). *J. Clin. Pharmacol. J. New Drugs* **8**, 54-9.
- Pillersdorf, A., Kuhn, L. P. and Bowman, R. E. (1968). U.S. Patent 3,398,007.
- Science Journal* (1968). **4**, (10), 27.
- Smith, E. (1960). U.S. Patent 2,942,991.
- Tice, L. F. and Abrams, R. E. (1953). *J. Am. Pharm. Assoc. (Practical Pharmacy Ed.)*, **14** (1).
- Tourtellotte, D. and Williams, H. E. (1958). In: "Recent Advances in Gelatine and Glue Research" (G. Stainsby, ed.) (pp. 246-51). Pergamon Press, London.
- Tutt, R. and Lane, L. B. (1961). *Tech. Assoc. Paper Pulp Ind. Monograph Ser.* **22**, 242-61.
- Twiss, D. F. and Neale, A. E. T. (1949). British Patent 621,046.
- Veis, A., Cohen, S., Cohen J. and Ranyl, C. A. (1967). U.S. Patent 3,317,434.

Chapter 14

Swelling, Adsorption and the Photographic
Uses of Gelatin

A. M. KRAGH

Ilford Limited, Ilford, Essex, England

I	Introduction	439
II	Properties of Gelatin—Importance in Photography	440
A.	Swelling of Gelatin	440
B.	Adsorption of Gelatin	447
III	General Constitution of Photographic-Films	460
IV	Use of Gelatin in Emulsion Technology	461
A.	Preparation of Emulsions	461
B.	Washing Emulsions	463
C.	Chemical Sensitization	463
D.	Final Additions, Coating and Drying	467
E.	Action of Gelatin on Exposure of Emulsions to Light	467
F.	Photographic Processing	468
G.	Properties of Dry Gelatin Films	469
H.	Photographic Paper	470
V	Conclusions	470
	References	471

I. INTRODUCTION

Light-sensitive photographic layers, incorrectly called 'emulsions' by long-standing convention, consist essentially of silver halide microcrystals embedded in a binding medium. Despite the advent of a wide range of synthetic water-soluble polymers, gelatin, which was used as the binding agent for photographic emulsions 100 years ago, is still the principal constituent of the binder in almost all commercial photographic films and papers.

Some reasons for this will become apparent from Section IV of this chapter where the ways in which gelatin is employed in the various stages

of the manufacture and use of photographic materials are described. In Section II the swelling and adsorption of gelatin, which are of particular importance in photographic use, are described.

Only "conventional" photographic processes are considered here, that is to say those based on the use of silver halide. However, the range of different photographic products based on silver halide is very wide and includes materials to which some generalizations that are valid for most products do not apply.

II. PROPERTIES OF GELATIN—IMPORTANCE IN PHOTOGRAPHY

The photographic process makes use of many of the physical properties of gelatin, such as its protective-colloidal, setting, swelling and film-forming properties. In general these do not set critical limits on the choice of gelatins: gelatins with a wide range of physical properties can be used at all stages of the process. The choice between gelatins for photographic use depends mainly on the photographic effect of small quantities of impurities. In particular it is important that substances that produce fog be absent and that the concentration of substances that effect photographic sensitivity be controlled.

The impurities that are important in photography are referred to in Sections IV C, V C and D. Most of the physical and chemical properties of gelatin are described in Chapters 3, 4 and 6. The swelling and adsorption of gelatin are considered in detail in this section.

A. The Swelling of Gelatin

The swelling of gelatin can be compared with the swelling of non-ionized crosslinked polymers but the effects are complicated by charge interactions and also by the structure of the gelatin network. A crosslinked polymer such as synthetic rubber, swells in benzene because in the absence of crosslinks the polymer chains would go into solution. Thus the increase in free energy when the crosslinked polymer swells is related initially to the free energy of solution of the uncrosslinked polymer, which is due to an entropy increase. (Heat is normally absorbed.) The resultant swelling forces are balanced at equilibrium by the tension in the network which corresponds to a decrease in configurational entropy as the chains become stretched. The theory of swelling of polymers has been treated by Flory (1953). For high degrees of swelling Flory and Behner (1943) give the simplified equation

$$v = [Z\epsilon V/M(1 - 2\mu)]^{3/5} \quad (1)$$

where v is the volume-fraction of polymer at equilibrium, V the molal volume of solvent, ϵ the density of pure polymer, μ an interaction constant between solvent and polymer and M the average molecular weight of the chains between crosslinks. Thus swelling depends primarily on the number of crosslinks and the interaction constant of polymer and solvent.

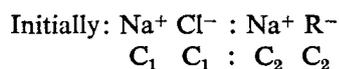
The swelling of gelatin differs from that of non-ionized polymers both in the molecular characteristics of the network that restrain the swelling and in the factors that promote swelling. In the first place the network of a gelatin gel does not consist of short crosslinks with single flexible chains between them; the crosslinks involve multiple interactions between chains which form fibrils (Tomka, 1974). This reduces swelling as compared with simple polymer networks. The effective crosslinks have a wide range of stabilities (Pouradier, 1967). Furthermore the number is not constant: it varies with temperature and, at high temperatures in particular, it varies with time.

Gelatin swells in water for the same reason that polymer gels swell but the swelling pressure is increased by the ionized groups on the molecule. The free energy of solution of gelatin is due to a negative heat of solution as a result of the hydration of the ionized groups as well as to the entropy effect. But a more important factor is that the swelling pressure is dependent not only on the free energy of solution but also on the Donnan equilibrium, which increases swelling.

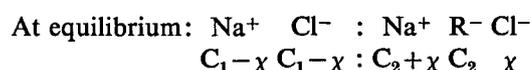
1. Factors affecting swelling pressure

(a) *The Donnan equilibrium* (i) Simple Theory. A gelatin gel may be imagined to have swollen to a state in which the swelling pressure corresponding to the free energy of solution is balanced by the contractile forces of the network. This however is not the equilibrium state since there is an additional swelling pressure due to the Donnan equilibrium which produces a further extension of the network. This can most simply be regarded as an osmotic pressure at the semipermeable membrane of the gel surface. Since the gelatin molecules cannot diffuse, and due to considerations of electric neutrality cannot lose their counter ions, there will be a nett excess of ions within the gel phase and hence an osmotic pressure.

The effect can be considered simply according to classical theories in terms of a solution of concentration C_2 of a high-molecular-weight sodium salt (with a non-diffusing anion) separated by a semi-permeable membrane from a solution of sodium chloride at a concentration C_1 .



Suppose a concentration χ of NaCl passes through the membrane, electric neutrality being maintained.



The chemical potential must be the same for sodium chloride on both sides of the membrane so, if ionic concentration is equated with ionic activity, $[\text{Na}^+][\text{Cl}^-]$ is equal on both sides of the membrane

$$\begin{aligned} (C_1 - \chi)^2 &= \chi(C_2 + \chi) \\ \chi/C_1 &= C_1/(C_2 + 2C_1) \end{aligned} \quad (2)$$

Thus the proportion of salt diffusing through the membrane χ/C_1 depends on the proportion of C_1 to C_2 . It can readily be calculated that the difference in the total number of ions inside and outside the membrane, on which the osmotic pressure depends, decreases with increasing C_1 .

The chemical nature of the ions is unimportant on simple theoretical considerations and modern theories treat the Donnan equilibrium in terms of the total increase in the number of counter-ions and decrease in the number of co-ions produced by the non-diffusing ion (Overbeek, 1956). When however one of the counter-ions is hydrogen and, as with gelatin, the degree of ionization depends on pH, the Donnan equilibrium varies with pH since it depends on the net charge on the gelatin molecule. Swelling therefore increases on either side of the iso-electric point. It decreases again at extreme pH values due to the effect of increased ionic concentration (C_1 in Equation 2). Simple quantitative theories relating the effects of net charge and ionic strength have been given by Proctor and Wilson (1916), Proctor and Burton (1916) and Bolam (1932). Equation 2 can however be applied if C_2 is taken as the total concentration of excess positive or negative charges on the gelatin molecule obtained from the titration curve (see p. 93) and C_1 as the concentration of diffusible electrolyte.

(ii) Electrostatic Charge Interactions. The general shape of the curves of swelling against pH at different salt concentrations, for example those shown in Fig. 1 (Jordon Lloyd and Pleass, 1927), can be explained as shown above in terms of the Donnan equilibrium.

The general shape of the curves is also similar to those given in Chapter 4 relating changes in the viscosity of gelatin with pH and ionic strength. These were explained in terms of the effects of attraction and repulsion of charges on molecular configuration. Swelling can be treated in an analogous

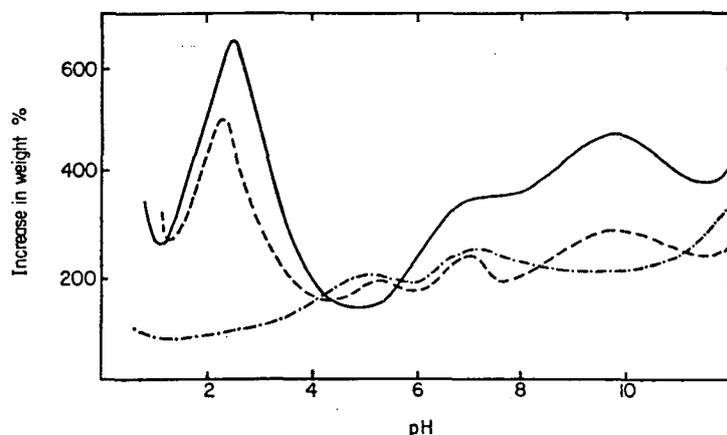
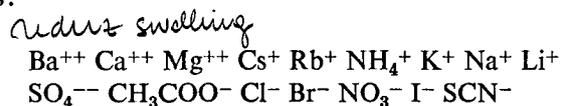


FIG. 1. Swelling of gelatin as a function of pH and salt content at 18°C. — no added salt; --- 0.05 M NaCl; -.-.-. 1.0 M NaCl (Jordan Lloyd & Pleass, 1927).

manner. Although conceptually different, this method of treating the effects of ionized groups on swelling is equivalent to the Donnan theory and leads to the same conclusions. However, the effect of salts in increasing swelling at the iso-electric point can most readily be explained in terms of a reduction in the attraction between oppositely charged groups on the gelatin chains, since there is no Donnan effect at the iso-electric point.

(iii) More Exact Theories. For quantitative calculations of Donnan equilibria the activities of ions rather than their concentrations need to be used. The predicted effects on swelling will then be smaller than those calculated from concentrations. The use of Debye-Hückel activity coefficients may be appropriate for swollen gelatin gels in which the fixed charges can be assumed to be distributed more or less uniformly, though with true polyelectrolytes the local concentration of ions can be high even at low dilutions so that the Debye-Hückel and related theories cannot be applied. More recent theories, described by Overbeek (1956) relate the Donnan equilibrium to the Gouy-Chapman theory of the electric double layer according to which the electric potential decreases exponentially from regions of high charge density. The Donnan effect is then summed for regions of different electric potential. (In the simple Donnan theory given above the electro-chemical potential was assumed to be constant throughout the two phases.) In this way the lowering of the ionic activity in regions of high charge density is allowed for without the use of activity coefficients. However, the theory has been worked out quantitatively only for plane charged surfaces.

(b) *Specific ion effects.* In relation to the Donnan equilibrium and the theory of the electric double layer it is normally sufficient to consider co-ions and counter-ions regardless of their nature. The valency of the ion is however very important. Divalent ions are much more effective than monovalent ions in screening charge interactions. For example, divalent counter-ions are about 50 times as effective as monovalent counter-ions in flocculating hydrophobic sols. Nevertheless differences are observed in the effects produced by ions of the same valency and these can be arranged in approximately the same order with regard to their effects on a variety of colloid-chemical phenomena, including flocculation of hydrophobic sols, salting out of proteins, increase of surface tension of solutions, ion exchange in clays, as well as the swelling of gelatin. This order is known as the Hofmeister or lyotropic series and is as follows:



The reason why the action of ions is in this order is only understood in part. With monovalent cations the order is the same as that of the increasing degree of hydration and as that of the ionic radius. Specific-ion effects occur in the electric double layer at a surface because the distribution adjacent to the surface (in the Stern layer) depends on the ionic radius. Furthermore specific-ion effects can be produced by adsorption since adsorption depends on the polarizability and hence the size of the ion.

The lyotropic series was initially related to the swelling of gelatin and the ions listed above on the left are the most effective in reducing swelling at pH values away from the iso-electric point. The main differences however are between the monovalent and divalent ions. There appears to have been no comprehensive investigation of the comparative effects of different salts on swelling at pH values both above and below the iso-electric point of the gelatin but it is apparent from the early work of Loeb (Jordan Lloyd, 1926) that the valency of the counter-ion is more important than that of the co-ion. For example sulphate ions are more effective in reducing swelling at pH 3.3 when the molecule has a net positive charge than at pH 9.3 when it has a net negative charge. At pH 3.3 sulphate ions are about ten times as effective as chloride ions in reducing swelling. Such effects can be related to the normal non-specific effects of valency. The specific effects are less easily explained but are due in part to various effects of ion binding. Ions to the right of the lyotropic series tend to act, in sufficiently high concentrations, as hydrogen-bond breakers and hence reduce the cohesion of the network. This applies particularly to lithium and thiocyanate. Sulphate ions on the other hand increase gel rigidity and presumably have some kind of crosslinking action.

This may in part explain their effectiveness as co-ions in reducing swelling in alkaline solution. Ion binding can also effect swelling by changing the ionization of the gelatin molecule and hence the iso-electric point.

2. *The restraint on swelling due to the gel network*

A swollen gelatin gel consists of a three-dimensional network of chains that is under tension so that it balances the swelling pressure of the aqueous swelling medium. The structure of the network, and hence the degree of swelling it permits, can vary widely depending on the conditions of setting and drying of the gelatin and on the temperature and time of swelling.

(a) *Rate of swelling.* Much work on swelling has been carried out with thin films attached to a support as in photographic usage. Under these conditions the gelatin dries with movement in one dimension only to produce a very anisotropic film which, even if subsequently stripped from the base, swells mainly in the direction of concentration.

The rate of swelling has been studied in detail by Libicky and Bermane (1972). Most results for both normal gels and covalently crosslinked gels were found to correspond approximately to a second-order equation of the form

$$K = \frac{1}{t} \frac{L L_{\infty}}{L_{\infty} - L}$$

where L indicates the swelling at time t , and L_{∞} the equilibrium swelling attained for $t \rightarrow \infty$. The authors point out that there is no theoretical justification for such an equation.

With films of gelatin about 10μ thick, such as those used for photographic films, swelling is rapid, and, providing the temperature is low, a plateau in the curve of swelling with time is obtained after several minutes. In the plateau region a slow increase in swelling with time is observed due to relaxation of strained bonds within the network. At 25°C and above, swelling continues to increase with time corresponding to the increased rate of relaxation and no very clear plateau region is observed. With thick gelatin films, which swell slowly, the initial swelling and the subsequent stress-relaxation stage cannot be clearly separated.

(b) *The effect of drying temperature and conditions of storage.* Jopling (1956) examined the effects of drying temperature and conditions of storage on the swelling in water of films of isoelectric gelatin coated about 10μ thick on a rigid support. The drying times varied between 1 and 12 hours. The results are shown in Figure 2. It can be seen that swelling was at a minimum for films

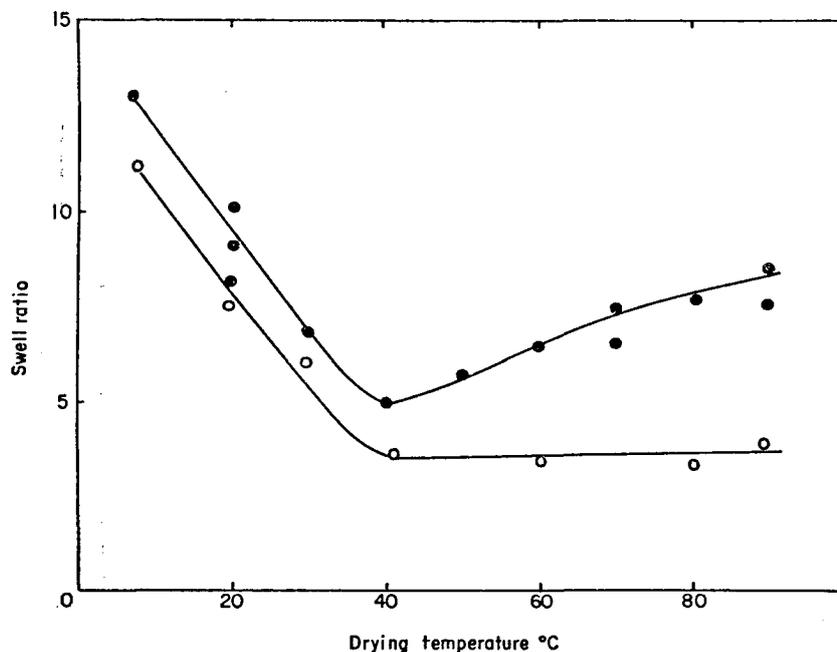


FIG. 2. Swelling in water at 20°C of iso-electric gelatin as a function of drying temperature. ● before conditioning; ○ after conditioning for 24 hours at RH 90% 20°C. (Jopling 1956).

dried at 40°C. The swelling of films dried above this temperature was greatly reduced by conditioning the film at 90% RH and 20°C for one day. The effect of the same treatment on the films that were dried below 40°C was much smaller. It was also found that six days further conditioning produced only a small additional effect.

These swelling effects can be related to the differences in structure between "hot-dried" and "cold-dried" gelatin (Chapter 8) and can be explained as follows. The swelling of a particular gelatin under given conditions of pH and salt concentration depends on the structure of the gel network. This varies according to the conditions of drying. Gelatin, dried at low temperatures from a well-matured dilute gel, has a regular structure of associated molecular chains with strong multiple hydrogen bonds between them. This structure becomes increasingly strained as drying proceeds. As the structure collapses, further linkages between chains and fibrils can occur, but these tend to be weak so that many of them break again on reswelling. Thus the swelling of gelatin tends to increase as the concentration at setting decreases. For concentrated gels set at room temperature the effect is small (Frieser *et al.*, 1964) and, except at high swelling temperatures, dry gelatin

swells to a concentration that is considerably lower than the original setting concentration because of the additional network structure formed during drying. Increasing the temperature of drying towards 40°C enables the structure formed at low concentrations to break and reform at higher concentrations so that swelling is reduced. The optimum conditions for obtaining low swelling, which occur according to Jopling (1956) at about 40°C, is when the formation of hydrogen bonds is delayed until the final stages of drying so that the gel structure is formed at a high concentration and is then most effective in restricting swelling. Further increases in drying temperature lead to a reduction in the total number of hydrogen bonds until at about 80°C the dry layer contains few, if any. In such layers swelling is largely unrestricted by hydrogen bonds which form only during swelling. Under these conditions thin layers will dissolve completely in rapidly stirred cold water. However on humidification of the gelatin films at 90% RH, 20°C, sufficient water is absorbed for the gelatin chains to be able to rearrange to form a network structure at a very high gelatin concentration (about 75%). This gives minimum swelling. The RH for rehumidification is fairly critical: changes occur rapidly at 90% RH but very slowly at 80% RH. Humidification has only a small effect on cold-dried films since they are already strongly structured and the temperature is not high enough for much bond-breaking to occur, (Taylor and Kragh 1974).

The effects of the relaxation of hydrogen bonds during drying is clearly shown by changes in the anisotropy of the films. Films dried at low temperature and then stripped off the support swell almost entirely in one direction, the direction of contraction. As the temperature of drying is increased, the ratio of lateral to vertical swelling is increased (Simms and Blake, 1960).

B. The Adsorption of Gelatin

Much of the work on the adsorption of polymers and polyelectrolytes at surfaces is relevant to the adsorption of gelatin and is considered here first. Some of this work was reviewed earlier by Kragh (1964). It has been reviewed more comprehensively by Stromberg (1967).

1. Synthetic polymers

(a) *Theories of adsorption.* The earlier theoretical studies of Simha *et al.* (1953) and Frisch and Simha (1954) were based on the assumption that isolated polymer molecules were adsorbed in a configuration similar to that of the random coils in solution except that chains meeting the surface were assumed to be "reflected". This led to the conclusions that only a small proportion of the polymer segments were in contact with the surface, that the adsorbed

layer was thick and of low density, and that the amount adsorbed increased with the square root of the molecular weight.

Silberberg (1962) concluded that polymer molecules would undergo a complete rearrangement at the surface to give a structure in which free loops or chains in solution alternated with runs of segments attached to the surface. On this theory the proportion of adsorbed segments was much higher than that predicted by the theory of Simha *et al.* (1953), the density of the portion of the adsorbed layer adjacent to the surface was much greater, and the layer thickness was independent of molecular weight. More recent theories (Hoeve *et al.*, 1965; Hoeve, 1965, 1966; Roe, 1965) treat the theory of adsorption in a related manner, but unlike Silberberg (1962) predict a distribution of loop lengths and a lower proportion of adsorbed segments. However, Silberberg (1967) and also Hoeve (1965, 1966) have shown that theories on the configuration of isolated molecules on a surface are not applicable to conditions of full surface coverage. As adsorption proceeds molecules already adsorbed on the surface "push up to make room" so that the free loops are extended with increasing surface coverage. Silberberg (1967) predicts that under these conditions the thickness of the layer will be proportional to the square of the molecular weight as in the theory of Simha *et al.*, which was based on isolated random-coiled molecules.

(b) *Experimental results.* Experimental results have been obtained with a wide variety of systems. They have however a number of features in common that are distinct from those obtained with small molecules. The results considered refer only to non-porous surfaces since porous surfaces complicate adsorption because of molecular sieving.

(i) *Reversibility of Adsorption.* Equilibrium takes a long time to establish and there is a marked hysteresis in adsorption and desorption to the extent that polymer often cannot be removed by washing with pure solvent (Frisch *et al.*, 1959). This does not indicate irreversibility in the thermodynamic sense; the individual segments of the polymer chains may be in an equilibrium state of adsorption and desorption but the molecule as a whole remains at the surface because of the small chance of all segments being desorbed together.

(ii) *Adsorption Isotherms.* Except when adsorption is very weak, the amount of polymer adsorbed increases at first very rapidly with increasing concentration in solution and then reaches a constant value. The data are often consistent with a Langmuir isotherm though this has no theoretical significance since the initial portion of the isotherm is normally too steep for the Langmuir isotherm to be differentiated from other isotherms and because equilibrium is often not attained in the shoulder region of the isotherm.

(iii) Temperature Dependence. The temperature dependence of adsorption is small and may be either positive or negative (Koral *et al.*, 1958; Jenkel and Rumbach, 1951; Perkel and Ullman, 1961; Ellerstein and Ullman, 1961).

An increase in adsorption with temperature may be explained on the assumption that if, as is to be expected, the proportion of segments adsorbed decreases with rising temperature the length of the free loops will increase and there will be room on the surface for more molecules.

(iv) Effect of Solvent. More polymer is adsorbed from a poor solvent than from a good one (Koral *et al.*, 1958; Jenkel and Rumbach, 1951; Perkel and Ullman, 1961; Kolthoff *et al.*, 1951). In a poor solvent polymer coils are tightly bunched together and may be expected to form more layers that are more closely packed. A fuller treatment of solvent effects is given in Chapter 8, II C.

(v) Saturation Adsorption. In general it has been found in accord with theory, that the amount of material adsorbed at saturation is several times the amount that could be adsorbed if all segments of the polymer were adsorbed on the surface in a close-packed layer.

(vi) Thickness of the Adsorbed Layer. The earlier theory of Simha *et al.* (1953) suggested that the thickness of adsorbed polymer layers should be of the same order as the diameter of the random coil in solution. More recent theories (Hoeve *et al.*, 1965; Hoeve, 1965, 1966) indicate rather thinner layers but also indicate that the density decreases sharply with the distance from the surfaces so that at large distances only a few polymer chains are found. The thickness cannot easily be measured experimentally. Probably the best method is based on ellipsometry with which values of the order of 50 nm have been obtained for the root-mean-square average thickness of high-molecular-weight polymers—the values varying with molecular weight (Stromberg, 1966). Much greater thicknesses have been obtained by hydrodynamic measurements. These thicknesses have been calculated from viscosity anomalies with very dilute solutions on the assumption that these anomalies are due to the narrowing of the capillary tube by adsorption of polymer onto the walls. Oehrns (1958) for example, calculated a thickness of 150 nm for polystyrene of molecular weight 800,000 adsorbed from toluene. This thickness is nearly three times the average dimensions of the random coil in solution (Koral *et al.*, 1958). It seems likely that thicknesses obtained in this way are considerably larger than that of the root-mean-square average.

2. Gelatin

The adsorption of polyelectrolytes including gelatin depends on the same considerations as the adsorption of polymers but is complicated by charge interactions in two ways. Firstly, the intramolecular charge interactions which

effect the configuration of the random coil in solution and hence the viscosity (Chapter 4), have corresponding effects in the adsorbed layer. Secondly, adsorption is affected by charge interactions between the molecule and the surface; with polyampholytes such as gelatin some segments of the molecule may be repelled and others attracted. When a small proportion of segments have a much stronger tendency to be adsorbed than the others, the length of the free loops is likely to be greater than with homogeneous polymers (Fontana, 1963; Hesselink, 1971).

Published work on the adsorption of gelatin falls into three main classes: firstly stabilization of dilute sols, in particular gold sols; secondly flocculation of concentrated mineral suspensions; and thirdly adsorption on silver bromide. Because of the intimate connection between some of the work and the effects of gelatin on the stabilization and flocculation of sols and suspensions these phenomena will be considered briefly.

(a) *Adsorption on gold sols.* Zsigmondy (1901) started systematic investigations on gold sols and introduced the principle of "gold number". This was an arbitrary unit which depended on the minimum amount of a protective substance that would prevent a gold sol being flocculated by NaCl solution. Among a number of naturally occurring macromolecular substances investigated, gelatin was found to have the lowest gold number.

Zsigmondy and Joel (1924) showed that when sufficient gelatin was present, a protective action was obtained, irrespective of the charge on the gelatin molecule. When however the concentration of the gelatin was low enough the gelatin "sensitized" the sols to flocculation so that they were flocculated by lower concentrations of electrolytes (Overbeek, 1938). Similar sensitizing effects have been observed with emulsions of paraffin oil droplets and gelatin (Limberg, 1926).

(b) *Adsorption on quartz suspensions.* The adsorption of gelatin on quartz suspensions was studied by Kragh and Langston (1962) and Kragh (1964), with particular reference to the mechanism of flocculation and the influence of pH on flocculation. Warm dilute gelatin solution was added to about 100 times the volume of a 4 per cent quartz suspension at room temperature and the mixture agitated gently. The rate of sedimentation of the boundary between floccules and supernatant liquid was used as a measure of the degree of flocculation. The amount of gelatin adsorbed was determined by measurement of the concentration remaining in the supernatant liquid. At low degrees of surface coverage this was independent of the time of agitation after about half a minute.

The initial portion of the adsorption isotherm was very steep and with additions of gelatin up to two thirds the value at saturation, over 99% of the gelatin was adsorbed.

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 451

The saturation adsorption was greatest in the region of the iso-electric point of the gelatin and was about 2.1 mg/m^2 for a high-grade gelatin based on a surface area determined by nitrogen on the dry powder. At pH 6.5 the adsorption was 1.4 mg/m^2 . At low degrees of surface coverage (e.g. 0.5 mg/m^2) almost all the added gelatin was absorbed at all pH values between 2 and 7, but adsorption fell off slightly at high pH values. The adsorption showed only a slight dependence on molecular weight and temperature.

(c) *Adsorption on silver bromide.* Pouradier and Roman (1952) were the first to make a systematic study of the adsorption of gelatin and concluded, before it was suggested by theoretical studies, that the molecules were not adsorbed flat on the surface. Their work is supported by a fuller investigation by Curme and Natale (1964) who determined the adsorption of gelatin at 40°C on silver bromide sols whose surface area was determined by adsorption of a cyanine dye and by electronmicrography. Gelatin was determined

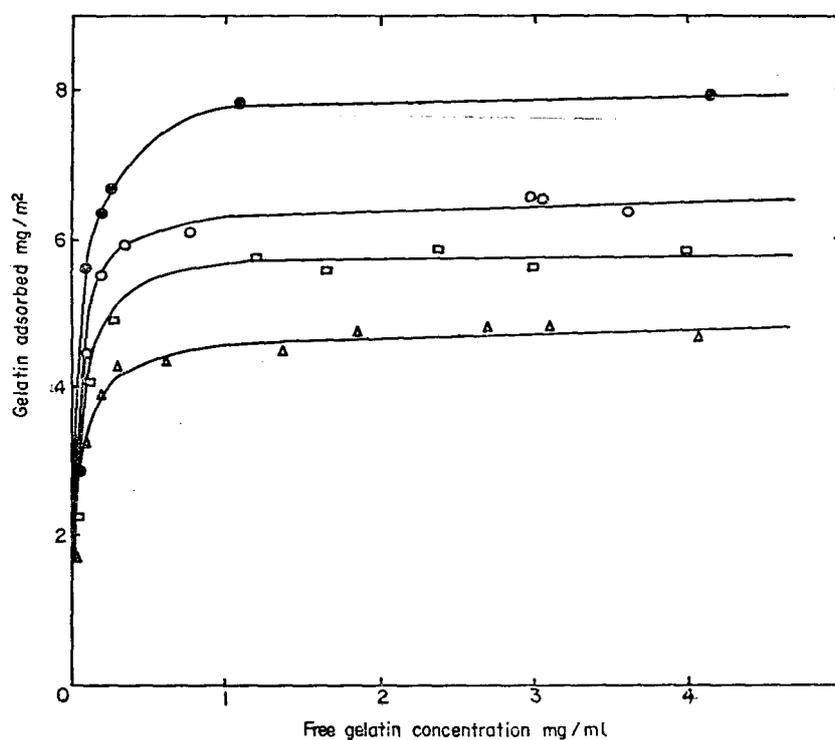


FIG. 3. Adsorption of gelatin on silver bromide as a function of pH and ionic strength. Δ pH 3.6, $\mu=0.0635$; \square pH 6.9, $\mu=0.0635$; \circ pH 4.7, $\mu=0.0635$; \bullet pH 4.7, $\mu=0.0185$ (Curme & Natale 1964).

by a Biuret method. The results obtained for the effects of pH on ionic strength are shown in Figure 3.

Adsorption increased with molecular weight. For three gelatin fractions of molecular weight 80,000, 263,000 and 450,000 (obtained by light scattering) the saturation adsorption in solutions of ionic strength 0.0185 were respectively 5.3, 6.8 and 7.8 mg/m².

(i) Saturation Adsorption. The surface coverages obtained by Curme and Natale (1964) are similar to those reported by Pouradier and Roman (1952) and are several times the values for monolayer coverage obtained with spread films on mercury or water for which values of about 0.95 (Keenan, 1929) and 0.9 mg/m² (Pouradier and Roman, 1952) have been obtained respectively. Nevertheless the isotherms show no sign of multilayer adsorption. The results are thus similar to those obtained for many polymers and are consistent with the theoretical model of a looped structure with only a proportion of molecular segments attached to the surface (Section I). This is also suggested by the molecular-weight dependence; according to the results of Curme and Natale (1964) given above, the adsorption is proportional to Mw^n where $n = 0.23$.

(ii) Effect of Electrostatic Interactions. Curme and Natale (1964) showed that the changes in saturation adsorption with pH and ionic strength varied inversely with the dimensions of the random coil in solution, as shown by viscosity (Figure 3). The adsorption was at a maximum at the iso-ionic point at which pH adsorption decreased slightly with increasing ionic strength. At pH values on either side of the iso-electric point where there was a net charge on the molecule, adsorption was lower than at the iso-electric point and was increased by increasing ionic strength. These effects are as would be expected from intra- and intermolecular charge interactions, and correspond qualitatively with the effects of pH and ionic strength on viscosity (Chapter 4). Thus it may be assumed that under conditions where there is electrostatic repulsion between gelatin chains the molecular loops can pack less closely in the adsorbed state. However the adsorption, like the intrinsic viscosity, cannot be related quantitatively to the net charge on the molecule. A further, though less important factor is the relation between the charge on the gelatin molecule and the charge on the surface. Curme and Natale (1964) and Kragh and Peacock (1967) showed that adsorption on a negatively charged surface was greater for negatively charged gelatin molecules than for positively charged molecules with the same net charge. The significance of this is considered below.

(iii) Desorption of Gelatin—Shape of Adsorption Isotherm. It is widely recognized that adsorption is largely irreversible. Pouradier and Roman (1952) measured only the gelatin adsorbed after washing; Curme and Natale (1964) found that adsorption was irreversible though they did not state at

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 453

what pH. However the isotherms they obtained showed a considerable shoulder which would indicate reversibility if the isotherms corresponded to equilibrium conditions. Kragh and Peacock (1967) made some precise measurements of adsorption on silver bromide, determining the gelatin by ultra-violet absorption. Both adsorption and desorption isotherms were obtained. The shoulder on the adsorption isotherm decreased when the adsorption time was increased from 5 to 18 hours and was very small. At pH 3.5 the isotherm was horizontal down to a concentration of gelatin in solution of 0.2 mg/ml. The desorption isotherm however remained horizontal down to even lower concentrations. However at pH 6.5, above the isoelectric point, when the charge on the gelatin molecule was the same as on the surface there was a slight shoulder in the desorption isotherm (Figure 4) corresponding to desorption of 16% of the gelatin. The equilibrium isotherm is likely to be between the experimental adsorption and desorption isotherms. At pH 9 under the same conditions 30% of the gelatin was desorbed. It is noteworthy that after a proportion of the gelatin had been desorbed desorption proceeded much more slowly suggesting that the number of segments adsorbed per molecule decreased with increasing surface coverage. This is consistent with the theory of Silberberg (1967) (Section 1a).

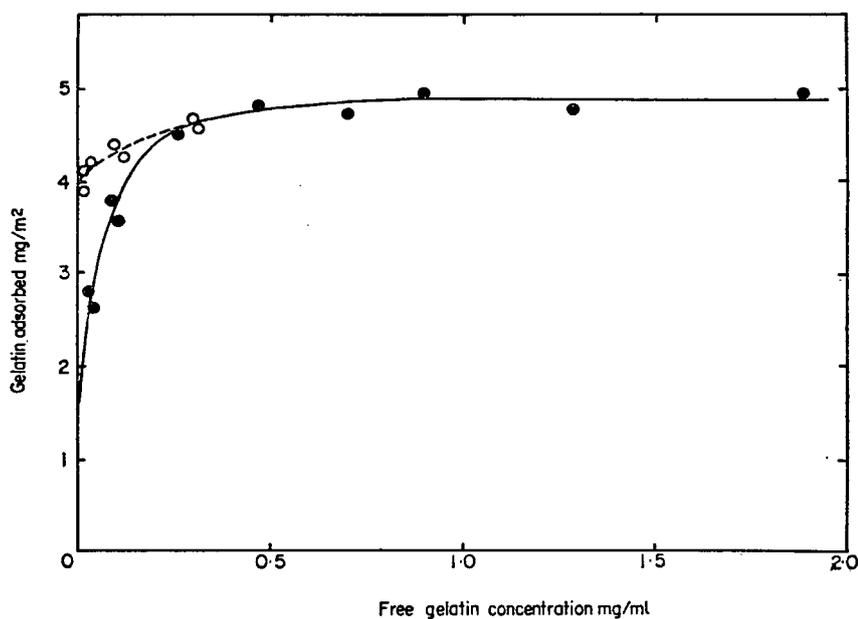


FIG. 4. Adsorption of gelatin on silver bromide at 40°C pH 6.5. ● adsorption; ○ desorption (Kragh & Peacock, 1968).

(iv) Groups Adsorbed. By analogy with the adsorption of cationic dyes and wetting agents (Herz and Helling, 1966; Ottewill and Rastogi, 1960) it would be expected that the ionized amino and guanidino groups would be strongly adsorbed and that the hydrocarbon portion of the molecules would also tend to promote adsorption. It might be expected that the ionized carboxyl groups would if anything be repelled from a negatively charged surface. However, Zimkin and Klyuchevitch (1968) have concluded that the carboxyl groups of glutamic acid are adsorbed. After hydrolysis, with enzymes, including trypsin, of the gelatin adsorbed to the surface, analysis of the amino acids remaining on the surface revealed mainly glutamic acid. This implies irreversible adsorption of glutamic acid residues. Some plausibility is given to this view by the fact that the amino acid β alanine, which has a similar structure to the amino acid *residue* of glutamic acid, was also found to adsorb strongly to silver bromide. However the results do not seem self-consistent since it is known that trypsin only cleaves the gelatin molecule between certain amino acids and unless it cleaves the molecule immediately on either side of the glutamic acid residue the large preponderance of glutamic acid remaining on the surface cannot be explained. The results therefore seem inconclusive.

Lanza and Lippolis (1969) studied the adsorption of gelatin acetylated on the amino groups and of gelatin esterified on the carboxyl groups but the results are difficult to interpret. The acetylated gelatin showed the same adsorption as normal gelatin at pH 2 but with no maximum at the iso-electric point (not stated but presumably about pH 4) and a sharp decrease between pH 4 and pH 8 to give much lower adsorption than normal gelatin. This can be interpreted as being due to the higher net charge on the molecule producing more extended chains and lower adsorption. The results with esterified gelatin show no adsorption below pH 6 and, taken at their face value, would indicate that it is necessary for there to be non-ionized basic groups before adsorption can occur. However results of Klein *et al.* (1971) show clear effects of adsorbed esterified gelatin at pH value below 6.

(v) Number of Groups Adsorbed. The total amount of gelatin adsorbed when the surface is saturated provides very little information on the proportion of groups on the gelatin molecule that are adsorbed to the surface. This may be expected to vary considerably without there being a marked change in saturation adsorption, though for non-ionized polymers the saturation adsorption increases as the number of groups adsorbed per molecule decreases. Kragh and Peacock (1967) attempted to obtain a measure of the proportion of groups adsorbed by determining the rate of dissolution of silver bromide in sodium thiosulphate. This was slowed down when gelatin was present by a factor varying between 5 and 100 depending on the pH and temperature. On the assumption that this effect correlated with the

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 455

number of groups adsorbed, and that these groups hindered dissolution, it could be concluded that the number of groups adsorbed decreased considerably with increasing temperature and also with increasing pH. Thus the results suggested that above the iso-electric point of the gelatin when the net charge on the molecule is negative, less groups were adsorbed on a negative surface. This result, which would be expected theoretically, is also consistent with the fact that a proportion of the gelatin was desorbed above the iso-electric point (Section iii). This would only occur if there were very few points of contact with the surface.

A corresponding investigation has been made by Klein *et al.* (1971) who studied the effect of pH on the Ostwald ripening of silver halide crystals in gelatin solution under conditions of minimum solubility at pAg values in the region of 3 and 9 when the silver halide has respectively a positively and negatively charged surface. Under these low-solubility conditions gelatin has more effect in inhibiting Oswald ripening (Romer and Sidorowicz, 1967). The results of Klein *et al.* (1971) correspond at pAg 9 with those obtained by Kragh and Peacock (1967) for the rate of dissolution of silver halide and the results at pAg 3 are complementary (Figure 5). These results therefore provide good evidence for the importance of ionic interaction with the surface as a mechanism of adsorption. Other results by Klein *et al.* (1971) with modified gelatins are consistent with this view.

More-direct evidence that more molecular segments are absorbed when the net charge on the gelatin is opposite to that of the surface is provided by

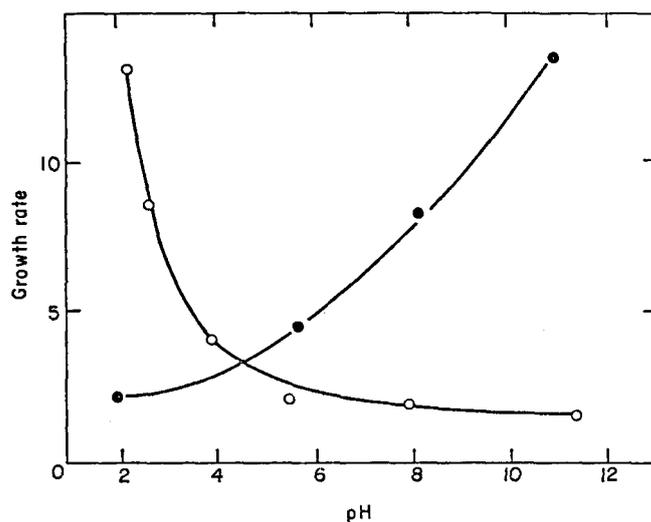


FIG. 5. Growth rate of silver halid crystals in gelatin solution (arbitrary units) as a function of pH. ○ pAg~3; ● pAg~9 (Klein *et al.*, 1971).

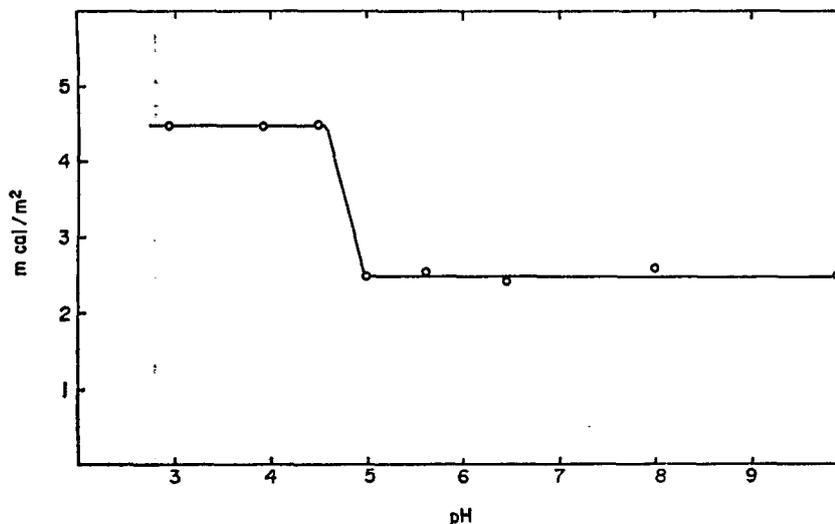


FIG. 6. Heat of adsorption of gelatin as a function of pH (Berendsen & Borginon, 1968).

the work of Berendsen and Borginon (1968) who determined heats of adsorption. They showed that the heat of adsorption changed sharply at the iso-electric point (Figure 6) and that it was higher below the iso-electric point than above, although the total amount of gelatin adsorbed was less. Another very significant effect observed was that the heat of adsorption had attained a constant value when only about 0.8 mg/m^2 of gelatin had been adsorbed compared with saturation figures of 3.3 and 5.5 mg/m^2 at pH values of 3.0 and 6.5 respectively. This supports the view originally put forward by Pouradier and Roman (1952) that at low surface coverage, molecules are adsorbed in a more extended configuration than at high surface coverages. Therefore as adsorption proceeds the proportion of segments adsorbed per molecule decreases in proportion to the number of molecules adsorbed. It should be noted that these results were obtained under conditions where there was a large shoulder on the adsorption isotherm and thus apparently not under conditions of equilibrium. A surprising feature of these results is that the heat of adsorption changes so little with pH except in the region of the iso-electric point. This suggests that any changes with pH in the proportion of segments adsorbed per molecule is exactly compensated for by the changes in the number of molecules adsorbed so that the total number of segments adsorbed remains constant. But on this assumption the sharp change at the iso-electric point is not easily explained.

Conclusions based on the proportion of segments adsorbed indicated by effects on Ostwald ripening and crystal dissolution suggest that when

saturation has been obtained the adsorbed layer should be fairly thick. Since they also suggest that charge interactions with the surface are the important factor it would be expected that on a negatively charged surface the layer would be thinner below the iso-electric point of the gelatin than above. However recent work suggests that such effects are not large. Maternaghan (1972) measured the adsorption of gelatin onto optically smooth polycrystalline silver bromide surfaces by ellipsometry. By this method, the changes in the polarization of light reflected from the surface are measured (Stromberg, 1967). Initially, measurements are made in the solvent so that the optical constants of the surface can be determined *in situ* and then the solvent is exchanged for the absorbate solution and the measurements repeated. In this way, the optical thickness and refractive index of the adsorbed layer can be determined and then the amount adsorbed calculated.

The results obtained at 40°C as a function of pH, with an alkali-processed ossein gelatin with an iso-electric point of 4.8 are shown in Figure 7. The solution phase contained 0.5 g per litre of gelatin and was adjusted to pBr 4. The root mean square thickness of the layer was of the order of 50 nm, similar to results obtained for polystyrene (Stromberg, 1967) and indicating an extended adsorbed layer. The work of Janus and Darlow (1962) with the viscometric technique (Section II, B, (b), (vi)) suggested similar thickness for adsorbed gelatin on glass.

Maternaghan's results in Fig. 7 show that the sharp decrease in adsorption below the iso-electric point of the gelatin is not matched by a corresponding decrease in layer thickness.

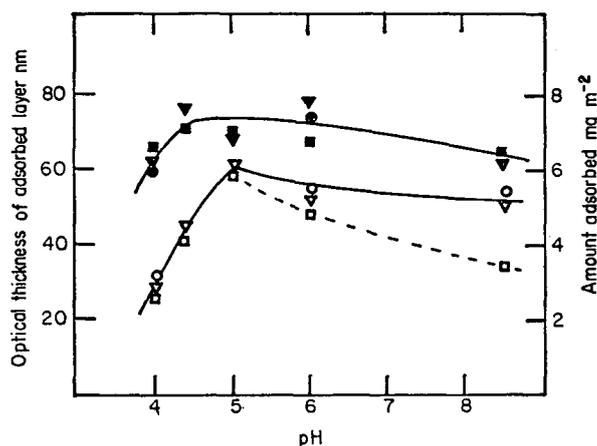


FIG. 7. Adsorption of gelatin in silver bromide: thickness of adsorbed layers as a function of time at 40°C. Gelatin concentration 0.5 g per litre; pBr 4. Thickness measured optically: ■ after 2 hr; ▼ after 12 hr; ● after 1 day. Amount adsorbed: □ after 2 hr; ▽ after 12 hr; ○ after 1 day.

It seems therefore that the only interpretation that is consistent with both these and the earlier results is that on a negatively charged surface, lowering the pH below the iso-electric point of the gelatin (and thereby increasing the net charge on the molecule) has the following effects: it extends the loops in the adsorbed layer due to charge repulsions between chains, giving a less concentrated adsorbed layer; it increases the number of adsorbed segments due to charge attraction between chains and surface without substantially reducing layer thickness. However the trend shown by results of Maternaghan for reduction in layer thickness may well become more substantial at lower pH values.

(vi) Multilayers. Gelatin molecules, which do not interact with one another at 40°C in solution, would not be expected to interact with a looped layer of adsorbed gelatin to form a multilayer; and in fact the adsorption isotherms for gelatin show no sign of multilayer formation. Multilayers of adsorbed polymers normally occur only in poor solvents where the formation of a condensed phase involves little change in free energy (Silberberg, 1972). Nevertheless at room temperatures gelatin molecules interact in solution to form hydrogen bonds so that the formation of multilayers under these conditions is likely to occur. On silver halide there is evidence that substantial skins of adsorbed gelatin are formed. These show up under electronmicroscopy after silver halide crystals on which they were adsorbed have been dissolved. Berg (1972) claims to have observed such skins on crystals that have never been cooled to room temperature and hence concludes that they are produced not by hydrogen bond formation but by interaction between gelatin and silver ions. This is supported by the work of Shinoda *et al.* (1972) who showed by scanning electronmicroscopy a structured surface on silver bromide, due apparently to gelatin. This could be removed by washing after treatment with 4-hydroxy-6-methyl tetra-azaindene, a well known complexing agent for silver ions. However existing knowledge on the interaction of silver ions with gelatin does not indicate how thick multilayers could be formed, and this phenomenon remains a puzzling one.

(d) *Electrokinetic experiments.* Reinder and Bendien (1928) showed by electrophoretic measurements that gelatin was adsorbed to negatively charged gold particles at a wide range of pH values both above and below the iso-electric point of the gelatin. Stable sols were produced independent of the lowering of zeta potential providing sufficient gelatin was present.

Kragh and Langston (1962) measured the electrophoretic mobility of gelatin on quartz at various pH values. They showed that the electrophoretic mobility depended largely on the charge on the gelatin molecule and was zero at the iso-electric point of the gelatin. Similar results have been obtained by Barr and Dickinson (1961) on silver bromide. The fact that the electro-

phoretic mobility does not correspond quantitatively to the net charge on the gelatin molecule in solution may be due to an effect of the surface charge of the solid or to differences in the position of the surface of shear as the configuration of the adsorbed layer changes with pH.

(e) *Effect of adsorbent.* Kragh and Langston (1962) found by experiments on flocculation that gelatin was adsorbed to almost any suspension. However quantitative measurements of adsorption have been reported only for silver bromide and quartz. The results of Kragh and Langston (1962) indicate that the saturation adsorption is only about half as great on quartz as on silver bromide. This is surprising since the inter- and intramolecular ionic interactions on which the saturation adsorption depends to a considerable extent, will not be affected by the adsorbent; furthermore the result would suggest that a higher proportion of groups were adsorbed on quartz than on silver bromide. This also seems unlikely since the most likely groups to be adsorbed on quartz are the cationic ones and these would not be expected to adsorb more strongly than on silver bromide. It is also not consistent with the large thicknesses suggested by the work of Janus and Darlow (1962). It is possible that the surface area, obtained by nitrogen adsorption on the dry quartz powder did not correspond to the area available to gelatin molecules in solution.

(f) *Flocculation with gelatin.* Although with gelatin there are many complicating factors due to charge interactions, it is clear that the main mechanism of flocculation does not depend on electric charge, since negative gelatin can flocculate negative suspensions, even though inefficiently. Furthermore, some neutral and negatively charged polymers flocculate some negatively charged suspensions better than gelatin.

The flocculation of various suspensions with polymers and poly-electrolytes of widely different character produces very similar effects and it is likely that the same basic mechanism applies. Most workers who have made detailed studies of flocculation with particular polymers have concluded that flocculation occurs when the surface of particles is only partially covered with adsorbed molecules, so that molecules attached by only a few segments to one particle can interact with the free remaining surface of other particles and form molecular bridges between them (Michaels and Morelos, 1955; Smellie and La Mer, 1958; Linke and Booth, 1959; Healy, 1961; Kragh and Langston, 1962; La Mer and Healy, 1963).

It was shown by Kragh and Langston (1962) that flocculation can occur in solutions of low ionic strength under conditions where the closest distance of approach of particles can be calculated to be several hundred angstrom units. It is therefore necessary for unadsorbed loops or chains of adsorbed gelatin molecules to extend some distance from the surface of the particles if bridges are to be formed.

Flocculation is therefore favoured by high molecular weight and occurs most readily with concentrated suspension where bridges can form before the molecules have reached an equilibrium state of adsorption. It seems reasonable to postulate that with very dilute suspensions and with dilute sols such as gold sols, gelatin molecules have time to be adsorbed more closely to their equilibrium configuration so that they extend less far from the surface when "collision" between particles occurs. Under these conditions there will be little or no flocculation if molecular bridges are unable to span the double-layer gap between particles. Similarly it can be postulated that the large decrease in flocculation produced by agitation is due to the fact that agitation of concentrated suspensions that have been flocculated with gelatin causes the breaking of molecular bridges formed under non-equilibrium conditions; this leads to progressively poorer flocculation since the bridging chains are likely to be adsorbed onto the particles. After deflocculation by agitation, the addition of salts, which lowers the closest distance of approach, then leads to reflocculation since bridges can again be formed. This is analogous to the coagulation of sensitized gold sols.

It is important to realize that flocculation by bridging leads to the formation of aggregates in which the individual particles remain separated by double layer repulsions. The floccules formed under these conditions are loose and have a large sedimented volume and flocculation is reversible. The classic mechanism of flocculation due to Van der Waals forces such as that obtained by salts on gold sols involves close contact between particles and produces flocculation that is much less easily reversed.

III. GENERAL CONSTITUTION OF PHOTOGRAPHIC FILMS

Photographic films may comprise up to a dozen different layers. In general there is a supercoat 1–2 μ thick consisting mainly of gelatin, which provides protection to the light-sensitive layers. These are coated on a plastic support which has previously been coated with one or more thin layers (subbing layers) that promote good adhesion between the emulsion and base. There is often also a backing layer on the other side of the base from the emulsion, which reduces the tendency of the film to curl and which may contain dyes to absorb light transmitted through the film. This layer may consist substantially of gelatin or of a synthetic polymer. Most films for use with X-rays are coated with emulsion on both sides of the base.

The emulsion layer contains microcrystals of silver halide in a binding medium which normally consists mainly of gelatin, though other polymers may be added. In monochrome films there may be one or two emulsion layers; in colour films up to six, as well as gelatin interlayers.

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 461

Fast emulsions contain mixed crystals of silver bromide and silver iodide, the iodide content being between 1 and 8%. Slower emulsions may consist of pure silver chloride or pure silver bromide but more often of mixtures of silver chloride and bromide in proportions that may vary widely.

The microcrystals of silver halide normally occupy between a quarter and a sixth of the volume of the dry layer, though in emulsions used for detecting nuclear radiation, they may occupy as much as half the volume. The average crystal size may vary between 0.04μ and 2μ , depending mainly on the speed of the emulsion. The crystal-size distribution and crystal shape also varies widely according to the type of film. A narrow crystal-size distribution produces an emulsion of high contrast. The thickness of the dry emulsion layer is commonly in the region of 10μ for negative and X-ray films.

IV. USE OF GELATIN IN EMULSION TECHNOLOGY

The production of photographic materials involves four processes in which different properties of gelatin are important; firstly the formation of the emulsion, that is the precipitation and growth of microcrystals of silver halide; secondly the washing of the emulsion to remove soluble salts; thirdly a chemical sensitization process during which the light sensitivity of the emulsion crystals is greatly increased; and fourthly the coating and drying of the emulsion on a base which may be glass, paper or plastic. Other properties of gelatin are important during processing after exposure.

A. Preparation of the Emulsion

1. *General*

Photographic emulsions are made by mixing a solution of silver nitrate with a solution of the halide, for example potassium bromide. Silver halide is then precipitated as crystals that are normally too small to be resolved with a microscope. The crystals are then grown, either following the addition of further silver halide, or by a process of Ostwald ripening whereby the larger crystals grow at the expense of the smaller ones which eventually disappear. Alternatively, the silver and halide solutions may be run into a stirred vessel at the same rate over a long period so that after an initial period of nucleation, growth occurs from a supersaturated solution of the silver halide. With all methods of producing emulsions the growth rate is usually increased by the addition of complexing agents for silver which increase the solubility of silver halide in solution. Excess bromide ions and ammonia are commonly used.

2. *Action of gelatin*

Very dilute solutions of silver nitrate and potassium bromide may be mixed to form stable sols, but at the concentrations used to produce photographic emulsions, often more than 1 M, a correspondingly high concentration of soluble potassium nitrate or other salt is formed by the reaction. In the absence of a protective colloid this salt would produce rapid aggregation of the microcrystals due to its action in narrowing the electric double layer round the crystals so that they can approach one another sufficiently close to be aggregated by Van der Waals forces. It is the primary function of the gelatin that is present at the mixing stage to prevent this process occurring. This protective-colloidal action of gelatin is well known for example in connection with the early work of Zsigmondy (1901) on the protection of gold sols (Section II B 2a). Gelatin, which is adsorbed in a relatively thick layer, prevents particles approaching one another closely enough for the attractive forces to be significant. The photographic literature nevertheless contains many references to the growth of silver halide by a coalescence process (Sheppard and Lambert, 1928; Arens, 1948; Malinowski, 1956; Hautot, 1958; Ammann-Brass, 1963; Perry, 1959; Klein *et al.*, 1971). Much of the evidence for "coalescence ripening" has been based on anomalous crystal size distributions which can now be explained by crystal twinning. The evidence from the recent work of Klein *et al.* (1971) is not easily assessed but cannot be so easily dismissed. Nevertheless coalescence ripening is difficult to reconcile with the known facts on protective-colloidal action and crystal morphology. However, under certain conditions of mixing the reagents in the preparation of photographic emulsions, some aggregation can occur before there has been time for gelatin molecules to be adsorbed. This effect has been investigated by Guttoff *et al.* (1963). There is no evidence that the type of gelatin is important in this process and in general it appears that there is little significant difference between the protective-colloidal action of different types of gelatin. The choice of gelatin for the mixing stage of emulsion making therefore depends mainly on the impurities present (Section C).

3. *Comparison of gelatin and other protective colloids*

There have been only a few published papers on the effect of protective colloids other than gelatin in the preparation of photographic emulsions (Evva, 1957; Dehio *et al.*, 1962; Saini *et al.*, 1964; Yano, 1968). However the exceptional qualities of gelatin are not merely dependent on its ability to prevent crystal aggregation, which can be matched by a number of water-soluble polymers; a more important factor is that gelatin does not interfere too greatly with the normal growth of silver halide crystals. In this respect polymers that behave similarly tend to be similar to gelatin with regard to

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 463

ionizable groups. For example acrylic copolymers with a proportion of amino or imino and carboxylic groups on side chains (Stonham, 1962; Yano, 1968). In contrast polyvinyl alcohol, which adsorbs strongly to iodobromide crystals and therefore prevents crystal aggregation, greatly inhibits crystal growth.

B. Washing Emulsions

With most emulsions, though in general not those used for photographic paper, the salts produced by double decomposition in the precipitation of silver halide, together with soluble halide salts or ammonia added for crystal ripening, need to be removed before sensitization of the emulsion and coating. This can sometimes be achieved conveniently by adding further gelatin and allowing the emulsion to set to a gel which is then shredded and washed with water. With this method the emulsion concentrations are necessarily fairly low due to the swelling of the gelatin gel in the wash water. When more concentrated emulsions are required the emulsion has to be coagulated before washing. A variety of agents that are well known for forming coacervates with gelatin can be used. These include salts such as sodium sulphate, inorganic solvents such as alcohol, surfactants such as dodecyl sulphate and a variety of polynuclear aryl-sulphonic acids. Chemically modified gelatins that form coacervates on lowering the pH below the iso-ionic point, may also be used without other coagulating agents.

The gel-forming properties of gelatin are clearly essential for the simple water-washing of emulsions but they are also important for the washing of emulsions after coagulation. Emulsion may be coagulated to give a high-concentration coacervate which is cooled to form a gel and then shredded and washed.

After washing, the emulsion may be set and stored in a refrigerated room before it is required for the next stage of the process.

C. Chemical Sensitization

Many photographic emulsions contain relatively little gelatin after precipitation and ripening and further gelatin is normally added. This gelatin is not necessarily of the same type as that used during the earlier stage of emulsion preparation and is selected for its suitability for the chemical sensitization process that follows.

1. Sulphur sensitization

(a) *General.* Towards the end of the 19th century after the replacement of collodion by gelatin it was discovered that heating photographic emulsions produced a large increase in light-sensitivity, though it soon became apparent

that this effect varied considerably with the gelatin used. It was not until 1925 however that Sheppard made his celebrated discovery of "sulphur sensitizers" in gelatin. It is now known that small traces of "active" sulphur compounds, mainly thiosulphate, present in the gelatin and derived largely from the sulphur-containing protein keratin, which occurs in hair and in the surface of the skin, are absorbed to the surface of the silver halide microcrystals and decompose when the emulsion is heated, to form silver sulphide. This process, which is called photographic digestion or, more confusingly, chemical ripening, greatly increases the light sensitivity of the emulsion.

It is a remarkable fact that gelatin, which was used for quite other reasons, contained thiosulphate in suitable amounts for the sulphur-sensitization process. The great majority of photographic emulsions are sulphur sensitized but it is no longer necessary to select and blend gelatins so that they contain a suitable concentration of naturally occurring sensitizers. Although emulsions are still made in this way with "fast" gelatins there is an increasing tendency to use "inert" gelatins and add the required amount of sodium thiosulphate or other sulphur sensitizers. The amount varies considerably depending on the type of emulsion but is of the order of 50 p.p.m. of thiosulphate ion in the gelatin present during digestion.

During the digestion process, which may take up to an hour or more at 60°C, the adsorbed thiosulphate, which covers only a small proportion of the surface of the silver halide, breaks down slowly to form silver sulphide. This process results in the formation of "sensitivity centres" which promote the formation of latent image at the crystal surface on exposure. The constitution of these centres is not known but it is possible that they contain silver as well as silver sulphide. With prolonged digestion the sensitivity centres become fog centres, i.e. centres that promote development of the unexposed crystals.

(b) *Action of restrainers.* The rate at which the digestion process proceeds, and the maximum photographic speed obtainable, can vary widely when different gelatins are used even though the amount of sulphur sensitizer present is the same. This effect is ascribed to impurities in the gelatin that restrain the sensitization process, although the possibility that it is in part caused by digestion activators cannot be ruled out (Nawn, 1968). The restraining power of gelatin is generally attributed to substances that adsorb strongly to silver halide and interfere with the formation or activity of the sensitivity centres. The most important of these is DNA in a partially degraded form (Venet and Pouradier, 1955; Steigmann, 1964; Russell and Oliff, 1966). Cysteine-containing proteins have been reported to act as restrainers (Steigmann, 1934), but also as activators (Nawn, 1968). There is little doubt that the main restrainer in highly restrained hide gelatins is

DNA, but ossein gelatins, which contain very little DNA (Russell, 1967) also vary in their restraining action and presumably contain some other restraining compound.

Within limits, the effects of increased restrainer content in the gelatin used for digestion can be compensated for by the addition of more sulphur sensitizer, but when the restrainer content is too high, the speed of the emulsion is reduced. Emulsions can be made with gelatin which, as far as is known, is free of restrainers, but many emulsions are made with partially restrained gelatins. Gelatins that are entirely free from restrainers are in fact not readily obtainable and it is important to keep the restrainer content of photographic gelatins to standard values to avoid variable digestion times.

Although restrainers are often detected by their effects on crystal growth in emulsions that have a very high ratio of gelatin to silver halide (Ammann-Brass, 1971), the effects are less important during the crystal growth stage of emulsion-making.

2. *Reduction sensitization*

It has been postulated by Chibisov (1959) that the important factor in the process of sulphur sensitization is not the formation of silver sulphide but the reduction of silver ion to silver which occurs concurrently with this reaction. The small quantity of silver that may be formed in this way is not readily detected, but there is ample evidence that the fog centres formed by over-digesting emulsions contain silver (Bassett and Dickinson, 1963). The importance of a reduction process in normal sulphur sensitization is however still a matter of controversy. It has been shown that gelatin together with its microconstituents varies in its reducing properties and it might be expected that these would be relevant. However there is no clear evidence that reducing tests for gelatin are important in relation to the sensitization reaction.

The sensitization process can however be enhanced, particularly with emulsions not sensitized with gold, by the addition of reducing agents such as stannous chloride, or a number of patented reagents, before digestion. This process is what is normally understood by "reduction sensitization".

3. *Gold sensitization*

A large number of emulsions are sensitized with gold as well as with sulphur compounds. The gold may be added as gold chloride or gold thiocyanate before or after digestion. This treatment produces a substantial increase in speed without increase in graininess and is particularly effective when flash exposures are used. The mechanism of the effect is complex and not fully understood. It has been discussed by Mueller (1966) and by Faelens (1968). It appears to involve both the incorporation of gold into the sensitivity centres on the surface of the crystal and the reaction of adsorbed gold

complexes with the latent image after exposure (Spracklen, 1961). The main effect is to promote the development of small latent image centres. There is little information on the importance of gelatin in gold sensitization though it may influence the process in a number of ways. The interaction has been studied by Pouradier *et al.* (1972) and Borginon (1972). Narath and Tiilikka (1961) and Faelens (1958) showed that silver halide could be sensitized more rapidly by gold in the absence of gelatin. The reducing action of gelatin, which has been ascribed to methionine (Pouradier, 1966) and to micro-constituents in gelatin, may also be important in the modification of sensitivity centres by gold. Mueller (1966) states that restrainers including nucleic acid interfere with the gold-sensitization reaction by complexing the gold.

The action of gelatin during gold sensitization is thus not well understood. It is necessary therefore to select gelatins by testing them in gold-sensitized emulsions.

4. Action of gelatin

During the course of digestion both speed and fog increase, but under suitable conditions the speed approaches a maximum before the fog has risen to an unacceptable level. It is on the control of this process that the selection of gelatins for digestion is primarily based, although the growth of fog on storage of the coated emulsion has to be considered as well as the fog formed immediately after digestion. Fogging effects are varied and complex; although the first requirement is that a gelatin should not produce fog during the digestion process it is found that some gelatins that are satisfactory in this respect cause fog when used for emulsification or when added after digestion. The impurities in gelatin that are responsible for fog formation or fog inhibition are not in general known and gelatins have to be tested in photographic emulsions. However, different emulsions vary in their susceptibility to fogging; for example this depends on gold sensitization (Section 3); but in general gelatins that do not produce fog in the most sensitive emulsions are satisfactory in other emulsions.

The significance of macromolecular gelatin, as distinct from its micro-constituents, in the digestion process is uncertain since there is little published work showing comparisons between gelatin and other colloids. Sometimes at least, it is found that when emulsions are prepared in protective colloids other than gelatin, a better speed-to-fog ratio is obtained when gelatin is added for digestion than when the emulsion is digested with only the other colloid present. It appears therefore that gelatin is of particular value in the digestion process for inhibiting the formation of fog centres. This effect is probably due to macromolecular gelatin rather than to any micro-constituents, since good speed-to-fog ratios can be obtained with the purest gelatins available.

D. Final Additions, Coating and Drying

1. Final additions

After emulsions have been digested a number of additions are made before coating. These usually include more gelatin, wetting agents and a hardening agent, i.e. a compound that crosslinks gelatin. The gelatin must not produce adverse sensitometric effects but the requirements are less critical than for the gelatins used at the earlier stages of emulsion making.

2. Requirements of gelatin for coating

The rheological properties of gelatin are of particular importance during the coating and drying of the emulsion. These processes however can be carried out in a variety of different ways and it is not possible to generalize on the desirable properties of the gelatin. In general high gel-strength is required, but high viscosity, which is necessary for some coating processes, is a disadvantage in others.

The coating machine applies the liquid emulsion or other gelatin layers, one or more layers at a time, to the film base which is then passed through a series of chambers where the coating is set rapidly and dried in the gel state. The rapid-setting property of gelatin is particularly useful in that it enables relatively thick aqueous coatings to be kept at a uniform thickness during drying without the coating having to be maintained horizontal. Rapid setting of the gelatin is obviously desirable at high coating speeds and a high melting point is also desirable to avoid melting during drying.

E. Action of Gelatin on Exposure of Emulsions to Light

On exposure to light, silver halide decomposes to form silver and halogen. It is important for the liberated halogen to be removed otherwise it will tend to recombine with the silver. Adsorbed sulphur sensitizers can act as halogen-acceptors (Frieser and Ranz, 1965), but gelatin probably fulfils this function also, at least at high exposure levels where solarization (reduction of density as exposure increases) would otherwise occur. Although the reaction of gelatin with photolytic halogen cannot be demonstrated at normal exposures, the reaction after long print-out exposures has been demonstrated by electron micrography by Hamilton *et al.* (1956).

It has been postulated by Loening (1951), on the basis of work with silver halide sols prepared in the absence of gelatin, that gelatin increases light sensitivity. However it has been shown by Keller (1967) that this apparent effect is due to the fact that gelatin stabilizes the latent image. With the simple silver bromide sols used, the latent image faded within seconds whereas when gelatin was added immediately after exposure the latent image

was stable for several hours. It is reasonable to conclude therefore that gelatin performs an important function in stabilizing the latent image in photographic emulsions though there is no information in the literature concerning the comparative effects of different gelatins. Keller (1967) found considerable differences between gelatins with silver halide sols; he also found that polyvinyl alcohol and polyvinyl pyrrolidone, although they had some effect, were far less effective than gelatin in stabilizing the latent image.

F. Photographic Processing

1. *The effect of gelatin on photographic development*

A photographic developer is usually an alkaline solution containing a reducing agent which will reduce silver halide rapidly when the reaction is catalysed by the latent image formed on exposure, and very slowly when the silver halide has not been exposed. There is evidence that gelatin enhances the ability of a developer to distinguish between exposed and unexposed crystals, but it is difficult to separate such an effect from its effect in stabilizing the latent image. Gelatin certainly moderates the development process so that a developer, required to work optimally in the absence of gelatin, would have a different formulation from one used with gelatin present. Comparisons under optimum conditions have not been reported.

2. *The control of swelling*

(a) *General.* In the simplest form of photographic processing the film or paper is placed in an alkaline developer, at a pH usually between 8 and 12, which converts exposed silver halide to silver. After a brief wash it is transferred to a fixing bath which is normally a concentrated solution of sodium thiosulphate. This dissolves the remaining unexposed silver halide. The film is then washed. With colour films the processing involves further stages.

There is an increasing tendency for films to be processed in automatic machines in some of which the films have to pass between rollers at relatively high temperatures. During processing the emulsion layer has to swell so that processing solutions can penetrate the layer, but it has to withstand undamaged the stresses of handling and the rapid changes in swelling produced by the processing cycle. It must then dry down to a layer with a uniform undamaged surface. These stringent requirements often cannot be met when the binder consists of unmodified gelatin.

With the exception of certain colour films, which may contain very large proportions of colour couplers, and of films containing other binders mixed with gelatin, the swelling and hardness of coated emulsion layers can be considered in terms of the properties of the gelatin binder. The presence of

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 469

the silver halide, is not in general important except in so far as it affects the appearance of the surface of the dried film.

The swelling of gelatin is considered in Section II A where it is shown that the degree of swelling depends on the amount of hydrogen bonding in the layer, and in particular on the concentration of the gelatin at the time the hydrogen bonds were formed. Thus it depends on the thermal history of drying and storage of the layer. In the production of films it is not practicable to control the properties of films, except to a limited extent, by changes in coating and drying methods. Furthermore, gelatin gels in which the only links are hydrogen bonds do not stand up to high-temperature processing. The introduction of additional covalent crosslinks is therefore necessary.

(b) *Hardening*. The term hardening is used rather loosely in photographic technology and may be taken to refer to changes in more fundamental properties such as rigidity modulus, ultimate breaking strength and creep. It can also be considered in terms of the swelling of gelatin since clearly a hard layer is obtained much more readily when the water content is low. The hardness can be controlled by the addition to the solution before coating of crosslinking agents for gelatin such as formaldehyde or glyoxal. These conditions are normally such that only a small amount of the crosslinking occurs during coating and drying so that most of the hardening reaction takes place in the dry layer where it has the most effect in reducing swelling. The amount of hardening carried out in this way is limited by adverse effects on sensitometric characteristics, and with fast films the hardening is often insufficient to produce a complete covalent network of gelatin chains; that is to say the gelatin will still disperse when heated in water. This may however require a temperature of 60°C or more, rather than about 30°C which would be normal for uncrosslinked gelatin.

When films are to be processed at high temperatures, a crosslinking agent may be added to the developing solution. At the high pH this reacts very rapidly and since it acts on the swollen layer can produce a considerable increase in hardness without large changes in swelling. Inorganic crosslinking agents such as aluminium sulphate may also be added to the fixing bath.

The type of gelatin used is not very significant in relation to the hardening reaction although the rate of crosslinking in solution and the dispersion temperature of the dry layer are functions of the molecular weight of the gelatin.

G. Properties of Dry Gelatin Films

The properties of dry films of gelatin are less easily modified than the properties of swollen ones. For example crosslinking has comparatively little effect. Plasticizers may be used but only to a limited extent when, like glycerol, they act as humectants, since a high moisture content in the film produces

adverse sensitometric effects. It is well known that the properties of dry gelatin films differ according to the conditions of drying (Chapter 8), but drying above the melting point, which for example is essential for producing the tack in the glue for gummed paper, produces a film which is unstable in its properties.

The main disadvantage of gelatin is its change in moisture content with humidity which leads to curling of photographic films and papers and to charging with static electricity under dry conditions. The curling of films is often controlled by coating a layer of gelatin on the back. A variety of treatments may be used to modify the electrical properties.

H. Photographic Paper

(a) *General.* Most of the foregoing description of the use of gelatin in photographic emulsions applies also to emulsions used for coating on paper, and in general the same type of gelatins are used.

Most emulsions for paper contain silver chloride or silver chlorobromide rather than silver iodobromide and the excess ions present are chloride rather than bromide. Furthermore ammonia is not normally used for emulsion making. For these reasons, and also because the emulsions are coated on adsorbent paper, the emulsions do not normally have to be washed. Washing may however be necessary when the emulsions are coated on paper that has been made impermeable to water.

Another difference between film and paper emulsions is that the photographic papers are usually given long exposures and therefore there is little advantage in gold sensitization and this is not normally carried out.

The control of fog is particularly important in emulsions for paper since highlights must be as white as possible. However, the relatively slow emulsions used for paper are less susceptible to fog than the iodobromide emulsions used for fast films.

(b) *Baryta coating.* Before paper is coated with emulsion it is first coated with a suspension of barium sulphate in gelatin which gives a smooth bright surface to the paper. A gelatin hardener is also added. The volume of gelatin in the layer is smaller than the volume of barium sulphate.

The main requirement of the gelatin used for baryta coating is that it should not produce any adverse sensitometric effect in the emulsion layer coated above it.

V. CONCLUSIONS

It is apparent that gelatin is particularly well adapted for photographic use in the following respects:

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 471

It prevents flocculation of silver halide crystals in solutions of high ionic strength.

It has only a small effect in impeding crystal growth.

It facilitates washing because of its setting properties and because it is easily coagulated.

It enables emulsions to be set, stored and remelted without sedimentation of the silver halide.

It controls the digestion process so that high speed can be obtained with low fog.

It facilitates coating due to its property of setting rapidly.

It dries to a tough, coherent, transparent film.

It acts as a halogen acceptor during exposure.

It stabilizes the latent image.

It swells so as to permit processing.

Possibly also it enhances the selectivity of the developer in *distinguishing* between sensitivity and fog centres.

In many of these properties it is unique or rivalled only by polyelectrolytes that have a similar distribution of charged groups.

If the earlier history of emulsion making is considered, the adaption of gelatin to the photographic process seems even more remarkable since it contains the sulphur sensitizers later found essential for obtaining high sensitivity. It also contains DNA as a restrainer though the value of this is less well established. In fact it might be thought that gelatin had been designed with the photographic process in mind.

The reason for this is in part that the photographic process has been developed in conjunction with the use of gelatin over a period of a hundred years and has become increasingly dependent on its properties. Gelatin is relatively cheap and increasingly is being manufactured pure and reproducibly, and development of a special binder for photographic use is likely to be expensive. It seems more probable therefore that in future silver halide emulsions will continue to be based on gelatin although increasingly incorporating other polymers. Photographic systems not based on silver halide do not normally involve the use of gelatins and are not likely to. However at present, processes based on silver halide are by far the fastest and most flexible, and non-silver processes have advantages only for certain specific uses.

REFERENCES

- Ammann-Brass, H. (1963). *In* "Photographic Science" (W. F. Berg, ed.) Focal Press, London.
- Ammann-Brass, H. (1972). *In* "Photographic Gelatin" (R. J. Cox, ed.) pp. 251-67, Academic Press.

- Arens, H. (1948). *Z. wiss. Photograph.* **43**, 120-38.
- Barr, J. and Dickinson, H. O. (1961). *J. Photograph. Sci.* **9**, 2-9.
- Berg, W. F. (Private Communication).
- Bassett, R. A. and Dickinson, H. O. (1963). *J. Photograph. Sci.* **11**, 239-48.
- Berendsen, R. and Borginon, H. (1968). *J. Photograph. Sci.* **16**, 194-8.
- Borginon, H. (1972). In "Photographic Gelatin" (R. J. Cox, ed.) pp. 183-92, Academic Press.
- Bolam, T. R. (1932). "The Donnan Equilibria". Bell, London.
- Chibisov, K. V. (1959). *J. Photograph. Sci.* **7**, 42-54.
- Curme, H. G. and Natale, C. C. (1964). *J. phys. Chem.* **68**, 3009-16.
- Dehio, H., Polla-Mattiot, G., Gillio-Tos, M. and Saini, G. (1962). *J. Photograph. Sci.* **10**, 302-5.
- Ellerstein, S. and Ullman, R. J. (1961). *J. Polymer Sci.* **55**, 123-35.
- Evva, F. (1957). *Z. wiss. Photograph.* **52**, 1-24, 64-74, 237-47.
- Faelens, P. A. (1968). *Photogr. Korr.* **104**, 137-45.
- Flory, P. J. and Behner, J. (1943). *J. chem. Phys.* **11**, 513-20.
- Flory, P. J. (1953). "Principles of Polymer Chemistry". Cornell University Press, New York.
- Fontana, B. J. (1963). *J. phys. Chem.* **67**, 2360-2.
- Frieser, H. and Ranz, E. (1965). *Ber. Bunsenges. phys. Chem.* **68**, 389-99.
- Frisch, H. L. and Simha, R. (1954). *J. phys. Chem.* **58**, 507-12.
- Frisch, H. L., Hellman, N. Y. and Lundberg, J. L. (1959). *J. Polymer Sci.* **38**, 441-49.
- Guttoff, F. B., Roth, P. H. and Steigmann, A. E. (1963). *J. phys. Chem.* **67**, 2366-73.
- Healy, T. W. (1961). *J. Colloid Sci.* 609-17.
- Hamilton, J. F., Hamm, F. A. and Brady, L. E. (1956). *J. appl. Phys.* **27**, 874-85.
- Hautot, A. (1958). "Photographic Scientifique", Etablissements Ceuterick, Louvain.
- Herz, A. H. and Helling, J. O. (1966). *J. Colloid Sci.* **22**, 391-403.
- Hesselink, F. Th. (1971). *J. Phys. Chem.* **75**, 65-71.
- Hoeve, C. A. J., Dimarzio, E. A. and Peyser, P. (1965). *J. Chem. phys.* **43**, 539-47.
- Hoeve, C. A. J. (1966). *J. Chem. phys.* **44**, 1505-9.
- Janus, J. W. and Darlow, R. L. (1962). *Nature, Lond.* **194**, 1075-6.
- Jenkel, E. and Rumbach, B. (1951). *Z. Elektrochem.* **55**, 612-18.
- Jopling, D. W. (1956). *J. appl. Chem.* **6**, 79-84.
- Jordon Lloyd, D. J. (1926). "Chemistry of the Proteins". Churchill, London.
- Jordon Lloyd, D. J. and Pleass, W. B. (1927). *Biochem. J.* **21**, 1352-67.
- Keenan, A. L. (1929). *J. phys. Chem.* **33**, 371-80.
- Keller, H. E. (1967). *Photogr. Korr.* **103**, 85-91, 104-12.
- Klein, E., Moisar, E. and Roche, E. (1971). *J. Photograph. Sci.* **19**, 55-8.
- Kragh, A. M. (1964). *J. Photograph. Sci.* **12**, 191-201.
- Kragh, A. M. and Langston, W. B. (1962). *J. Colloid Sci.* **17**, 101-23.
- Kragh, A. M. and Peacock, R. (1967). *J. Photograph. Sci.* **15**, 220-5.
- Kolthoff, I. M., Gutmacher, R. G. and Kahn, A. (1951). *J. phys. Chem.* **55**, 1240-46.
- Koral, J., Ullman, R. and Eirich, F. R. (1958). *J. phys. Chem.* **62**, 541-50.
- La Mer, V. K. and Healy, T. W. (1963). *Rev. pure appl. Chem. (Aust.)* **13**, 112-33.
- Lanza, P. and Lippolis, M. T. (1969). *J. Photograph. Sci.* **17**, 155-6.

14. SWELLING, ADSORPTION AND THE PHOTOGRAPHIC USES OF GELATIN 473

- Libicky, A. and Bermame, D. (1972). In "Photographic Gelatin" (R. J. Cox, ed.) pp. 29-48, Academic Press.
- Limberg, H. (1926). *Rec. trav. chim.* **45**, 875-89.
- Linke, W. G. and Booth, R. B. (1959). A.I.M.E. Convention, San Francisco. (American Cynamid Co. Stamford).
- Loening, E. E. (1951). In "Fundamental Mechanisms of Photographic Sensitivity" pp. 149-60. Butterworths, London.
- Malinowski, J. (1956). *Z. wiss. Phot.* **51**, 186-200.
- Maternaghan, T. (Private communication).
- Michaels, A. S. and Morelos, O. (1955). *Ind. Engng. Chem.* **47**, 1801-09.
- Mueller, F. W. H. (1966). *Photogr. Sci. Engng.* **10**, 338-43.
- Narath, A. and Tiilikka, A. (1961). *J. Photogr. Sci.* **9**, 303-11.
- Nawn, G. H. (1968). *Photogr. Sci. Engng.* **12**, 108-16.
- Oehrn, O. E. (1958). *Ark. Kemi.* **12**, 397-436.
- Ottewill, R. H. and Rastogi, M. C. (1960). *Trans. Faraday Soc.* **56**, 866-92.
- Overbeek, J. Th. G. (1938). *Chem. Weekbl.* **35**, 117-21.
- Overbeek, J. Th. G. (1956). In "Progress in Biophysics and Biophysical Chemistry". Vol. 6, pp. 57-84. Pergamon Press, London.
- Perkel, R. and Ullman, R. (1961). *J. Polymer Sci.* **54**, 127-48.
- Perry, E. S. (1959). *J. Colloid Sci.* **14**, 27-35.
- Pouradier, J. and Roman, J. (1952). *Sci. Inds fotogr.* **23**, 4-13.
- Pouradier, J. (1966). *J. Chim. phys.* **63**, 469-75.
- Pouradier, J. (1967). *J. Chim. phys.* **64**, 1616-20.
- Pouradier, J., De Cugnac-Pailliotet, A. and Gadet, M. C. (1972). In "Photographic Gelatin" (R. J. Cox, ed.) pp. 175-82, Academic Press.
- Proctor, H. R. and Burton, E. F. (1916). *J. Soc. chem. Ind., Lond.* **35**, 404-9.
- Proctor, H. R. and Wilson, W. H. (1916). *J. chem. Soc.* **109**, 307-19.
- Reinder, W. and Bendien, W. M. (1928). *Rec. trav. chim.* **47**, 977-88.
- Roe, R. J. (1965). *J. Chem. phys.* **43**, 1591-8.
- Romer, R. and Sidorowicz, A. (1967). *J. Photogr. Sci.* **15**, 115-23.
- Russell, G. and Oliff, D. L. (1966). *J. Photogr. Sci.* **14**, 9-22.
- Russell, G. (1967). *J. Photogr. Sci.* **15**, 236-40.
- Saini, G., Polla-Mattiot, G., Leoni, A. and Marini, M. (1964). *J. Photogr. Sci.* **12**, 307-11.
- Sheppard, S. E. and Lambert, R. H. (1928). In "Colloid Symposium Monograph" (H. B. Weiser, ed.) Vol. 6, pp. 265-82. Chemical Catalogue Co., New York.
- Silberberg, A. (1962). *J. phys. Chem.* **66**, 1872-83, 1884-907.
- Silberberg, A. (1967). *J. Chem. phys.* **48**, 2835-51.
- Silberberg, A. (1972). *J. Colloid and Interface Sci.* **38**, 217-26.
- Simha, R., Frisch, H. L. and Eirich, F. R. (1953). *J. phys. Chem.* **57**, 584-9.
- Simms, W. M. and Blake, J. N. (1960). *Nature*, **187**, 998. Also in "Research Panel Paper No. 39", Gelatine and Glue Research Association.
- Smellie, R. H. and La Mer, V. K. (1958). *J. Colloid Sci.* **23**, 589-99.
- Spracklen, D. M. (1961). *J. Photogr. Sci.* **9**, 145-50.
- Steigmann, A. (1934). *Photogr. Korr.* **70**, 184-5.
- Steigmann, A. (1964). *Sci. Inds fotogr.* **35**, 145-57.
- Stonham, J. P. (1962). British Patent 967208.
- Stromberg, R. R. (1967). In "Treatise on Adhesion and Adhesives" (R. L. Patrick, ed.), Vol. 1, pp. 69-118. Edwin Arnold, London.
- Taylor, D. J. and Kragh, A. (1974). *J. Photogr. Sci.* **22**, 223-7.

- Tomka, I., Bohonek, J., Spuehler, J. and Ribeaud, M. (1975). *J. Photogr. Sci.* **23**, 97-103.
- Venet, A. M. and Pouradier, J. (1955). *Bull. Soc. chim. Fr.* pp. 922-8.
- Yano, T. (1968). *Photogr. Korr.* **104**, 121-26.
- Zimkin, E. and Klyuchevitch, V. (1968). *J. Photogr. Sci.* **16**, 154-6.
- Zsigmondy, R. (1901). *Z. analyt. Chem.* **40**, 697-719.
- Zsigmondy, R. and Joel, E. (1924). *Z. phys. Chem.* **113**, 299-312.

Chapter 15

The Chemical Examination of Gelatins

A. A. LEACH

The Brewers' Society, London, England

AND

J. E. EASTOE

*Department of Dental Science, Royal College of Surgeons of England,
London, England*

I Introduction	476
II Measurement of the Gelatin Content of Solids and Solutions ..	476
A. Balance Sheet for Gelatin; the Significance of the Moisture and Ash Content	476
B. Methods of Measuring Gelatin Content	477
III Amino Acid Analysis of Gelatins	481
A. Significance and Limitations of Amino Acid Composition ..	481
B. Hydrolysis of Gelatin	481
C. Chromatographic Analysis	482
D. Direct Measurement of Hydroxyproline	483
E. Direct Measurement of Amino Acids	485
F. Direct Measurement of Amide Nitrogen	485
G. Amino Acids Derived from Impurities	485
IV Examination of the End Groups of Protein Chains	486
A. Introduction	486
B. N-Terminal Groups	486
C. C-Terminal Groups	487
V Examination of Side Chain Groups	488
A. Computation from Amino Acid Composition	488
B. Titration Curves	488
C. Direct Measurement	489
VI Examination of Other Constituents of the Gelatin Molecule ..	491
A. Introduction	491
B. Carbohydrate	492
C. Other Residues	493
VII Examination of Chemically Modified Gelatins	493

A. Introduction	493
B. Titration Curves	494
C. Formol Titration	494
D. Spectrophotometric Method	494
E. Ninhydrin Colorimetric Procedure	495
F. Chemical Methods	495
VIII Gelatin Contaminants	496
A. Inorganic Components	496
B. Proteins and Mucosubstances	500
C. Lipids	501
D. Other Minor Components	501

I. INTRODUCTION

This chapter briefly describes the methods employed in the chemical examination of gelatins and pays particular attention to their limitations or advantages. Owing to space considerations most experimental details are not included. However the references to the methods are noted, the majority of which are readily available. Some of the methods can be applied to collagens and some are also applicable to other proteins.

II. MEASUREMENT OF THE GELATIN CONTENT OF SOLIDS AND SOLUTIONS

A. Balance Sheet for Gelatin; the Significance of the Moisture and Ash Content

The moisture and ash contents of gelatins together do not normally exceed 20% of the weight of the solid. The estimations of these components, whilst simple, require care before reliable values can be obtained. All other analyses must be related back to dry and ash-free material so that it is vital that these values shall be as accurate as possible. Whilst carrying out a programme of investigation on a sample of gelatin, regular checks on the moisture content must be carried out as it is likely to vary if the sample is subjected to repeated exposure to the atmosphere. Before carrying out such a programme it is worthwhile equilibrating the gelatin with the atmosphere.

The British Standard (1975) defines moisture content as the percentage loss in weight of the sample when a thin and evenly distributed film of gelatin is dried at 105°C for 18 ± 1 hours. A lid is placed on the dish which is then cooled in a desiccator, reweighed and the loss in weight recorded as moisture. Eastoe and Williams (1959) have shown the procedure to be reliable and also that if the gelatin powder is finely divided then the dissolution and evaporation stages can be omitted. The present authors have found that

provided the oven has adequate circulation of air the gelatin plus water can be placed in the oven directly. Earlier Hitchcock (1931) showed that constant weight was obtained after about three days at 105°C. However the first mentioned authors showed that this value is only 0.1 to 0.3% higher than that obtained by the standard procedure. Severe or prolonged heating conditions must be regarded as suspect because of the loss of water resulting from the decomposition of the protein which becomes apparent from its increasing insolubility.

Langston *et al.* (1965) studied the British Standard (1959) procedure for measuring the ash content of gelatins and found it to be both time consuming and unreliable. They also demonstrated that ashing in a muffle furnace frequently yielded low and non-reproducible values unless the sample was sulphated before or after ashing. Sulphating usually results in a slight overestimate of the ash content because the equivalent weight of sulphate is higher than those of most of the other radicles present in gelatins in significant amounts.

B. Methods of Measuring Gelatin Content

1. Total N content

Where gelatin occurs in the absence of other nitrogenous substances, its concentration can be accurately measured by determining the total nitrogen content. An accuracy of $\pm 0.2\%$ is possible and the method can be made very sensitive by evaporating a sufficiently large volume of solution, acidified with sulphuric acid to prevent loss of ammonia. The total N contents of dry, ash free alkali- and acid-processed gelatins are 18.15 and 18.30% respectively and corresponding factors of 5.51 and 5.46 should be used to convert weight of nitrogen to gelatin. The factor 6.25, often employed to calculate the protein content of foodstuffs, is generally misleading where the protein can be identified and its N content measured.

Total N is most easily determined by the Kjeldahl method. The procedure of Chibnall *et al.* (1943) in which 0.1 g of protein is digested with 20 ml of sulphuric acid and 5 g of potassium sulphate-catalyst mixture, gives accurate results for gelatin. The digestion time can be reduced to 2 hours with complete recovery of nitrogen as ammonia, if an equal weight of mercuric sulphate is substituted for copper sulphate in the catalyst mixture (Eastoe and Eastoe, 1954). Sodium thiosulphate (5% w/v) should be incorporated in the 30% (w/v) sodium hydroxide solution added at the distillation stage. Ammonia is steam-distilled from a suitable small portion of the sulphuric acid digest in the apparatus of Markham (1942) or, more conveniently, that of Yuen and Pollard (1953). The ammonia is absorbed in 1% (w/v) aqueous boric acid and titrated with 0.01 N-hydrochloric acid using methyl red screened

with methylene blue as indicator. The detailed procedure is described by Eastoe and Courts (1963) who also give a micro-Kjeldahl technique, based on Nesslerization, suitable for 0.1 mg of gelatin. Where metals are likely to interfere with the Nessler reagent the ammonia may be distilled in a stream of nitrogen in a micro-still and the Nessler or ninhydrin reaction applied to the distillate (Eastoe, 1968).

2. *Hydroxyproline*

The determination of hydroxyproline, which is described in Section IIID can be used to measure the concentration or proportion of gelatin or collagen in the absence or presence of other substances including proteins. The calculation is on the basis of the yield of hydroxyproline per unit weight of gelatin. The minimum concentration of hydroxyproline which can be measured with precision is about 5 μg per ml which is equivalent to about 35–42 μg commercial gelatin. The method can be applied most accurately when the type of gelatin or collagen is known because of the variation of the proportion of hydroxyproline in collagens and gelatins (Eastoe, 1967). The hydroxyproline content of alkali-processed gelatins is higher than those produced by the acid-process (Courts and Kragh, 1960). By careful examination of the published data for the proportion of hydroxyproline present in the type of gelatin or collagen under investigation, the errors from such a variation can be minimized.

3. *Reaction with ninhydrin*

(a) *After total hydrolysis.* The reaction with ninhydrin to give Ruhemann's purple of the amino acids and ammonia, produced by hydrolysis of a protein, provides the basis for a sensitive method of protein determination (Eastoe and Courts, 1963; Eastoe, 1965). The colour produced per unit weight varies somewhat for different proteins according to their amino acid composition. Although the variation is much smaller and more predictable than with the Lowry method (see next section), it is nevertheless important that calibration should be carried out with a protein as similar in composition as possible to the unknown. This is readily achieved for gelatins by choosing, as standard, a sample manufactured under similar conditions to the unknown. The difference in amide content will affect the colour density to a maximum extent of approximately 5% for extremes of isoionic point (see Chapter 3, Section IIIA1). Gelatin and collagen give approximately 20% less colour than the majority of proteins as the imino acids, which are present in relatively large amounts, produce yellow colours which have negligible absorption at 570 nm, where the purple colour is measured. Approximately 2.5 μg of gelatin correspond to an optical density of 0.1 with a final volume of 8 ml and 2 cm optical path (method of Moore and Stein, 1948). A five-fold

increase in sensitivity is possible by a corresponding reduction in reagent volumes and modification of the spectrophotometer for small samples (Eastoe, 1961).

Hydrolysis is carried out with 6N-hydrochloric acid (see Section IIIB), which is subsequently removed by evaporation in vacuo. Where the ratio of gelatin to acid is high, the evaporation step may be avoided by careful neutralization of the hydrolysate to pH 5. The hydrolysate is dissolved in pH 5.0, 0.2 M-sodium citrate buffer and diluted to a suitable volume, such that 1 ml portions taken for analysis correspond to 10–15 μg of gelatin. Blank and standard determinations should be run and used to calculate the gelatin content of the unknown. The ninhydrin reagent should be fresh, or if stored under nitrogen and in the dark it may be used until it is about two weeks old.

(b) *Without hydrolysis stage.* The ninhydrin colorimetric procedure described in Section VIIE can be used to estimate the concentration of gelatin by means of its ϵ -amino groups. The measurements must be carried out in the absence of other proteins, ammonia and other ninhydrin positive substances. Ideally the gelatin used in the studies should be employed as the reference material. The major difficulty with this method is that when low grade gelatins or degraded gelatin are under examination the contribution of α -amino groups to the intensity of colour becomes significant and can vary widely so that an estimation using another gelatin as a reference can be greatly in error. The method is best confined to the estimation of gelatin of high molecular weight in say physicochemical studies where degradation of the molecules does not take place.

4. *The Lowry method*

The method of Lowry *et al.* (1951) is based on preliminary treatment of the unhydrolysed protein with an alkaline copper reagent, followed by reaction with Folin and Ciocalteu's (1927) phenol reagent. The amount of colour formed varies considerably for different proteins depending on the tyrosine and tryptophan contents and the sequence of certain amino acids with functional groups especially histidine, arginine and glutamic acid. Although preliminary digestion or hydrolysis is avoided the comparative insensitivity of the method for gelatin and the possibility of variation between gelatins of different tyrosine contents must be regarded as potential disadvantages.

5. *Refractive index*

Measurement of refractive index enables the approximate gelatin content of fairly concentrated aqueous solutions to be rapidly determined and is

suitable for control purposes during manufacture. The refractive index is referred to that of water. If the Abbé refractometer is used, it is necessary to maintain the platform at constant temperature (Kenchington, 1952). The double cell Pulfrich refractometer enables the difference in refractive index to be determined directly, water being placed in one compartment and the gelatin solution in the other, so that the need for close temperature control is less critical (Krane and Watson, 1929). A refractive index increment of 0.0018 is given by 1% of gelatin at 34.5°C with white light in the Abbé instrument, the difference between true and measured percentage concentrations of gelatin being approximately $\pm 0.15\%$. Inorganic salts, in the concentrations usually present, cause no significant error.

6. *UV absorption*

The absorption of radiation in the far ultraviolet region by the peptide bond can be used for the direct estimation of the concentration of gelatin in solution provided no interference from other substances exists (Tombs *et al.*, 1959). Measurement at a wavelength of 210 nm is usually the most convenient. However with many spectrometers this is a region of low sensitivity and errors may also arise from stray radiation. Measurements can be made in the phenylalanine and tyrosine absorption regions (see Section III E) but the sensitivity is considerably reduced. The above type of measurement is most useful as a means of following fractionation procedures.

7. *Protection of gold sols*

Addition of electrolytes to sols of colloidal gold causes a change in colour from red to blue, which results from the coagulation of the gold particles. Proteins protect the sol from this change, the degree of protection depending on the concentrations of protein and electrolyte. This has been used for the detection and approximate determination of low concentrations of proteins, including gelatin in solution (Davis, 1956; method given in Eastoe and Courts, 1963). Colours are matched visually against those produced by dilutions of a 1 p.p.m. gelatin solution, under specified experimental conditions. A number of trials may be required to obtain the correct dilution range. Ideally a match is made between solutions mid-violet in colour, a tint which can be produced by 1.5 μg of alkali-processed or 3 μg of acid processed gelatin in a final volume of 14 ml in this test. Red colours correspond to too high and blue colours to too low concentrations of the protective colloid.

8. *Miscellaneous methods*

Various methods have been suggested for detecting and determining gelatin in foods, in which interfering organic substances are present. To

detect gelatin in dairy products, other proteins are first removed with alkaline basic lead nitrate (Jacobs and Jaffe, 1932) or mercuric nitrate (Ferguson and Racicot, 1936), after which tannic acid or picric acid gives a precipitate when gelatin is present. As little as 0.025% of gelatin can be identified in milk products by its protective effect on the formation of a cadmium sulphide precipitate which remains in colloidal solution (Braunsdorf, 1934). Přistoupil *et al.* (1956) described a method for the turbidimetric determination of gelatin with tannin. Gelatin in meat extracts may be separated as an insoluble complex with formaldehyde, the total nitrogen of which is determined (Analytical Methods Committee, 1953). Remington and McRoberts (1926) determined gelatin in ice cream by precipitation with alum and 90% ethanol, after removal of casein by isoelectric precipitation.

III. AMINO ACID ANALYSIS OF GELATINS

A. Significance and Limitations of Amino Acid Composition

For those proteins which can be prepared in a pure and homogeneous state, the data from amino acid analysis can be used to determine exactly the number of residues of each amino acid in the protein molecule. Even though the analytical values are subject to errors, the knowledge that whole numbers of residues are present in every identical protein molecule enables the most probable values to be assessed with reasonable certainty, except for the most abundant residues in proteins of high molecular weight.

The heterogeneous nature of gelatin as regards molecular weight and the composition of different molecules (see Chapter 3) makes it impossible to determine any precise relationships based on whole numbers of residues. The amino acid data must therefore be regarded as empirical and, as it represents an average for molecules of different composition, decimal parts persist. Accuracy is therefore entirely limited by the analytical methods themselves, because it is not possible to check the data by independent methods available for homogeneous proteins. At best, accuracy will be approximately $\pm 1\%$ for the more abundant amino acids, but perhaps only $\pm 20\%$ for the rarest residues such as tyrosine. Thus, while amino acid data for gelatin are useful to provide an overall picture to assess its nutritional value or for comparative purposes with collagen or between gelatins, they are more limited as a guide to molecular structure.

B. Hydrolysis of Gelatin

Complete hydrolysis of proteins for amino acid analysis is usually carried out by heating a solution in 6N-hydrochloric acid at a constant temperature,

usually between 100 and 115°C for a period between 24 and 72 hours. The most labile peptide bonds break down rapidly so that a solution of dipeptides, which are comparatively stable, is soon formed (see Eastoe, 1966b). Most of the hydrolysis period is required for the comparatively slow breakdown of residual dipeptides. Their stability varies with composition, peptides containing valine, leucine or isoleucine being particularly slow to hydrolyse, especially valylvaline (Synge, 1945). Fortunately gelatin and collagen contain only small amounts of amino acids which form stable peptide bonds and probably none of the most stable dipeptide sequences. Gelatin is therefore hydrolysed more readily and under milder conditions than many proteins. This is advantageous as decomposition of the more labile amino acids during hydrolysis is minimized. It is usually necessary to correct the values for the hydroxy amino acids, serine and threonine, for hydrolysis losses of 5 and 3% respectively for 24 hours and 10 and 5% respectively for 48 hours at 100°C (Rees, 1946; Eastoe, 1955). The amide value should be correspondingly reduced. The methionine value should be corrected for oxidation by adding on the values for the sulphoxide and sulphone peaks, where these are observed. Bidmead and Ley (1958) intentionally oxidize methionine to the sulphone and cystine to cysteic acid before hydrolysis.

Substantially complete recovery of weight and nitrogen of gelatin as amino acids is obtained after 24 hours hydrolysis at 100°C, the recovery of leucine, isoleucine and phenylalanine being slightly increased by amounts of the same order as the experimental error by extending the hydrolysis period to 48 hours (Eastoe, 1955). Piez *et al.* (1960) obtained similar results for hydrolysis of calf skin collagen at 106°C except for a 10% increase in the valine value on extending the hydrolysis from 24 to 72 hours.

Hydrolysis is preferably carried out with at least 100 times the sample weight of 6N- i.e. 20% (w/w) redistilled hydrochloric acid in a sealed borosilicate glass tube either evacuated or in an atmosphere of nitrogen, to minimize oxidation of methionine. If carbohydrate is present more acid should be used (up to 1000-fold excess) to minimize reactions between sugars and amino acids (Dustin *et al.*, 1953; Eastoe, 1966b). At the end of the hydrolysis period the hydrolysate is cooled and diluted to a known volume. Hydrochloric acid is removed from suitable aliquots either (i) by evaporation to dryness in small beakers *in vacuo* over P₂O₅ and KOH in a desiccator at room temperature, (ii) in a rotary vacuum evaporator at slightly elevated temperature, or (iii) by freeze-drying (see Blackburn, 1968).

C. Chromatographic Analysis

Selection of particular methods and practical details of techniques are outside the scope of this chapter and the reader is referred to reviews and standard text books (Bailey, 1962; Eastoe and Courts, 1963; Gordon and

Eastoe, 1964; Eastoe, 1966a, 1966b; Blackburn, 1968). Development of ion-exchange chromatography and the ninhydrin reaction as a quantitative colorimetric method (Moore and Stein, 1948) has made possible the separation and determination of virtually all the protein amino acids in a single procedure. Ion-exchange chromatography is at present far the most widely used method for precise amino acid analysis. Although both gas chromatography of volatile derivatives and mass spectrometry appear promising, technical difficulties have so far prevented their wide adoption.

The original ion-exchange manual methods with stepwise (Moore and Stein, 1951) and gradient (Moore and Stein, 1954) elution need a minimum of specialized equipment (fraction collector and colorimeter or spectrophotometer) but are very slow, requiring a great deal of attention from the operator for periods of up to two weeks. They are suitable mainly where occasional analyses are required. A semi-micro modification permits the analysis of 0.5 mg of protein (Eastoe, 1961). Introduction of automatic methods by stepwise (Spackman *et al.*, 1958) and gradient (Piez and Morris, 1960) elution permit a complete analysis in 24 hours. The main disadvantage is the comparative expense of the apparatus where there are insufficient analyses to keep it in continuous use. Recent developments with short columns, fine resins and fast flow rates enable an analysis to be finished in 2-4 hours.

Variations from standard procedures may be required in the amino acid analysis of gelatin, as a result of its content of glycine, hydroxyproline and hydroxylysine. Owing to the large proportion of glycine it may be necessary, with the automatic method, to run an additional chromatogram, with a lower loading of amino acids, to keep the glycine peak on the recorder scale. Results for hydroxyproline may be less accurate than for other amino acids owing to the low colour intensity produced by the ninhydrin reaction. Methods for the determination of hydroxyproline without chromatographic separation are considered in sub-section D. Hydroxylysine gives two peaks attributed to the normal and allo diastereoisomers, which usually precede lysine in the basic region of the chromatogram. To prevent overlapping with histidine, it may be necessary to advance or retard its elution position by slightly increasing or reducing the pH of the eluting solution.

D. Hydroxyproline

Basically there are three methods used for the estimation of hydroxyproline, i.e. (i) reaction with ninhydrin to form a yellow pigment, (ii) reaction with ninhydrin to yield a red pigment, and (iii) the oxidation of the imino acid to give a pyrrole compound which then reacts with p-dimethylaminobenzaldehyde to give a red pigment. Reaction (i) which was originally demonstrated

by Grassman and von Arnim (1934) is not very sensitive but is of value in the estimation of hydroxyproline during chromatographic procedures based on that of Moore and Stein (1951). The present authors have found that using this method with the above authors' own ninhydrin reagent (Moore and Stein, 1948) yielded reliable values. The method cannot be used for the estimation of hydroxyproline in the presence of other ninhydrin positive materials.

The unstable red pigment which is an intermediate in the formation of the yellow one of reaction (i) can be stabilized and used for the estimation of hydroxyproline (Troll and Cannon, 1953). A correction for the contribution of pigment from proline is described. However for the greatest accuracy the separation of the imino acids is necessary (Rogers *et al.*, 1954). Other amino acids do not interfere with the estimation.

Reaction (iii) has been widely employed because the hydroxyproline content of gelatin hydrolysates can be measured simply and without prior removal of other amino or imino acids. Although popular the method requires many precautions before accurate results can be obtained and in consequence numerous papers have appeared on the subject. The methods mentioned briefly below are regarded by the present authors as amongst the most reliable. Neuman and Logan (1950) developed the oxidation procedure of McFarlane and Guest (1939), and it now forms the basis of many methods. In this technique the hydroxyproline is oxidized by hydrogen peroxide in the presence of alkaline copper sulphate, the solution heated to destroy the excess peroxide, cooled, acidified, p-dimethylaminobenzaldehyde added and the colour developed by heating. Improved reproducibility and yields of colour can be obtained if the oxidation and peroxide destruction stages are combined (Leach, 1960c). The replacement of hydrogen peroxide with chloramine-T also results in improved accuracy (Stegemann, 1958; Stegemann and Stalder, 1967).

Tyrosine yields 1.5–2.0% (Neuman and Logan, 1950; Miyada and Tappel, 1956) and tryptophan 1.3% (Miyada and Tappel, 1956) of the colour at the wavelength of measurement as the same weight of hydroxyproline, but they do not contribute any colour in the Stegemann procedure (Stegemann, 1958). Hydroxyproline can be separated from large amounts of tyrosine by a simple chromatographic procedure (Leach, 1961b). Methods for overcoming contributions from the above amino acids, "humins" pigments and other possible interfering substances are (a) the isolation of the pyrrole compound by steam distillation and subsequent reaction with the colour reagent (Serafini-Cessi and Cessi, 1964), and (b) a lengthy but reliable procedure in which there are three stages in which interfering substances can be removed (Prockop and Udenfriend, 1960). The solution is treated with charcoal and a cation exchange resin to remove unwanted pigments, the oxidation carried

out, further interfering substances removed by extraction, the solution heated to convert pyrrole-2-carboxylic acid to pyrrole which is then extracted into toluene and reacted with the colour reagent.

E. Direct Measurement of Amino Acids

All of these procedures depend upon the reactivity of the side chain groups and therefore are described under section VC.

F. Direct Measurement of Amide Nitrogen

The amide groups of proteins are readily hydrolysed by mineral acids with the release of ammonia. Gordon *et al.* (1941) found that hydrolysis of protein amide groups was complete after 10 days, treatment with 12 N-hydrochloric acid at 37°C. Identical results are obtainable more quickly for wool (Leach and Parkhill, 1955) and gelatin (Eastoe *et al.*, 1961) by boiling with 2 N-hydrochloric acid under reflux for 1 hour.

The ammonia released can be determined, after steam distillation from the alkaline solution into 1% aqueous boric acid, by direct titration with 0.01 N-hydrochloric acid (see also sub-section IIB 1). Prior to this step the cooled gelatin digest is neutralized to pH 5.0 by addition of sodium hydroxide and a suitable portion transferred to the distillation apparatus and made alkaline with an equal volume of 0.05 M-sodium tetraborate-0.15 N-sodium hydroxide solution. This borate buffer does not cause further decomposition of certain amino acid constituents which happens if the solution is made alkaline with sodium hydroxide alone. It also minimizes distillation of carbon dioxide which would result in an inaccurate end-point. This occurs if sodium borate solution alone is added (Eastoe *et al.*, 1961).

Good results are obtained with this method except when there is rapid darkening of the acid hydrolysate during hydrolysis, followed by deposition of a brown precipitate and development of a caramel odour. In such instances erroneously high results are obtained, probably due to carbohydrate impurities reacting with amino acids to produce coloured products and free ammonia (see Blake and Plaster, 1950). The determination of amide content for systems containing protein and carbohydrate is discussed by Marshall and Gottschalk (1966). A method for gelatin involving distillation of ammonia *in vacuo* at 55°C is described by Cassel and McKenna (1953).

G. Amino Acids Derived from Impurities

Cystine may be present in small amounts of impurities (see Chapter 3, VB). Its determination is complicated by liability to decomposition during

hydrolysis. Substantial amounts of cystine give rise to a peak in the neighbourhood of valine, its position being particularly pH-sensitive. Very small amounts are best investigated by oxidation of the intact protein with performic acid prior to hydrolysis. This converts cystine to the strongly acidic cysteic acid, which is eluted from cation-exchange resin as a sharp peak at zero retention volume (Partridge and Davis, 1955). Cystine and cysteine in gelatins are mainly of photographic interest, in which connection they are determined polarographically. Zahn and Wegerle (1955) describe a method for the detection of lanthionine sulphoxide.

Tryptophan is generally considered to be absent from gelatins. Its determination at low levels in the presence of amino acids such as serine is difficult owing to its instability. Alkaline hydrolysis causes less destruction than acid and is employed in the widely used method of Spies and Chambers (1949, see also Section VC4).

IV. EXAMINATION OF THE END GROUPS OF PROTEIN CHAINS

A. Introduction

Although the original end groups of the collagen macromolecule are presumably represented in gelatin, additional ones, both N-terminal and C-terminal result from hydrolysis of peptide bonds during manufacture (Chapter 3). A greater variety of terminal groups is therefore present in gelatin than in proteins produced by direct biosynthesis. Comments concerning the empirical nature of amino acid analysis in relation to gelatin therefore apply with more force to end-group analysis. Greater reliance has to be placed on the quantitative accuracy of the analytical procedures than when only a single kind of end group has to be identified. Information concerning N- and C-terminal groups is valuable in pointing to those linkages of collagen which are most labile under particular conditions of gelatin manufacture.

B. N-Terminal Groups

The method of Sanger (1945) in which 1-fluoro-2,4-dinitrobenzene (FDNB) reacts under mild conditions with free terminal amino groups has been most widely used. Courts (1954) described suitable reaction conditions for gelatin in which a 2% (w/v) solution in 8% (w/v) aqueous sodium bicarbonate is shaken with excess FDNB for 24 hours (see also Eastoe and Courts, 1963). On subsequent neutralization the dinitrophenyl (DNP) gelatin is precipitated as a yellow gel, which is dried and washed with acetone to remove excess

reagent. The bond between the DNP and imino group is fairly stable to the acid hydrolysis (5.5 N-HCl for 16 hours at 100°C) employed to release the terminal DNP-amino acids prior to their separation, identification and quantitative determination. This depends on the absorption given by these yellow substances in the region of 430 nm. The stability of the DNP-imino group bond is variable and depends upon the amino acid involved. Once the identity of the end groups is established the stability of the DNP derivatives of the appropriate amino acids under the hydrolysis conditions employed is measured. As DNP compounds are sensitive to light, procedures should be carried out in subdued lighting to ensure quantitative recoveries. The DNP-amino acids can be separated for identification on columns of silica gel (Porter and Sanger, 1948), kieselguhr (Mills, 1952), buffered kieselguhr (Matheson, 1963), Celite (Courts, 1954) and silicic acid-Celite (Green and Kay, 1952), and also by paper chromatography (Blackburn and Lowther, 1951; Biserte and Osteux, 1951; Levy, 1954).

The reaction with phenylthiohydantoin is also used for detection of N-terminal groups. Successive removal of amino acids from the N-terminal ends of peptides (Abderhalden and Brockmann, 1930) makes sequence studies possible, the method having been first fully exploited by Edman (1950). The thiohydantoins eventually formed are hydrolysed by alkali to free amino acids, which are identified by paper chromatography. Sjöquist (1953) devised a method for the direct paper chromatography of phenylthiohydantoins, which can be quantitatively determined by their absorption at 270 nm (Edman and Sjöquist, 1956). Unfortunately the Edman degradation method is not easily applicable to gelatin because of the variety of N-terminal residues and sequences.

C. C-Terminal Groups

Methods for the determination of C-terminal groups are on the whole more cumbersome and less satisfactory than for N-terminal groups. The hydrazinolysis method of Akabori *et al.* (1952) which has been applied to gelatin (Heyns and Legler, 1957) results in cleavage of all peptide bonds and the conversion of amino acids (except the C-terminal) to their hydrazides. The C-terminal amino acid is liberated in the free state, separated and identified.

Methylation of proteins, followed by reduction with lithium boron hydride results in the conversion of the terminal carboxyl group to a carbinol group (Chibnall and Rees, 1958). The C-terminal amino acid is thus converted to a β -amino alcohol which is separated from the hydrolysate and identified by column or paper chromatography (Bailey, 1962). Side reactions, which result in cleavage of the polypeptide chain, cause serious interference for proteins of high molecular weight.

Another chemical method, which has been applied to gelatin, involved protection of the free amino group, followed by reaction with acetic anhydride and ammonium thiocyanate, which convert the C-terminal residue to the corresponding thiohydantoin (Waley and Watson, 1951).

Carboxypeptidases, though valuable reagents for the determination of sequences near the C-terminal ends of homogeneous proteins (Folk and Gladner, 1958), are probably of limited value for gelatin owing to the difficulty of interpreting the significance of products liberated from a multiplicity of C-terminal sequences.

V. EXAMINATION OF THE SIDE CHAIN GROUPS

A. Computation from Amino Acid Composition

The groups under consideration are tyrosyl, phenyl, imidazolyl, guanidino, ϵ -amino, hydroxyl other than the phenolic hydroxyl group, carboxyl and amide. One can compute the number present from the amino acid composition if it is assumed that none, apart from carboxyl, is involved in chemical combination with some other group or compound. The first four listed can be calculated from the number of residues of the appropriate amino acid present, the ϵ -amino from the sum of lysine and hydroxylysine together with ornithine, if present, and hydroxyl from the sum of hydroxylysine, hydroxyproline, serine and threonine. The number of amide groups can be calculated from the amide-N content, and on subtracting this value from the sum of aspartic acid and glutamic acid residues, the number of carboxyl groups is obtained. Values calculated from reliable amino acid analyses agree closely with the values determined directly by the methods described below. The agreement is such that it can be concluded that very few side chains can be involved in chemical combination.

B. Titration Curves

Kennington and Ward (1954) studied the titration curves of gelatins and showed that reasonably accurate determinations of acidic and basic groups could be achieved if the following criteria were employed with a temperature of 40°C: pH 1.5–6.5 represents carboxyl; pH 6.5–8.0, α -amino plus imidazole and pH 8.0–11.5, ϵ -amino. By selecting the pH 6.5 boundary the small amount of carboxyl titrating above this value is offset by a similar amount of α -amino plus imidazole titrating below it. Similarly at the pH 8.0 limit the small proportion of α -amino plus imidazole titrating above this value is offset by the small amount of ϵ -amino groups of lysine and hydroxylysine

titrating below it. The ϵ -amino range includes a contribution from phenolic groups, but Kenchington and Ward (1954) have shown that in the case of alkali-processed gelatins of low tyrosine content the error is small. The guanidino groups can also be measured by titration. The titre from the isoionic point to the maximum (about pH 1.5) is numerically equal to the titre of all the basic groups, and thus by subtracting the titration due to the other basic groups that of arginine remains. Full experimental details of these methods are given by Kenchington and Ward (1954) and Kenchington (1960). Similar studies have been made by Salvinien and Combet (1956). Methods of estimating side chains by titration in the presence of formaldehyde are mentioned in Section VIIC. The total basic groups in collagen and gelatin can be estimated by direct titration with perchloric acid using anhydrous acetic and formic acids as solvents (Ellis and Pankhurst, 1952). The end point is determined potentiometrically or conductimetrically.

C. Direct Measurement

1. Guanidino

The Sakaguchi colour test, which is the reaction of the guanidino group in the presence of alkali and hypohalite with α -naphthol, has been used (Janus, 1956) to estimate these groups in gelatin. Particular care is required to avoid the destruction of the groups by excess hypohalite before they can react with the reagent. The method is equally applicable to gelatins which have been guanidated (Davis, 1958).

2. Imidazole

Pauly showed that the imidazole group of histidine reacts with diazotized sulphanilic acid in the presence of sodium carbonate to give a red colour. Hitch (1956) has used the Weiss and Ssobolew (1913–1914) modification of this reaction and showed it to be applicable to intact proteins. Tyrosine also yields the red colour and Hitch describes a correction procedure which depends upon the knowledge of the tyrosine content of the protein. Details of the procedures are more readily available in an article by Hamilton (1960).

3. ϵ -amino

The ϵ -amino groups can be measured by the ninhydrin procedure described in Section VIIE. It is important to establish the yield of colour per ϵ -amino group in terms of glycine equivalent for the particular type of gelatin under investigation for the yield appears to vary with type. Thus calculating the number of groups from the amino acid composition the present authors found that a limed ox-hide gelatin yielded 0.84 and an acid-processed pig skin gelatin yielded 0.72 glycine equivalents of colour per group. This may reflect

the lack of availability of some of the lysines in the latter type of gelatin. Allowance must be made for the contribution from the α -amino groups. The method only has value if sufficient numbers of analyses are required to warrant the work involved in establishing the method. These disadvantages do not apply to the examination of modified gelatins where the method is used in a comparative fashion (see Section VIIE).

Titration data (Section VB) can be used to estimate the ϵ -amino groups but the phenolic groups of tyrosine also contributes to the titre in this region. Similarly the formol titration (see Section VIIC) can be used to measure these groups, but again the phenolic groups also contribute. Kenchington (1960) describes in detail the pH and temperature criteria, the limitations of the method and the application of both forms of titration to the estimation of these groups.

The procedure of Van Slyke (1927) depends upon the reaction of nitrous acid with the amino groups to yield nitrogen. A reliable direct micro form of this method is described by Kainz (1953) and is also given in detail by Hamilton (1960) who also usefully summarizes the defects of the procedure. The most notable defects are the reaction of other groups with nitrous acid to yield nitrogen.

The 1-fluoro-2:4-dinitrophenyl- ϵ -N-lysine can be isolated and measured colorimetrically (Courts, 1954). Unfortunately some of the ϵ -amino groups in certain types of gelatin are not available for reaction with the reagent (Harding, 1965). Acid-processed gelatins are notable in this respect and thus a similar situation exists to that with the ninhydrin reaction.

4. Indolyl

From a study of the absorption spectra of gelatin Goodwin and Morton (1946) were able to deduce that a small amount of tryptophan was present. Similarly Piez (1965) has obtained evidence for its presence in the α -chains from codfish skin collagen. The estimation was approximate only as the maximum proportion present was about one residue per 1000 total residues. The number of indolyl side chains cannot be computed from the usual amino acid analysis as tryptophan is destroyed during acid hydrolysis.

The minute amounts of tryptophan present in intact gelatins have been measured by its condensation with p-dimethylamino-benzaldehyde in the presence of 19N sulphuric acid to give a colourless product which is then converted to a blue compound by the action of sodium nitrite (Spies and Chambers, 1949).

5. Tyrosyl

Cobbett *et al.* (1962) made an extensive study of the estimation of tyrosine in gelatins and obtained a good correlation between the values from spectro-

photometric and colorimetric procedures and the chromatographic data of Eastoe (1955, 1960). The colorimetric procedure depends upon the reaction of free or combined tyrosine with α -nitroso- β -naphthol (Gerngross *et al.*, 1933) in the presence of nitric acid to give a coloured derivative. The first named authors were able to obtain accurate results provided (i) a solution containing a small amount of derivative was used to promote the reaction, (ii) care was taken to minimize exposure to light, (iii) the delay before measurement was minimized, and (iv) phenolic preservatives were absent. The spectrophotometric method (Goodwin and Morton, 1946; Beavan and Holiday, 1952) depends upon the absorption of light by tyrosine in the UV region. The measurements were made at 292 nm (λ max.) and 304 nm in alkaline solution. The latter reading is less influenced by the other compounds present. Errors arise if preservatives which absorb UV light are present or if the gelatin contains large amounts of naturally occurring impurities.

6. *Carboxyl, hydroxyl and phenyl*

There does not appear to be any reliable method for the direct measurements of these groups apart from the titration of the carboxyl groups mentioned above. Although the phenyl side chain has an absorption spectrum in the UV region the molar extinction coefficients are too low (Beavan and Holiday, 1952) to form the basis of a method of measurement particularly in the presence of tyrosine and tryptophan.

VI. EXAMINATION OF OTHER CONSTITUENTS OF THE GELATIN MOLECULE

A. Introduction

While gelatin molecules consist primarily of amino and imino acids, joined in peptide linkage, other features may be present, derived from comparatively sparsely distributed components of the collagen macromolecule. Such constituents or linkages may be either left intact or altered during manufacture. Other organic constituents of the raw material may interact with gelatin, introducing modifications. Where very low levels of reactive groups are concerned it may not be clear whether they are true components of gelatin or are associated with impurities. In the following subsections various practical methods are briefly considered, which may prove useful for the characterization of commercially produced gelatin, without necessarily implying that all the constituents detected are intrinsic parts of the gelatin molecule.

B. Carbohydrate

Anthrone is a suitable reagent for sugars, especially hexoses, in the presence of a large excess of amino compounds (see Eastoe and Courts, 1963). The method of Dreywood (1946), as modified by Bangle and Alford (1954) is suitable for approximate determinations. Two volumes of 0.16% (w/v) anthrone in 96% sulphuric acid are poured into 1.5–30 mg of unhydrolysed protein in one volume of water. The heat of dilution promotes the reaction, sugars producing a blue-green colour with an absorption maximum at 620 nm and uronic acids a less intense pink with a maximum at 545 nm. Yemm and Willis (1954) give details of the rate of colour formation with different sugars to provide a basis for obtaining optimum sensitivity. Helbert and Brown (1955, 1956) suggest that for greatest accuracy both anthrone and the material examined should be dissolved in 27.5 ± 0.1 N-sulphuric acid and the mixed solutions heated in a water bath for a specified combination of time and temperature to obtain optimum colour density for any particular sugar. Gelatin retards colour formation with anthrone, the reaction proceeding at 100°C but not at 40°C, although the final optical density reached is not affected (Wood, 1958a).

Bial's orcinol reagent in conjunction with ferric chloride gives with pentoses a green colour with a maximum at 670 nm. The quantitative procedure of Albaum and Umbreit (1947) has been widely used. However Dische (1955) considers that this is liable to interference by hexoses and prefers the earlier technique of Dische and Schwarz (1937), with a shorter heating period for which interference is claimed to be negligible.

Hexosamines are mainly derived from mucopolysaccharides and glycoproteins. They partially survive acid hydrolysis (Leach, 1960b) and, where present, appear as peaks in amino acid analysis, since they react with ninhydrin (Eastoe, 1954; Eastoe and Courts, 1963). Fractions containing hexosamines can be distinguished from amino acids, as hexosamines condense with acetylacetone to give a product which yields a red colour with p-dimethylaminobenzaldehyde (Elson and Morgan, 1933; Palmer *et al.*, 1937). A variety of hexosamines may be separated from one another and from amino acids by chromatography on cation-exchange resins eluted with hydrochloric acid (Crumpton, 1959).

Hexuronic acids, which are more labile than hexosamines, are also derived from mucopolysaccharides. The carbazole reaction (Dische, 1947) is reasonably specific for uronic acids, other sugars giving less than 7% of the colour on an equimolar basis. Steigmann (1958a) claims to have detected glucuronic acid in alkali-degraded gelatin by means of thiobarbituric acid. Venet *et al.* (1957) have also studied their occurrence in gelatins.

Sialic acids, which are N-substituted derivatives of neuraminic acid, occur

naturally as terminal groups of the oligosaccharide side chains of glycoproteins. They are labile under acid conditions and their determination is reviewed by Neuberger and Marshall (1966). The direct Ehrlich method (Werner and Odin, 1952) is preferable to diphenylamine (Dische, 1929) which is interfered with by sugars and gelatin (Leach, 1959). The reaction with 2-thiobarbituric acid (Aminoff, 1959; Warren, 1959) now appears to be most widely used, and is more specific than the methods mentioned above.

C. Other Residues

Aldehydes in gelatins may be related to a specific type of cross-linkage, derived from lysine and present in collagen (see Chapters 1 and 3) or derived from carbohydrates. A test for gelatin aldehydes ("reductones") based on reaction with cold *p*-aminobenzoic acid is described by Steigmann (1958b) who also discusses "browning" products derived from carbohydrate-containing proteins.

Methods for nucleic acids in gelatin are described by Russell and Oliff (1966) including the determination of deoxyribose, ribose and the base adenine. The latter appears to be associated with nucleic acid impurity, rather than gelatin itself.

Procedures for the determination of ester-like linkages, γ -glutamyl linkages and crosslinkages of various types require detailed study and careful control. References to key papers are given in sub-section IVF of Chapter 3.

VII. EXAMINATION OF CHEMICALLY MODIFIED GELATINS

A. Introduction

The chemical modification of side-chain or end-of-chain groups of gelatin may take the form of a simple substitution or of a complete change of the group. The degree of modification achieved can be measured by a variety of methods, some of which apply to a single type of group and others to a combination of groups. The major types of method are based on the measurement of (i) the changes in the titration curve of the gelatin, (ii) the decrease of the formol titration, (iii) the increase of the absorption of light when the substituent contains a suitable chromophore, (iv) the loss of the ability to take part in colour forming or other reactions, or vice versa, and (v) the substituent chemically, usually after its removal from the protein.

B. Titration Curves

Using the criteria described in Section VB and measuring the reductions of the appropriate titrations, Kenchington (1957) estimated the degree of modification achieved during the esterification of carboxyl groups, the acetylation of α - and ϵ -amino plus imidazole groups and the guanidation of ϵ -amino groups.

C. Formol Titration

The formol titration has been applied (Cobbett *et al.*, 1964; Clark *et al.*, 1965; Leach, 1966) to the estimation of the degree of modification achieved at the amino groups in gelatin. By using different pH values before the addition of the formaldehyde Leach (1966) was able to distinguish between the substitution achieved at the α - and ϵ -amino plus imidazole groups and the ϵ -amino groups alone. For the former groups the initial pH of the gelatin solution was adjusted to 6.5 (see Section B), formaldehyde added and the solution titrated to pH 9.0. The reduction of this titration on modification of the groups was used to assess the degree of modification achieved. By using a similar procedure but with an initial pH of 9.0 the modification at the ϵ -amino groups alone was examined. In the latter method all the ϵ -amino groups are not titrated as some of them are already titrated during the initial pH adjustment. However the effect is eliminated by using a direct comparison between the parent and modified gelatins. In both methods the phenolic group of tyrosine contributes a small titration below pH 9.0 both in the presence and absence of formaldehyde. However, errors from this source must be negligible as there is a good correlation between the formol titrations and the ninhydrin colorimetric procedure (Leach, 1966). The methods have been applied to gelatins modified with aryl and alkyl sulphonyl chlorides (Cobbett *et al.*, 1964; Clark *et al.*, 1965; Leach, 1966), cyanate (Leach, 1966), *s*-triazine dichloride and its derivatives and with a combination of these reagents (Clark *et al.*, 1965).

D. Spectrophotometric Method

This method depends upon the substituent possessing a chromophoric group. The wavelength at which the measurement is carried out must be sufficiently removed from that of the tyrosine and phenylalanine already present in the gelatin in order to avoid interference. This approach has been used to estimate the substitution of the α -plus ϵ -amino groups of gelatin by benzene sulphonyl chloride (Cobbett *et al.*, 1964). It was found that errors could arise from the reaction of the phenolic group of the tyrosine with the

reagent. Two types of error arose, (i) the absorption correction obtained for the tyrosine region in the original gelatin was larger than that in the modified protein, and (ii) the absorption from the derivative of the reaction of the phenolic groups with the reagent. The errors are more serious with acid-processed gelatins which contain larger proportions of tyrosine.

E. Ninhydrin Colorimetric Procedure

Slobodian *et al.* (1962) have shown that both α - and ϵ -amino groups in proteins react quantitatively with ninhydrin, the ϵ -amino groups yielding 65–75% of the colour obtained from an equivalent number of leucine α -amino groups. When the ϵ -amino groups are on N-terminal or free lysine the yield is reduced to 7–10%. Imidazole and modified amino groups do not react. By measuring the diminution of the yield of colour following modification Cobbett *et al.* (1964) estimated the degree of modification achieved at the α -plus ϵ -amino groups of gelatin. This procedure was modified to allow for the hydrolysis of the protein which takes place during colour development (Leach, 1966). This method has been applied to gelatins modified with aryl sulphonyl chlorides (Cobbett *et al.*, 1964; Leach, 1966), and cyanate (Leach, 1966).

F. Chemical Methods

1. Total and O-acetyl groups

Bello and Vinograd (1956) modified the procedures of Blackburn and Phillips (1944) for the estimation of total and O-acetyl contents of acetylated gelatins. In order to measure the former the modified protein is refluxed for 4 hours with 5N.H₂SO₄ and the acetic acid formed isolated by distillation and titrated with alkali. The O-acetyl procedure is based on the observation (Wolfram *et al.*, 1936; Syngé, 1939) that O-acetyl groups are readily hydrolysed by cold dilute NaOH whereas the N-acetyl groups are stable under these conditions. The derivative is allowed to stand in contact with a known quantity of 0.02 N NaOH for 24 hours at room temperature and the amount of alkali consumed measured. Similarly Kenchington (1957) measured the quantity of alkali required to overcome the drift of pH when the acetylated gelatin was maintained at a fixed pH in the range pH 9–10.

2. Guanidino groups

Eastoe and Kenchington (1954) estimated the degree of guanidation of the ϵ -amino groups of lysine and hydroxylysine by measuring the reduction in the proportion of these residues following guanidation and also by measuring the amounts of homoarginine and hydroxyhomoarginine formed. All four

amino acids are separated chromatographically and then estimated with ninhydrin.

3. Esterified carboxyl groups

Kenchington (1957) estimated these groups in a modified gelatin by the procedure of Belcher *et al.* (1955). The method depends upon the conversion of $-\text{O}-\text{CH}_3$ groups by hydriodic acid to methyl iodide which is then changed by bromine in potassium acetate/acetic acid solution to iodic acid. This is measured iodometrically after the addition of potassium iodide.

VIII. GELATIN CONTAMINANTS

A. Inorganic Components

1. Metals

The determination of metallic elements in gelatin is important as they affect its properties, especially with regard to colour and photographic use. Toxic substances need to be detected in gelatins for use in food. Upper limits for the contents of arsenic, copper, lead and zinc in edible gelatins have statutory force in Great Britain (Statutory Instruments: 1951).

Choice from the variety of published analytical methods largely depends on the accuracy required, the number of samples examined and the measuring apparatus available. Reference should be made to standard text books and original papers for general and more specific aspects of procedures. For the determination of low concentrations of metals, an absorptiometric method (Sandell, 1944) probably offers the best combination of reasonable accuracy, high sensitivity and wide application, without need for expensive equipment.

For many procedures it is necessary first to separate the metals from the gelatin to prevent the latter from interfering. Arsenic can be separated as one of its volatile compounds, while for most other metals, dry oxidation by ashing is suitable if the furnace temperature is controlled. Ashing gelatin is something of an art and a capital investment in platinum dishes offers dividends in conveniently rapid ashing with little liability to contamination. The availability of radioactive isotopes has greatly facilitated checking recoveries of small amounts of elements (Gorsuch, 1959). Removal of metals from gelatin by mixed-bed ion-exchange resins (Janus *et al.*, 1951) and addition of a known amount of metal, permits an overall check of an analytical procedure.

(a) *Arsenic*. The statutory limit in edible gelatin is 1 p.p.m. of elementary arsenic, so a moderately sensitive method is necessary. It has long been considered that arsenic is largely volatilized during dry ashing, though the

findings of Gorsuch (1959) cast some doubt on this. Arsenic is usually separated as a volatile derivative—the trichloride, hydride or diethyl hydrogen arsenide (Remenec, 1936). The first British Standard (B.S., 1959) method, having a wet oxidation of gelatin by nitric acid and double separation of arsenic as chloride and hydride, appears to the authors to be unduly complicated in relation to the limited accuracy of the Gutzeit finish and the possibility of high blanks from the large amounts of reagents used. The second B.S. method, derived from that of Eastoe and Eastoe (1953) is simpler to carry out, as it involves only preliminary acid hydrolysis of gelatin, followed by evolution of arsenic hydride. The molybdenum blue absorptiometric finish is sensitive, accurate and not subjective. The separation and estimation of arsenic added to deionized gelatin permits calibration and testing of overall accuracy.

(b) *Calcium*. This is one of the commonest ions found in gelatin and is easily determined after dry ashing at 550°C and dissolving the residue in hydrochloric acid. It can be precipitated on a small scale (Marsden, 1941) from slightly acid solution as oxalate, the precipitate being dissolved in hot dilute sulphuric acid and titrated with permanganate. Titration in alkaline solution with EDTA using the fluorescent indicator, fluorescein-bismethylene-iminodiacetic acid (calcein) is suitable for small amounts of calcium (Eastoe, 1965). The direct potentiometric determination by means of a calcium fluoride electrode was applied to a study of calcium binding in gelatin by Tenderloo (1936). Calcium electrodes are commercially available and enable the free calcium ion concentration to be read directly in the neutral pH region.

(c) *Copper*. The statutory limit for copper in edible gelatin is 30 p.p.m. The British Standard method involves extraction of yellow copper diethyldithiocarbamate from slightly alkaline solution with carbon tetrachloride and measurement at 432 nm. The procedure should be carried out in subdued light to prevent fading. Ames and Davidson (1948) proposed a rapid limit test based on formation of a blue colour with gum guaiacum. Russell and Hart (1958) found that 2,2-biquinoline is suitable for the determination of small amounts of copper in gelatin, if any ferric ions which are present are complexed with tartaric acid. Polarographic methods for copper in photographic gelatins have been studied by Michel and Maron (1950) and Walther (1961). Dry ashing at 450°C is recommended by most investigators, but Gorsuch (1959) found that addition of sulphuric acid is advisable.

(d) *Iron*. There are several sensitive and moderately specific reagents which are applicable to solutions of gelatin ash, the iron being oxidized or reduced to ferric or ferrous state. Steigmann (1946) used *p*-phenylenediamine hydrochloride or phenyl-*p*-phenylenediamine hydrochloride with hydrogen peroxide in strongly acid solution. Eastoe and Eastoe (1951) obtained a linear calibration for up to 70 p.p.m. of ferrous iron, using *o*-phenanthroline at

pH 4.6 with hydroxylamine hydrochloride as reducing agent. Losses at 590°C were negligible, even in the presence of chloride.

(e) *Lead*. This element is quantitatively recovered after dry-ashing at 450–550° for 15 hours, but substantial losses occur at 650°C (Gorsuch, 1959). The statutory limit is 10 p.p.m. and the B.S. method, based on that of the Analytical Methods Committee (1954) is sufficiently sensitive and specific for accurate determinations. Use of “lead-free” reagents is essential to avoid high blanks. The dithizone complex of lead is extracted with chloroform from an ammoniacal aqueous solution containing cyanide. Excess dithizone is removed and the optical density of the red organic phase is measured at 520 nm. The method is free from interference by other heavy metals under these conditions. Earlier methods based on elaborate separations of heavy metals, followed by turbidimetric comparison of lead sulphide (Ames and Davidson, 1948; Scott-Dodd, 1959) are of questionable accuracy without substantial analytical experience. Michel and Maron (1950) describe polarographic techniques for lead and zinc.

(f) *Zinc* is not lost on ashing at 500°C for 16 hours. The statutory limit is 100 p.p.m. In the B.S. procedure, based on that of Strafford *et al.* (1945), copper is removed by preliminary extraction with diethylammonium diethyldithiocarbamate in chloroform but it appears to be assumed, probably with justification, that the lead content of gelatin is too low to cause significant interference. The solution is adjusted to pH 7 with ammonium acetate buffer and shaken with successive 1 ml portions of standard dithizone solution until the green colour fails to change to pink. Byčichin *et al.* (1950) ash at 800°C, extract with dithizone at pH 9 and match visually with permanent standards. They attribute high zinc content to “galvanized” plant.

(g) *Chromium* can be determined satisfactorily by atomic absorption spectroscopy or colorimetrically with diphenylcarbazide and a good correlation between the results from the two methods obtained (Coupet and Cabanis, 1969).

2. Anions, etc.

(a) *Chloride*. Most procedures are based on precipitation of silver chloride. Pouradier and Chateau (1951) suggest boiling a solution of gelatin in 20% nitric acid with excess silver nitrate, filtering off the silver chloride, dissolving it in 20% ammonium hydroxide and titrating potentiometrically with 0.0067 M-thiourea using a silver sulphide electrode. Internal adsorption indicators are unsuitable for direct titration of chloride in gelatin solutions. Instead the solution, slightly acidified with nitric acid and containing a coil of stout silver wire, forms a half-cell connected by a potassium sulphate-agar bridge to a similar reference half cell containing deionized gelatin or without gelatin, to which 1 drop of standard silver nitrate solution is added. The two

silver wires are connected to a galvanometer. The unknown solution is titrated with silver nitrate until the initial direction of deflection is just reversed. Walther (1962) describes the direct polarographic determination of chloride in photographic gelatins using a low resistance mercurous sulphate electrode. The polarographic curve is strongly affected by pH.

(b) *Phosphate*. Orthophosphate in solutions of gelatin ash in dilute sulphuric acid is conveniently determined by one of the several modifications of the molybdenum blue method. The procedure of Fiske and Subbarow (1925) makes use of a fairly stable reducing agent, 1-amino-2-naphthol-4-sulphonic acid. The modification of Yuen and Pollard (1951) provides a slower rate of colour increase at room temperature. By heating the test solution for 10 minutes at 100°C a stable colour is obtained with a five-fold increase in sensitivity (Eastoe, 1965). The optical density of the blue solution is best measured at its absorption maximum in the near infra-red (815 nm) but an absorptiometer with a red filter is also suitable.

(c) *Sulphite*. Sulphur dioxide is sometimes used as a preservative in gelatins, the statutory limit for edible gelatin being 0.1%. Except for photographic purposes the two B.S. methods, which involve distillation of sulphur dioxide into hydrogen peroxide are sufficiently sensitive and accurate. The first B.S. method involves boiling gelatin solution under reflux in a current of carbon dioxide for one hour and appears unnecessarily tedious and the apparatus too complicated for routine use. The second British Standard method (see also Francis and Pilgrim, 1944) involving steam distillation is more suitable and an equivalent distillation apparatus can easily be improvised with standard conical joints. The recommended titration with sodium hydroxide of the sulphuric acid formed is simpler than either iodometry or back titration of excess peroxide with permanganate.

Bartos and Sezerat (1965) reduce sulphur dioxide in gelatin solution to hydrogen sulphide by means of titanous chloride and measure the darkening of a lead acetate paper, suspended above the solution, in a closed flask. Sulphur dioxide in gelatin dissolved in 0.1 M citric acid can be determined polarographically (Stefan, 1962). The wave height is proportional to concentration and since the wave is suppressed by adding peroxide, the method can be used for rapid control of either excess sulphur dioxide or hydrogen peroxide during gelatin manufacture. Wood (1955) determines the sulphur dioxide content of gelatin solutions by direct titration with iodine, a correction being made for other reducing substances present by subtracting the titration obtained for a second portion, treated with formaldehyde. This method gives lower results than distillation, possibly because the latter causes decomposition of organic sulphur compounds.

Matschiner *et al.* (1970) determine sulphite iodometrically using a biamperometric end-point. The results compare favourably with those obtained

polarographically and the determination can be carried out in the presence of nitrite.

(d) *Thiosulphate*. The determination of thiosulphate in gelatin is mainly of photographic interest, as it may be a natural sensitizer (Wood, 1952). Polarographic methods are applicable directly to gelatin solutions (Štefan, 1960) or after extraction (Grainger and Mathewson, 1965). Janus and Nellist (1967) have reviewed the methods available and favour a direct polarographic determination after tryptic hydrolysis, with precautions to prevent interference by sulphite, chloride and surface-active substances. Concentration of thiosulphate with ion-exchange resin increases sensitivity but interference results from concentration of other anions. Warburton and Przybylowicz (1967) determine thiosulphate with an adaption of the methylene blue test.

(e) *Other sulphur-containing ions* including sulphide and various polythionates as well as various forms of "labile" and "active" sulphur are almost exclusively of photographic interest and their investigation is dealt with in Chapter 7.

B. Proteins and Mucosubstances

For the reasons mentioned in Chapter 3 proteins and mucosubstances are combined in a single section. Three methods are employed for the estimation of these substances in gelatins.

1. *Isolation of a coagulum following an acid treatment of the gelatin solution* (Sheppard *et al.*, 1931; Leach, 1960b; Williams, 1961; Zimkin and Paronik, 1960, Leach, 1961a)

This is a simple method but measures only a certain fraction of the mucoproteins and includes some gelatin in the isolated material (Leach, 1960b, 1961a). However the estimation does give a guide to gelatin or glue quality, (Leach, 1960b, 1961a; Williams, 1961) and allowance for the gelatin content of the complex can be made by assuming that its hydroxyproline content represents gelatin.

The most rapid procedure is the modification due to Williams (1961) in which gelatin (10 g) is dissolved in water (90 ml), adjusted to pH 4 with 20% w/w glacial acetic acid and heated at 85°C for 24 hours. The coagulum is isolated by centrifugation, washed with water and reisolated. This stage is repeated three times and coagulum transferred to a tared moisture dish, dried at 105°C for 16 hours and weighed. This method does not contain a correction for the small amount of ash present. If the coagulum is required for further analysis larger quantities are employed and the bulk of the water is removed from the coagulum by an alcohol and ether treatment (Leach, 1960b). The moisture, ash and hydroxyproline content of the material can then be measured and the appropriate corrections applied.

2. *Adsorption onto charcoal*

This procedure is not recommended by the originator (Maron, 1958) for quantitative work because the material is not removed completely from the gelatin solution by adsorption onto the charcoal. The method does however include a prolonged washing stage and results in the isolation of a material containing very little gelatin complexed with it.

3. *Removal of the gelatin by its adsorption onto an ion-exchange resin*

This method was not designed for routine use; it isolates the materials not adsorbed by an ion-exchange resin at pH 5.0 in the presence of a buffer. The adsorptive capacity of the resin is very low so that only a small amount of gelatin can be removed from the solution and in addition all the other experimental conditions governing the complete removal of the gelatin are very critical (Leach, 1960a).

C. Lipids

The bulk of lipids present in gelatins and glues is derived from depot fat and survives through the gelatin and glue manufacturing processes as a result of the emulsifying action of the protein. The bulk of the fat can be isolated along with the mucosubstances (see Section VIII B), very little of it existing in the readily extractable form (Leach, 1961a). Poorly degreased raw material can give inferior quality glues in which globules of free lipid can be seen with the aid of a microscope, or sometimes even with the naked eye. Methods (Leach, 1961a; B.S., 1959) of measuring fat content involve the destruction of the protecting action of the protein followed by the isolation of the free fatty acids which are derived from both triglycerides and the original free fatty acids. No correction can be applied for any glycerol lost during the process.

D. Other Minor Components

Most of the investigations of the minor constituents of gelatins have been in terms of their photographic properties (see Chapter 5). Of the organic components the reducing sugars and aldehydes have been of particular interest. In general the substances have not been isolated, but their presence inferred from colour reactions and the intensity of the colour formation related to the photographic properties of the gelatins (see Section VI B).

Wood (1958b) isolated sugars from gelatin following a hydrolysis procedure, but the method is not quantitative and furthermore the nature of the sugars isolated suggests that they may have been formed from the mucosubstances during hydrolysis.

REFERENCES

- Abderhalden, E. and Brockmann, H. (1930). *Biochem. Z.* **225**, 386–408.
- Akabori, S., Ohno, K. and Narita, K. (1952). *Bull. Chem. Soc. Japan*, **25**, 214–8.
- Albaum, H. G. and Umbreit, W. W. (1947). *J. Biol. Chem.* **167**, 369–76.
- Ames, W. M. and Davidson, E. (1948). *Food* **17**, 200–1.
- Aminoff, D. (1959). *Virology* **7**, 355–7.
- Analytical Methods Committee (1953). *Analyst* **78**, 134–5.
- Analytical Methods Committee (1954). *Analyst* **79**, 397–402.
- Bailey, J. L. (1962). In "Techniques in Protein Chemistry", Elsevier, Amsterdam.
- Bangle, R. and Alford, W. C. (1954). *J. Histochem. Cytochem.* **2**, 62–76.
- Bartos, J. and Sezerat, A. (1965). *Ann. Fals. Expert. Chim.* **58**, 39.
- Beavan, G. H. and Holliday, E. R. (1952). *Advan. Protein Chem.* **7**, 319–86.
- Belcher, R. Fildes, J. E. and Nutten, A. J. (1955). *Anal. Chim. Acta*, **13**, 16–22.
- Bello, J. and Vinograd, J. R. (1956). *J. Am. Chem. Soc.* **78**, 1369–72.
- Bidmead, D. S. and Ley, F. J. (1958). *Biochim. Biophys. Acta*, **29**, 562–7.
- Biserte, G. and Osteux, R. (1951). *Bull. Soc. Chim. Biol.* **33**, 50–63.
- Blackburn, S. (1968). "Amino Acid Determination—Methods and Techniques", Edward Arnold, London.
- Blackburn, S. and Lowther, A. G. (1951). *Biochem. J.* **48**, 126–8.
- Blackburn, S. and Phillips, H. (1944). *Biochem. J.* **38**, 171–8.
- Blake, J. N. and Plaster, F. H. (1950). *J. Soc. Leather Trades' Chemists*, **34**, 177–86.
- Braunsdorf, K. (1934). *Z. Unters. Lebensmittel*, **67**, 326–32.
- British Standard 757 : 1975. Methods of Sampling and Testing Gelatines.
- Byčichin, A., Halámek, C. and Hlaváček, K. (1950). *Tech. Hlidka Kožel*, **25**, 36–41.
- Cassel, J. M. and McKenna, E. (1953). *J. Am. Leather Chemists' Assoc.* **48**, 142–54.
- Chibnall, A. C. and Rees, M. W. (1958). *Biochem. J.*, **68**, 105–22.
- Chibnall, A. C., Rees, M. W. and Williams, E. F. (1943). *Biochem. J.* **37**, 354–9.
- Clark, R. C., Cobbett, W. G., Gibbs, J. A., Jones, R. T., Leach, A. A., Pratt, A. N. and Sutton, D. A. (1965). *J. Appl. Chem. (London)*, **15**, 479–88.
- Cobbett, W. G., Gibbs, J. A. and Leach, A. A. (1964). *J. Appl. Chem. (London)*, **14**, 296–302.
- Cobbett, W. G., Kenchington, A. W. and Ward, A. G. (1962). *Biochem. J.* **84**, 468–77.
- Coupet, M. and Cabanis, J. C. (1969). *Trav. Soc. Pharm. Montpellier*, **29**, 27–33.
- Courts, A. (1954). *Biochem. J.* **58**, 70–4.
- Courts, A. and Kragh, A. M. (1960). *Anal. Biochem.* **1**, 88–91.
- Crumpton, M. J. (1959). *Biochem. J.* **72**, 479–86.
- Davis, P. (1956). *J. Appl. Chem. (London)*, **6**, 413–15.
- Davis, P. (1958). In "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), pp. 225–30. Pergamon Press, London.
- Dische, Z. (1929). *Mikrochemie*, **7**, 33–68.
- Dische, Z. (1947). *J. Biol. Chem.* **167**, 189–98.
- Dische, Z. (1955). *Methods of Biochemical Analysis*, **2**, 313–58, Interscience Publishers, New York.
- Dische, Z. and Schwarz, K. (1937). *Mikrochim. Acta*, **2**, 13–19.
- Dreywood, R. (1946). *Ind. Engng. Chem. Analyt. Edn.* **18**, 499.
- Dustin, J. P., Czajkowska, C., Moore, S. and Bigwood, E. J. (1953). *Anal. Chim. Acta*, **9**, 256–62.
- Eastoe, J. E. (1954). *Nature*, **173**, 540–1.

- Eastoe, J. E. (1955). *Biochem. J.* **61**, 589-602.
- Eastoe, J. E. (1960). *J. Appl. Chem. (London)*, **10**, 393-4.
- Eastoe, J. E. (1961). *Biochem. J.* **79**, 652-6.
- Eastoe, J. E. (1965). *Congrès et Colloques Univ. Liège*, **31**, 265-74.
- Eastoe, J. E. (1966a). *Brit. Med. Bull.* **22**, 174-9.
- Eastoe, J. E. (1966b). In "Glycoproteins: Their Composition, Structure and Function" (A. Gottschalk, ed.), pp. 112-50, Elsevier, Amsterdam.
- Eastoe, J. E. (1967). In "Treatise on Collagen", Vol. 1 (Ramachandran, G. N., ed.), pp. 1-72. Academic Press, London.
- Eastoe, J. E. (1968). *J. Dent. Res.* **47**, 982-3.
- Eastoe, J. E. and Courts, A. (1963). *Practical Analytical Methods for Connective Tissue Proteins*, Spon, London.
- Eastoe, J. E. and Eastoe, B. (1951). *Br. Gelat. Glue Res. Ass., Res. Rep. Ser. B.*, No. 2.
- Eastoe, J. E. and Eastoe, B. (1953). *J. Sci. Food Agr.* **7**, 310-21.
- Eastoe, J. E. and Eastoe, B. (1954). *Biochem. J.* **57**, 453-9.
- Eastoe, J. E. and Kenchington, A. W. (1954). *Nature* **174**, 966-7.
- Eastoe, J. E., Long, J. E. and Willan, A. L. D. (1961). *Biochem. J.* **78**, 51-6.
- Eastoe, J. E. and Williams, A. P. (1959). *Mfg. Chemist* **30**, 374-5.
- Edman, P. (1950). *Acta Chem. Scand.* **4**, 277-93.
- Edman, P. and Sjöquist, J. (1956). *Acta Chem. Scand.* **10**, 1507-9.
- Ellis, S. C. and Pankhurst, K. G. A. (1952). *Biochem. J.* **52**, 350-2.
- Elson, L. A. and Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824-8.
- Ferguson, S. and Racicot, P. A. (1936). *J. Assoc. Offic. Agr. Chemists* **19**, 476-8.
- Fiske, C. H. and Subbarow, Y. (1925). *J. Biol. Chem.* **66**, 375-400.
- Folin, O. and Ciocalteu, V. (1927). *J. Biol. Chem.* **73**, 627-50.
- Folk, J. E. and Gladner, J. A. (1958). *J. Biol. Chem.* **231**, 379-401.
- Francis, A. C. and Pilgrim, A. J. (1944). *Analyst* **69**, 90.
- Gerngross, O., Voss, K. and Herfeld, H. (1933). *Chem. Ber.* **66B**, 435-42.
- Goodwin, T. W. and Morton, R. A. (1946). *Biochem. J.* **40**, 628-32.
- Gordon, A. H. and Eastoe, J. E. (1964). "Practical Chromatographic Techniques". George Newnes, London.
- Gordon, A. H., Martin, A. J. P. and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369-87.
- Gorsuch, T. T. (1959). *Analyst* **84**, 135-73.
- Grainger, F. and Mathewson, H. D. (1965). *J. Phot. Sci.* **13**, 269-72.
- Grassman, W. and von Arnim, K. (1934). *Ann. Chem.* **509**, 288-303.
- Green, F. C. and Kay, L. M. (1952). *Anal. Chem.* **24**, 726-32.
- Hamilton, L. D. G. (1960). In "Analytical Methods of Protein Chemistry" Vol. 2 (Alexander, P. and Block, R. J., eds.), pp. 61-100, Pergamon Press, London.
- Harding, J. J. (1965). *Advan. Protein Chem.* **20**, 109-190.
- Helbert, J. R. and Brown, K. D. (1955). *Anal. Chem.* **27**, 1791-96.
- Helbert, J. R. and Brown, K. D. (1956). *Anal. Chem.* **28**, 1098-1100.
- Heyns, K. and Legler, G. (1957). *Hoppe-Seylers Z. Physiol. Chem.* **306**, 165-72.
- Hitch, S. F. (1956). Ph.D. Thesis, University of London.
- Hitchcock, D. I. (1931). *J. Gen. Physiol.* **15**, 125-37.
- Jacobs, M. B. and Jaffe, L. (1932). *Ind. Engng. Chem. Analyt. Edn.* **4**, 418-20.
- Janus, J. W. (1956). *Nature*, **177**, 529.
- Janus, J. W., Kenchington, A. W. and Ward, A. G. (1951). *Research (London)* **4**, 247-8.

- Janus, J. W. and Nellist, D. R. (1967). *J. Phot. Sci.* **15**, 270-6.
- Kainz, G. (1953). *Mikrochim. Acta* **4**, 349-65.
- Kennington, A. W. (1952). *Bull. Br. Gelat. Glue Res. Ass.* **3** (1), 2-4.
- Kennington, A. W. (1957). *Biochem. J.* **68**, 458-68.
- Kennington, A. W. (1960). In "A Laboratory Manual of Analytical Methods of Protein Chemistry, Vol. 2" (Alexander, P. and Block, R. J., eds.) pp. 353-88. Pergamon Press, London.
- Kennington, A. W. and Ward, A. G. (1954). *Biochem. J.* **58**, 202-7.
- Krane, J. G. and Watson, H. E. (1929). *Chem. Ind. (London)* **T48**, 335-6.
- Langston, W. B., Leach, A. A. and Willan, A. L. D. (1965). *Mfg. Chemist. Aerosol News* **36** (11), 78 and 81.
- Leach, A. A. (1959). *Br. Gelat. Glue Res. Ass.*, Res. Rep. Ser. B. no. 11.
- Leach, A. A. (1960a). *Biochem. J.* **74**, 61-9.
- Leach, A. A. (1960b). *J. Appl. Chem. (London)* **10**, 367-72.
- Leach, A. A. (1960c). *Biochem. J.* **74**, 70-1.
- Leach, A. A. (1961a). *J. Appl. Chem. (London)* **11**, 10-19.
- Leach, A. A. (1961b). *Anal. Biochem.* **2**, 529-34.
- Leach, A. A. (1966). *Biochem. J.* **98**, 506-12.
- Leach, S. J. and Parkhill, E. M. J. (1955). *Proc. Intern. Wool Textile Res. Conf. Australia* **92-101**.
- Levy, A. L. (1954). *Nature* **174**, 126-7.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265-75.
- Markham, R. (1942). *Biochem. J.* **36**, 790-91.
- Maron, N. (1958). In "Recent Advances in Gelatin and Glue Research" (Stainsby, G. ed.), pp. 221-4, Pergamon Press, London.
- Marsden, A. W. (1941). *Chem. Ind. (London)* **T60**, 20-3.
- Marshall, R. D. and Gottschalk, A. (1966). In "Glycoproteins, Their Composition, Structure and Function" (Gottschalk, A. ed.), pp. 151-7, Elsevier, Amsterdam.
- Matheson, N. A. (1963). *Biochem. J.* **88**, 146-51.
- Matschiner, H., Siemroth, J. and Wolff, R. (1970). *Mikrochim. Acta* **321-6**.
- McFarlane, W. D. and Guest, G. H. (1939). *Can. J. Research* **17B**, 139-42.
- Michel, G. and Maron, N. (1950). *Analytica Chim. Acta* **4**, 542-50.
- Mills, G. L. (1952). *Biochem. J.* **50**, 707-12.
- Miyada, D. S. and Tappel, A. L. (1956). *Anal. Chem.* **28**, 909-10.
- Moore, S. and Stein, W. H. (1948). *J. Biol. Chem.* **176**, 367-88.
- Moore, S. and Stein, W. H. (1951). *J. Biol. Chem.* **192**, 663-81.
- Moore, S. and Stein, W. H. (1954). *J. Biol. Chem.* **211**, 893-913.
- Neuberger, A. and Marshall, R. D. (1966). In "Glycoproteins, Their Composition, Structure and Function" (Gottschalk, A. ed.) pp. 190-234, Elsevier, Amsterdam.
- Nueman, R. E. and Logan, M. A. (1950). *J. Biol. Chem.* **184**, 299-306.
- Palmer, J. W., Smyth, E. M. and Meyer, K. (1937). *J. Biol. Chem.* **119**, 491-500.
- Partridge, S. M. and Davis, H. F. (1955). *Biochem. J.* **61**, 21-30.
- Piez, K. A. (1965). *Biochemistry* **4**, 2590-6.
- Piez, K. A. and Morris, L. (1960). *Anal. Biochem.* **1**, 187-201.
- Piez, K. A., Weiss, E. and Lewis, M. S. (1960). *J. Biol. Chem.* **235**, 1987-91.
- Porter, R. R. and Sanger, F. (1948). *Biochem. J.* **42**, 287-94.
- Pouradier, J. and Chateau, H. (1951). *Sci. Ind. Phot.* **22**, 164-7.
- Přístoupil, T. I., Tomanová, V. and Nikl, J. (1956). *Chem. Listy* **50**, 386-7.

- Prockop, D. J. and Udenfriend, S. (1960). *Anal. Biochem.* **1**, 228–39.
- Rees, M. W. (1946). *Biochem. J.* **40**, 632–40.
- Remenec, M. (1936). *Chem. Listy* **30**, 96–100.
- Remington, R. E. and McRoberts, L. H. (1926). *Ind. Eng. Chem.* **19**, 267–9.
- Rogers, C. J., Kimmel, J. R., Hutchin, M. E. and Harper, H. A. (1954). *J. Biol. Chem.* **206**, 553–9.
- Russell, G. and Hart, P. J. (1958). *Analyst* **83**, 202–7.
- Russell, G. and Oliff, D. L. (1966). *J. Phot. Sci.* **14**, 9–22.
- Salvinien, J. and Combet, S. (1956). *Compt. Rend.* **242**, 114–16.
- Sandell, E. B. (1944). "Colorimetric determination of traces of metals". Interscience Publishers, New York.
- Sanger, F. (1945). *Biochem. J.* **39**, 507–15.
- Scott-Dodd, A. (1949). *Analyst* **74**, 118–19.
- Serafini-Cessi, F. and Cessi, C. (1964). *Anal. Biochem.* **8**, 527–8.
- Sheppard, S. E., Hudson, J. H. and Houck, R. C. (1931). *J. Am. Chem. Soc.* **53**, 760–5.
- Sjöquist, J. (1953). *Acta Chem. Scand.* **7**, 447–8.
- Slobodian, E., Mechanic, G. and Levy, M. (1962). *Science* **135**, 441–2.
- Spackman, D. H., Stein, W. H. and Moore, S. (1958). *Anal. Chem.* **30**, 1190–1206.
- Spies, J. R. and Chambers, D. C. (1949). *Anal. Chem.* **21**, 1249–66.
- Statutory Instruments : 1951, No. 1196. Emergency Laws, Food Standards (Edible Gelatine) Order, 1951. H.M.S.O., London.
- Štefan, V. (1960). *Chem. Prumysl.* **10**, 126–9.
- Štefan, V. (1962). *Chem. Prumysl.* **12**, 652–5.
- Stegemann, H. (1958). *Z. Physiol. Chem.* **311**, 41–5.
- Stegemann, H. and Stalder, K. (1967). *Clin. Chim. Acta* **18**, 267–73.
- Steigmann, A. (1946). *Food* **15**, 105–6.
- Steigmann, A. (1958a). *Sci. Ind. Phot.* **29**, 260–1.
- Steigmann, A. (1958b). *Sci. Ind. Phot.* **29**, 321–6.
- Strafford, N., Wyatt, P. F. and Kershaw, F. G. (1945). *Analyst* **70**, 232–46.
- Syngé, R. L. M. (1939). *Biochem. J.* **33**, 1913–34.
- Syngé, R. L. M. (1945). *Biochem. J.* **39**, 351–5.
- Tendeloo, H. J. C. (1936). *J. Biol. Chem.* **113**, 333–9.
- Tombs, M. P., Souter, F. and MacLagan, N. F. (1959). *Biochem. J.* **73**, 167–71.
- Troll, W. and Cannon, R. K. (1953). *J. Biol. Chem.* **200**, 803–11.
- Van Slyke, D. D. (1927). *J. Biol. Chem.* **73**, 121–6.
- Venet, A. M., Pouradier, J. and Landucci, J. M. (1957). *Bull. Soc. Chim. France* **1325**–9.
- Waley, S. G. and Watson, J. (1951). *J. Chem. Soc.* 2394–7.
- Walther, D. (1961). *Mitt. Forschungslab. Agfa Leverkusen-Muenchen* **9**, 207–17.
- Walther, D. (1962). *Z. Wiss. Phot. Phytophysik. Photochem.* **56**, 176–84.
- Warburton, C. D. and Przybylowicz, E. P. (1967). *J. Photogr. Sci.* **15**, 201–6.
- Warren, L. (1959). *J. Biol. Chem.* **234**, 1971–5.
- Weiss, M. and Ssobolew, N. (1913–1914). *Biochem. Z.* **58**, 119–29.
- Werner, I. and Odin, L. (1952). *Acta Soc. Med. Upsalien.* **57**, 230–41.
- Williams, A. P. (1961). *J. Appl. Chem. (London)* **11**, 100–3.
- Wolfrom, M. L., Konigsberg, M. and Soltzberg, S. (1936). *J. Am. Chem. Soc.* **58**, 490–1.
- Wood, H. W. (1952). *Sci. Ind. Phot.* **23**, 209–17.
- Wood, H. W. (1955). *J. Phot. Sci.* **3**, 144–9.

- Wood, H. W. (1958a). *J. Phot. Sci.* **6**, 91-6.
- Wood, H. W. (1958b). *J. Phot. Sci.* **6**, 170-5.
- Yemm, E. W. and Willis, A. J. (1954). *Biochem. J.* **57**, 508-14.
- Yuen, S. H. and Pollard, A. G. (1951). *J. Sci. Food Agr.* **2**, 36-42.
- Yuen, S. H. and Pollard, A. G. (1953). *J. Sci. Food Agr.* **4**, 490-6.
- Zahn, H. and Wegerle, D. (1955). *Leder.* **6**, 278-9.
- Zimkin, E. A. and Paronik, S. I. (1960). *Usp. Nauchn. Fotogr. Akad. Nauk S.S.S.R. Otd. Khim. Nauk*, **7**, 103-8.

Chapter 16

Physical Tests for Gelatin and Gelatin Products

F. W. WAINEWRIGHT

*Canada Packers Limited Research and Development Division,
2211 St. Clair Avenue West, Toronto, Ontario.*

I	Introduction	508
II	Methods for Determining Gelatin Gel Strength	508
	A. Bloom Gel Strength Method	508
	B. Quick Bloom Test	514
	C. Methods for Determining the Bloom Strengths of Small Gelatin Samples	515
	D. The Automatic Bloom Gelometer	516
	E. The Boucher Jelly Test	517
	F. Japanese Industrial Standard K6503 : 1955 Method	518
	G. Japanese P.A.G.I. Specification: 1959 Method	519
III	Dessert Gel Strength Tests	519
	A. The AOAC Test	520
	B. British Test	520
IV	Methods for Determining Viscosity	521
	A. BS757 : 1975 Method	522
	B. GMIA Method	523
	C. Factors Affecting Viscosity Determinations	524
V	Viscosity Degradation Test	525
VI	Methods for Determining Gelatine pH	527
	A. BS757 : 1975 Method	527
	B. GMIA Method	527
	C. 40°C Method	528
VII	Methods for Determining Gelatin Isoionic Point	528
	A. Rapid Method	528
	B. Ion Exchange Method	529
VIII	The Determination of Gel Melting Point	529
	A. BS757 : 1975 Method	529
	B. Factors Affecting the Method	530
IX	Gelation Time Tests	531
	A. Edible Gelatin Test	531
	B. Photographic Gelatin Test	531

C. Factors Affecting Gelation Rates	532
D. The Tecam Gelation Timer	532
References	533
Equipment and Apparatus Suppliers	534

I. INTRODUCTION

Although industrial gelatin production processes have existed for at least two centuries, methods for the comparative testing of gelatins have been evolved only in the last fifty or sixty years. At first, interest was focussed primarily on the strengths of the gels which samples could form under standard conditions. Later, the importance of other gelatin physical properties became recognized and included in gelatin specifications. Today, any laboratory concerned with the testing of gelatin and gelatin products must be equipped for a wide range of physical, chemical and microbiological tests. However, gel strength remains the property on which the commercial values of gelatins are principally based.

Within the last twenty years, the principal advance in gelatin gel strength testing has been the development of the Dummy Bloom, by Fysh and Ward. With this device, which is now in general use throughout the world, gelometers can be tested and accurately adjusted for correct performance. It is fair to say that the introduction of the Dummy Bloom resulted in a considerable improvement in the level of commercial gel strength testing. Despite this, the level is not consistently high. The reason for this is inadequate attention to all the factors affecting gel strength determinations, sometimes described as operator error. There is less excuse for the wide variations found to occur in the determinations of physical properties such as viscosity and pH.

It is hoped that the details in this chapter will contribute towards the improvement of the level of testing of gelatin physical properties. It will be noted that with most methods a recommendation is made to test under the specific conditions encountered in the use of the gelatins. Only in this way will manufacturers be able to supply, or users specify, the materials most suitable for specific applications. This can only benefit the entire industry.

II. METHODS FOR DETERMINING GELATIN GEL STRENGTH

A. Bloom Gel Strength Method

The physical property which principally determines the value of a commercial gelatin is the rigidity of the gel which it forms under standard conditions. The commercial value increases with increasing rigidity.

In 1925, Bloom developed an instrument to measure gelatin gel rigidity. The design of this instrument was such that it has remained basically unchanged and has become the standard instrument for gelatin testing throughout the world. Essentially, the Bloom gelometer determines the weight required to make a 0.5 in diameter, flat bottomed plunger depress the surface of a gelatin gel 4 mm. The weight is applied as a stream of lead shot, the total of which is subsequently weighed to the nearest gram. This weight is expressed as the "grams Bloom" or "Bloom" for the sample under test.

The method for determining the Bloom value of a gelatin sample is as follows. 7.5 g of the sample are placed in a Bloom bottle and well mixed with 105 ml of distilled water. This mixture is allowed to stand until the gelatin is completely swollen, then heated in a 65°C water bath until the gelatin is dissolved. The bottle is placed in a 10°C chill bath and matured for 16–18 hours, after which it is removed and tested with the gelometer. The Bloom values of commercial gelatins usually range from 80 to a maximum of about 320 g.

(i) Gelatin Concentration—Bloom strength is approximately proportional to the square of the solution concentration (Ferry, 1948a). Thus it is necessary, in routine Bloom determinations, to weigh to an accuracy of ± 0.01 g. The addition of 105 ml of water to the gelatin samples is conveniently accomplished with an automatic pipette. This should deliver 105 ± 0.1 ml.

(ii) Bottle Dimensions—The results of Sheppard and Sweet (1923) indicate that the Bloom test is dependent upon the dimensions of the specified bottles. Both British Standard (B.S.) 757 and the Standard methods of the Gelatin Manufacturers Institute of America (G.M.I.A.) specify bottles with internal diameters of 59 ± 1 mm. The overall height of the bottle is not critical except that the surface of the gel must be below the shoulder of the bottle.

It is essential that the bottoms of the bottles are flat. If uneven, the jars can rock during the gel strength determination and the test accuracy will be reduced.

The bottles should be fitted with rubber bungs which have small diameter holes drilled through them. These permit the expansion of air, when the samples are heated, while minimizing evaporation losses.

(iii) Sample Swelling—It is advisable, after thoroughly mixing a gelatin sample with distilled water, to allow the gelatin to swell and equilibrate. This will take from fifteen minutes to two hours, depending upon the particle size of the sample. Once fully swollen the gelatin will rapidly dissolve, with a minimum of heating and thermal degradation. In the absence of a swelling procedure, the gelatin particles tend to clump together when warmed in water. Prolonged heating is required to dissolve these clumps and this can result in severe thermal degradation.

(iv) Preparation of the Solution—After swelling, the gelatin samples are

conveniently dissolved by placing the bottles in a 65°C bath. In 15 minutes the samples will have reached 55–60°C and will dissolve with gentle swirling. Any undissolved gelatin can be seen rising from the bottom of the bottles in striations of swollen material, so the absence of these striations indicates complete solution. Care should be taken to avoid producing a layer of foam on the gelatin solutions. Any samples gelled with foam in the centre of the surface, where the gelometer plunger is placed, must be rejected.

After preparation of the solutions the bottles should be placed in room temperature water, for 15 minutes. This reduces the heat load placed in the gelation bath and lessens the risk of bottles cracking.

(v) Gelation—Both B.S. 757 and the G.M.I.A. state that the gelation bath temperature should be $10 \pm 0.1^\circ\text{C}$. The bath should have an adequate refrigeration reserve, to ensure a rapid return to 10°C when several bottles are placed in it, and a stable, horizontal shelf for the bottles to ensure that the gel surfaces will be horizontal. Sloping gel surfaces tend to throw the gelometer plunger from the vertical position during gel strength determinations, thus reducing test accuracy.

The process of gelation commences below c. 38°C (Levi, 1960), and as the temperatures of the samples continue to fall initial setting occurs and the rigidity of the gels increase rapidly. After temperature equilibration is complete at 10°C rigidity still increases, but at a declining rate, so that after 16 hours at 10°C the rigidity of a good quality gelatin is increasing by only about 0.5% per hour (Saunders, 1954). Therefore the specification of 16–18 hours maturing at 10°C is satisfactory for routine gel strength determination purposes.

(vi) Bloom Gelometer Adjustment—Before testing the samples, the Bloom gelometer should be checked to ensure that its platform is level, also that its suspension is hanging freely within the guides and adjusted so that the disc is just touching the bottom contact.

If the disc is suspended above this contact then the test results will be high because of the extra loading required to extend the spring. A slack suspension permits part of the suspension weight to contribute to the test and the results will be low.

Next, the gelometer should be checked with a Dummy Bloom (Fysh and Ward, 1953) for correct adjustment and operation. A Dummy Bloom consists of a stand and four calibrated spring steel strips approximating to Bloom values of 80, 100, 200 and 300 g. These steel strips simulate tests with gels and thus provide a reliable and convenient method of checking a gelometer. Preferably two strips should be used, their values spanning the expected values of the samples being tested. Erratic values for the strips indicate electrical or mechanical malfunctions, such as dirty contacts or the suspension fouling the gelometer. Consistently high or low values indicate that the gelo-

meter contact gap is incorrect. These faults should be rectified before proceeding to gel testing.

Use of a Dummy Bloom does not confirm that the gelometer is loaded with the correct quantity of lead shot, that the gelometer loading rate is correct or that the gelometer plunger is correctly dimensioned. Because gelatin gels do not exhibit perfect elasticity their Bloom values are dependent upon the gelometer loading rate. Therefore it is essential to check regularly that the instrument is filled with 800 g of lead shot which will pass through a 12 mesh but be retained by a 14 mesh screen and also that the rate at which this shot is loaded is 200 ± 5 g in 5 seconds.

Consistently high or low Bloom values, obtained despite the above adjustments, indicate erroneous plunger dimensions and these should be checked against those recommended in the section dealing with plungers.

(vii) Measurement of Bloom Strength—Bloom strength determinations should be made only when the gelometer has been checked and adjusted as recommended in the previous section. The only precaution necessary during the measurements is to ensure that the gelometer plunger makes contact with the gel surface at the approximate centre.

It is advisable to test the standard gelatin gels (Section AVIII) first. Occasionally these tests indicate that the gelometer disc and contacts should be cleaned although Dummy Bloom results have been satisfactory. This effect is attributed to possible differences in the speed at which the gelometer disc descends to the bottom contact. During Dummy Bloom measurements the disc descends through 4 mm at a uniform rate. During gel strength determinations the rate at which the disc descends possibly slows as resistance from the walls and bottom of the Bloom bottle increases. Thus, tests with gels are possibly more likely to be affected by dirty contacts than tests with a Dummy Bloom.

With proper sample preparation, the results for the standard samples should be within the limits quoted in Section Ax. Consistent results outside of these limits usually indicate changes in the moisture contents of the standard gelatin samples, if the gelometer is functioning normally, but this should be confirmed by a determination of moisture content.

After the completion of each test the gel surface should be examined for cracking or splitting, and the test result rejected where this has occurred. When this effect occurs normally it is only with low quality samples and the application of a thin smear of Vaseline to the bottom of the plunger can sometimes help in avoiding such failures.

In North America duplicate gelometer measurements are sometimes made on the same set gel. Because of delayed elastic effects such tests can give erroneous results unless the gel is allowed a few minutes to relax between measurements. This practice also increases the possibility of errors in sample

preparation being undetected, in comparison with the sounder practice of using duplicate gel preparations of each gelatin.

After completion of the Bloom strength determinations a further series of Dummy Bloom tests should be made to ensure that the gelometer has functioned properly throughout the test period.

(viii) Bloom Gel Strength Standards—It is necessary to employ gelatin standards when Bloom determinations are made, to ensure that the samples being tested have been properly prepared and matured, and that the gelometer is functioning correctly. Stable, homogenous gelatins with Bloom values between 100–280 g will be suitable, and preferably they should each be from a single extract rather than a blend to avoid segregation of the blend components.

For standardization, ten separate determinations of the sample's Bloom strength should be made, with a correctly functioning and adjusted gelometer. This number of determinations is required to obtain a mean value accurate to one gram. At the same time the moisture content of the sample should be determined. The results of duplicate moisture determinations should agree to within 0.2% and be averaged. A further set of Bloom measurements is advisable, at a later date, to confirm the result.

The moisture content of a standard should preferably be determined each time it is used, so that allowance can be made in its Bloom value for any variation in moisture content. Duplicate samples of standards approximating to, or spanning the expected Bloom values of the samples under test, should be prepared whenever Bloom strength tests are made. These can be used directly or to prepare secondary standards by the above technique.

(ix) Factors Affecting Bloom Strength Determinations—

(a) Moisture Contents of Samples—Ferry (1948a) has shown that the rigidity of gelatin gels is approximately equal to the square of the *protein* concentration. Thus, small changes in the moisture contents of gelatin samples can appreciably affect their Bloom strengths. As the moisture contents of commercial gelatins can range from 7 to 15% the importance of determining the moisture content of a sample, at the time of determining its Bloom strength is obvious. Suitable methods for the determination of gelatin moisture content will be found in Chapter 15.

An approximate formula (B.S. 757: 1975), for calculating the effects of moisture content change on Bloom strength is

$$\Delta B = - \frac{2 \times B_1 \times \Delta m}{100 - m_1}$$

where B_1 is the Bloom strength at % moisture content m_1 and Δm is the difference between m , and the standard moisture content.

Kramer and Rosenthal (1965) have developed a rather more complicated formula which gives similar results.

(b) pH—Kragh and Langston (1957), have shown that the Bloom strengths of all types of gelatins decrease below pH 5 and above pH 9. In the range pH 5-9 the Bloom strengths of alkali processed gelatins are almost constant, but those of acid processed materials increase slightly as the pH rises from 5 to 9. With acid extracted, alkali processed gelatins however, the trend is reversed, Bloom strength increasing from pH 9 to pH 5.

Although Bloom strength is pH dependent, the test is normally made at the pH of the sample which may be misleading as regards performance in particular uses. The values of the majority of commercial gelatins are in the range pH 4.5 to 7.

(c) Gelometer Plunger Dimensions—Angus and Wainwright (1962) have examined the effects of plunger size variations on Bloom strength determinations. They report that the maximum permissible tolerances on Bloom gelometer plunger dimensions are: diameter ± 0.0005 in, radius of curvature 0.014-0.017 in.

(x) The Accuracy of Bloom Strength Determinations—Fysh and Ward (1953) have made a careful study of the spread and deviation in mean values obtained with various sets of replicate Dummy Bloom and Bloom jelly strength determinations.

Their results, shown in Tables I and II, indicated that the Bloom gelometer is most accurate in the 100-300 g region.

The authors do not claim that greater accuracy can be obtained, but suggest that their results indicate levels of accuracy which any laboratory should attain with reasonable care, when testing homogenous samples which are relatively free from bacteria and enzymes.

TABLE I. The spread of results

	Number of replicates in set						
	2	3	4	5	6	7	8
Dummy Bloom							
Below 100 g	3	4	5	5	5	5	6
100-300 g	3	3	4	4	4	4	4
Above 300 g	3	4	5	5	5	5	5
Bloom Strength (with gels)							
Below 100 g	5	6	7	7	7	8	8
100-300 g	4	5	5	6	6	6	6
Above 300 g	6	8	9	9	10	10	10
(g, at 95% confidence level)							

TABLE II. The deviation in mean values

	Number of replicates in set						
	2	3	4	5	6	7	8
Dummy Bloom							
Below 100 g	2	2	2	2	2	1	1
100-300 g	2	2	1	1	1	1	1
Above 300 g	2	2	2	2	2	1	1
Bloom Strength							
Below 100 g	3	3	2	2	2	2	2
100-300 g	3	2	2	2	2	2	2
Above 300 g	4	3	3	3	2	2	2

(g, at 95% confidence level)

(xi) Differences between British Standard and North American Bloom Tests—The British and North American Bloom tests differ only in the dimensions of the plungers used. Both tests are performed with a 13 mm diameter, flat-bottomed plunger, but the British model has the bottom edge rounded to a radius of 0.4 mm.

The bottom edge of the North American model is sharp. Because of its greater gel-contacting area, Bloom values determined with the American plunger will be some 2% higher than those obtained with the British Standard model.

An older type of plunger, also 13 mm diameter, but with the bottom edge radiused 0.8 mm, is still occasionally used in North America. Bloom tests with this plunger will be some 5% lower than those obtained with the standard American plunger and 3% lower than those obtained with the English plunger.

B. Quick Bloom Test

The 16-18 hours maturing at 10°C required by the Bloom test can occasionally be inconvenient. Wootton and Gibbs (1960) have shown that the rigidities of Bloom test gels, after two hours maturing at 10°C, are linearly related to their Bloom strengths.

The shorter maturing time necessitates careful control of the solution temperature, prior to placing the samples in the 10°C bath, and of the rate at which the solutions cool. Thus, after dissolving the samples the solutions must be brought to 30°C. For control of the cooling rates the Bloom bottles must be of similar glass thickness. The requirement of an adequate cooling reserve in the 10°C bath is even more critical for this test than for the normal

Bloom test. If inadequate, the rates at which the samples cool and gel will be dependent upon the number of bottles placed in the bath.

After maturing, the samples are tested with a Bloom gelometer in the normal manner.

The results obtained by Wootton and Gibbs indicate that the accuracy of this test is similar to that of the standard Bloom test, also that English Bloom strength, B , is related to this "short" Bloom strength, B_s , as follows:

$$B = \frac{B_s - 2}{0.75}$$

Because the authors examined only eight gelatin samples and failed to detail the types used, the above formula must be considered approximate and possibly not applicable to all types of gelatins. However, their results do indicate that the Bloom test can be suitably shortened for all gelatins.

C. Methods for Determining the Bloom Strengths of Small Gelatin Samples

There is no direct method for determining the Bloom strengths of small gelatin samples, i.e. 1–2 g amounts. However, methods are available for testing small volumes of gelatin gels, the results of which can be translated into Bloom values.

Saunders and Ward (1954) modified the Kinkel and Sauer (1925) method to measure the rigidities of gelatin gels. In this modified method a cylinder of gelatin gel, matured for 16–18 hours at 10°C in a glass tube, is deformed by air pressure. The volume displaced by the free gel surface is measured by the movement of mercury or carbon tetrachloride in a capillary tube attached to the main tube. It is claimed that the method is accurate to $\pm 1\%$ and that the rigidity modulus of as little as 0.1 g of gelatin can be determined at the nominal $6\frac{2}{3}\%$ concentration.

A linear relationship between Bloom strength, B , and modulus of rigidity, G (dynes/cm²) is shown, in which $B = 20 + 2.86 \times 10^{-3} G$.

This test is not suitable for routine testing. Each rigidity tube requires careful calibration and the tests are time consuming. There is no doubt however, that it is a valuable research tool.

Timson and Kelly (1966) have modified the Saunders and Ward method. The gelatin solution is gelled and matured in a glass tube which is then incorporated into an apparatus for applying air pressure to the end of the tube and measuring gel deformation through the change in volume of water in a calibrated pipette.

Although these authors discuss the measurement of the rigidity of gelatin

gels they quote the standard method for determining the Bloom strength of animal glues and have used the concentration of this method ($12\frac{1}{2}\%$) in their experiments.

Wootton and Kenchington (1959) developed a method for measuring the gel strengths of small volumes of $6\frac{2}{3}\%$ concentration gels, using the Boucher Jelly Tester equipment with a 0.95 cm diameter ball plunger. This method requires only 12.5 ml of gelatin solution, i.e. 0.833 g gelatin. This volume of solution is gelled under the conditions of the Bloom test, in a jar 3 cm in diameter, 2.4 cm deep and 0.5 cm thick. Because of the small dimensions of the test jar special precautions are required to centre the plunger, during the test.

The results obtained by these workers, using four gelatins covering a Bloom range of 80–260 g indicate that the British Standard Bloom strength, B , is related to the result, B_0 , of this form of the Boucher Test, as follows:

$$B = \frac{B_0}{1.48}$$

The reproducibility of the test appears reasonably satisfactory.

D. The Automatic Gelometer

An English instrument manufacturer has, with the co-operation of the Gelatine and Glue Research Association, designed and produced an automatic gelometer. The name given to this instrument is The Boucher Electronic Jelly Tester. Basically it consists of a beam suspended by a torsioned wire running through its centre. A plunger is attached to one end of the beam and this is loaded by means of torque applied to one end of the wire. The torque applied to achieve a plunger travel of 4 mm is automatically recorded.

In practice a Bloom bottle is placed on the instrument platform and the instrument is switched on. The platform rises until the gel surface makes contact with the plunger, then torque is applied to the torsion wire until the plunger has depressed the gel surface 4 mm. At completion of the test the instrument platform returns to the starting position. The torque applied during the test is read from the digital recorder integral with the instrument.

The only feature of the torsion loading principle differing significantly with lead shot loading is that the rate of loading of the former is rather slower, being half the rate. This is by no means a disadvantage and in fact enlarges the versatility of the instrument by permitting an extension into weaker gel testing such as the dessert jelly (see Section E). A 25 mm plunger would be required for this purpose.

The "electronic jelly tester" was designed so as to offer two improvements over the Bloom gelometer. Firstly the speed of operation associated with

push button methods substantially increases the number of samples which can be measured per hour. Secondly a semi-skilled operator can obtain accurate readings with very little training, although this does not obviate the skills required in the preparation of the standard gel. In the latter stages of its development, the manufacture of the Bloom gelometer was discontinued in the U.K. so that this new instrument might well be regarded as a valid replacement.

It has been shown by Angus and Culshaw (1966) that the relationship between the two gelometer models is linear certainly up to about 300 g. For values above 300 g an inter-relating graph might be required. The design of the instrument makes it appear possible to modify it to give linearity above 300 g at the expense of this relationship at low Bloom values but no experimental evidence for this is so far available. The conformity with Bloom described with gel testing by Angus and Culshaw is confirmed by these authors using dummy Bloom test strips.

Electronic gelometers are now in current usage in several testing laboratories. The former Gelatine and Glue Research Association had taken an active part in the instrument's development. The first batches of machines were tested after manufacture, with the issue of their certificate of approval to the purchaser. Technical details and a comparison with other gelometers is given in Courts (1972).

E. The Boucher Jelly Test

The original type of Boucher instrument is in regular use in England for testing gelatin and dessert gelatin gels. It consists of a pivoted beam, at one end of which are adjustable weights to balance it. The other end of the beam is fitted with contacts, top and bottom, and a plunger. The beam operates between a contact gap, which determines the angle through which the beam pivots and hence the plunger travel distance. At a point equidistant between the beam pivot and the plunger is suspended a container. Water is fed to this container from a constant level reservoir, so providing the loading mechanism.

In practice the beam is balanced, then the gel bottle is placed on the instrument platform which is elevated until the gel makes contact with the plunger and raises the beam to the top of the contact gap. The flow of water to the beam container is commenced. When the beam reaches the bottom of the contact gap the water flow is automatically stopped. The volume of water in the container is measured in ml and expressed as the Jellogram value for the sample under test.

The standard plunger for this instrument is a flat bottomed metal disc, 13 mm in diameter, which operates through a distance of 5 mm. The gels are prepared from solutions of 5 g of gelatin in 100 ml of water and matured

for 17 ± 1 hours at 10°C . Koprowski (1951) recommends the use of test bottles, 56 mm internal diameter, 60 mm external diameter and 82 mm high. Although these dimensions are slightly smaller than those specified for the Bloom test, it is common practice to use Bloom bottles for this test.

The Boucher Jelly Tester can be standardized with a Dummy Bloom (Angus *et al.*, 1961). Since the plunger travel is $5/4$ times that of the Bloom gelometer and the instrument is loaded midway between plunger and pivot point, the dead-weight values (Wainewright, 1961) of the dummy strip readings should be multiplied by $2 \times 5/4 = 2.5$.

Gibbs *et al.* (1961) have examined the relationship between British Standard Bloom values, B , and Jellograms, B_j , and report the relationship

$$B = 0.6B_j + 26$$

Because of the longer plunger travel, the greater plunger area and the use of water loading at the midpoint between the beam pivot and plunger, tests with the Boucher Jelly tester are appreciably slower than those made with the Bloom gelometer. However, the principal disadvantage of the instrument is its dependence upon manufacturing accuracy. For good comparative testing there must be little variation in the distances between the pivot points, loading points and plungers for all Boucher instrument beams. A comparison of two of these instruments (Angus *et al.*, 1961b) revealed differences in beam construction sufficient to cause a 1% difference in test results.

F. Japanese Industrial Standard K6503: 1955

This standard details the apparatus and method for what is possibly the general Japanese gel strength test. The apparatus is essentially a balanced beam, pivoted at the mid-point and fitted with pans on top of each beam end and equidistant from the pivot point. The test method is as follows:

A jar containing the test gel is placed on one pan and the beam is balanced. A flat bottomed, 15 mm diameter rod is clamped over the gel so that it just touches the gel surface, then lead shot is loaded into the other pan until the gel has been raised 5 mm against the rod. This weight of shot, in grams, is the gel strength. The 5 mm elevation of the test gel is indicated by a pointer set vertically in the centre of the beam, with a scale mounted behind it.

The gel is prepared from 250 cc of a 10% gelatin solution which is matured for 20 minutes at 10°C and then 5 hours at 0°C in a glass test jar of 90 mm internal diameter and 65–70 mm high. The plunger rod, which can be metal or glass, should be chilled to 1°C before the test, and the test should be completed at an ambient temperature of 10°C .

The stipulated requirement for instrument loading is that the lead shot should be poured slowly, from an approximate height of 30 mm.

In this test the accuracy with which the gel is elevated 5 mm depends upon the ratio of the length of the pointer to the length of beam from the pivot point to a pan mounting point. In neither of two translations of the Japanese specification were these dimensions given. Experiments (Angus *et al.*, 1961a) with a ratio of pointer to beam length of 2 : 1 indicated the accuracy to be poor.

The rate at which the instrument is loaded will influence the test result, so the method will be subject to large operator errors.

It is probable (in view of the other uncertainties) that this test does not require an ambient temperature of 10°C, and while it may be necessary to pre-chill a metal plunger-rod it is doubtful whether the use of an uncooled glass rod would seriously affect test results.

An approximate formula for the relationship of British Standard Bloom strength, B, to J.I.S. units, J, is

$$B = \frac{J - 202}{2.47}$$

G. Japanese P.A.G.I. Specification: 1959

This specification details physical and chemical tests for photographic gelatins and emulsions. For gel strength determinations the instrument depicted closely resembles an old-style Bloom gelometer, but the test requires gels that are prepared from 120 ml of 5% solutions by maturing for three hours at 0–1°C. The specified test-bottle dimensions are the same as those of the standard Bloom test, as also are the plunger travel and rate of loading. However, the specification omits any reference to plunger size, so the relationship on the results of this test to those of the standard Bloom determination is not known.

The results of Wootton and Gibbs (1960) indicate that the specified gel-maturing period is satisfactory for reasonable test accuracy, but it is probable that the quoted maturing temperature limits are too large for satisfactory test reproducibility.

III. DESSERT GEL STRENGTH TESTS

Dessert jellies can have gelatin concentrations of 1–2%, pH values in the range 3–4 and sucrose contents of 10–20%. Also they are usually prepared at temperatures other than 10°C. Because these conditions are very different from those of the Bloom test, the Bloom strength of a gelatin sample is a poor guide to its value for use in desserts for the reasons briefly noted in A(ix). The most satisfactory method of determining this value is to test the sample under

the conditions of, or approximating to, the particular dessert formulation for which it is required.

A. The A.O.A.C. Test (1965)

Neither B.S. 757 nor the standard methods of the G.M.I.A. detail a gelatin dessert test, but the official methods of the Association of Official Agricultural Chemists (A.O.A.C.) include such a test. This test is as follows: A solution of 2.0 g of the gelatin dessert powder in 100 ml of water is prepared in a standard Bloom bottle, and matured for 17 ± 1 hours at $10^\circ \pm 0.1^\circ\text{C}$. The gel is tested with a Bloom gelometer equipped with a 1 in diameter, flat-bottomed plunger at the normal loading rate of 200 g per 5 sec. It should be noted that the bottom edge of the plunger is not radiused, also that a light-weight shot receiver is recommended. The results of this test will depend upon the formulation of the dessert and presumably the dessert manufacturer will define the test formulation and limits which he considers satisfactory.

The rigidities of these test gels are very low, in comparison with those of the normal Bloom test gels, hence the specification of a large diameter plunger and a light-weight shot receiver. For such weak gels the normal Bloom gelometer loading rate is too high. The plunger bounces during the test and thus test accuracy is lost. Borker *et al.* (1966) examined the use of lower loading rates, but with different sized plungers, so that the effects of the loading rates alone could not be assessed from their results. Not surprisingly, they reported that tests with a 26 mm plunger and loading rate of 45 g/5 secs were more consistent than tests with a 16 mm diameter and a loading rate of 75 g/5 secs.

A further possible source of error with this test lies in the use of a 26 mm diameter plunger with the standard Bloom bottle. Even with a 13 mm plunger the bottle dimensions affect the test. With the larger plunger wall effects will be very high and test accuracy accordingly reduced, if the dimensions vary or the centering is imperfect.

The reduction of Bloom gelometer loading rate can be achieved with a commercially available device which is attached to the gelometer shot spout. This device, which is designed for the American model gelometer, can fail to deliver a uniform flow of lead shot and is prone to block. These effects are sometimes due to worn or broken shot, which should be regularly sieved to ensure that it remains within the specified size limits.

B. British Dessert Gelatin Test

In this test, which is used widely in Britain, 7 g of the gelatin powder are dissolved in 300 ml of a 17.1 % sucrose solution containing 1 g citric acid. The

solution is matured in 300 ml capacity jars, $2\frac{1}{8} \pm \frac{1}{8}$ in internal diameter, for 18 hours at $16.5 \pm 0.1^\circ\text{C}$ then tested with the Boucher Jelly Tester equipped with a 26 mm diameter, flat-bottomed plunger. The bottom edge of this plunger is radiused to 1 mm. With this test the dessert manufacturer need only select a standard range appropriate to his requirements, within which results should fall.

Use of the Boucher instrument, with its water loading and mechanical advantage, enables the gels to be tested at low loading rates. This same advantage is available with the Boucher Electronic Jelly Tester. The results of this test will be less affected by wall effects, because of the larger test jar and greater gel volume, than tests using Bloom bottles.

With care the method can give satisfactory results, but it could possibly be improved by the incorporation of sodium citrate or other buffer into the formulation. This would aim in stabilizing the pH for comparative testing.

Because the effects of pH, concentration and temperature on the rigidities of gelatin gels vary with different gelatin samples, all gelatins should be assessed for dessert applications under dessert conditions. This can be done either by formulating the sample to the desired dessert and testing this under the appropriate concentration and temperature conditions, or testing the gelatin sample at pH, concentration and temperature levels related to the dessert application. Of these two methods the first is preferred, although it is more wasteful of materials.

As the gel strengths of dessert jellies are comparatively weak a large diameter plunger is required for most gelometers, to raise the test result to a level suitable for accurate determinations. A 26 mm diameter plunger appears satisfactory for this purpose, but the use of this size plunger increases the wall effects of the standard Bloom bottle; therefore the use of larger diameter test jars and gel volumes of at least 200 ml is recommended.

Even with 26 mm plunger the normal loading rate of the Bloom gelometer is too high for accurate dessert testing. As was noted in IIIA, a loading rate of 45 g/5 seconds appears suitable, but this entails the use of an adaptor which is not entirely satisfactory. Its flow rate can be erratic and it is prone to block. Either fault is sufficient to destroy test accuracy. For this reason the Boucher Jelly tester is preferred, although the faults inherent in such beam type gelometers should not be overlooked.

IV. VISCOSITY DETERMINATIONS

Viscosity is the second most commercially important physical property of gelatin samples. Low viscosity gelatins give short, brittle gels, while high viscosity gives tougher, extensible gels. For many applications gelatins of high

viscosity are preferred and command higher prices, other things being equal. The viscosities of gelatins are normally measured at 60°C and the nominal 6 $\frac{2}{3}$ % Bloom test concentration, in both Europe and North America. The results are expressed in millipoises.

A. British Standard 757: 1975 Method

This method recommends the use of a calibrated Ostwald viscometer conforming to B.S. 188. The No. 2 viscometer of the 1937 edition of B.S. 188, with a range of 5 to 40 cP and constant of about 0.05, was particularly suitable for gelatin viscosity determinations.

In the 1957 edition of B.S. 188 the recommended dimensions of the viscometers were changed. The viscometer most closely resembling the No. 2 viscometer is now the type C, which has a range of 6–45 cP and an instrument constant factor of about 0.01. The use of this latter viscometer for measuring gelatin solutions with viscosities below 6 cP, as many 6 $\frac{2}{3}$ % gelatin solutions are, will not result in any significant error providing the appropriate kinetic energy correction is applied.

Where the Bloom strengths of gelatin samples are also being determined it is sufficient to remelt the gels for viscosity determinations. For viscosity determinations alone a 6 $\frac{2}{3}$ % solution is prepared as for Bloom testing, with 7.5 g of gelatin and 105 ml of water. This solution should be prepared with the same accuracy as that required for the Bloom test.

For accurate viscosity determinations gelatin solutions should be free from particles capable of hindering the flow of solution through the viscometer capillary. Both filtration and viscometer filling are conveniently combined by using a small sinter glass funnel (Kragh, 1961) whose stem has been replaced by a length of glass tubing which can slide into the filling arm of the viscometer and reach the bottom bulb. A grade 1 sinter funnel is suitable. The tubing is attached to the funnel with a small length of rubber tubing which fits inside the stub of the funnel stem and over the tubing.

The viscometer should be held in the viscometer bath while being filled. The bottom bulb is filled to just above the fiducial mark and the solution equilibrated to the bath temperature. Temperature equilibration is rapidly achieved by sucking the solution into the top viscometer bulb and allowing it to flow back. Duplication of this procedure is usually sufficient. After equilibration any excess solution should be removed from the viscometer with a transfer pipette and the viscometer aligned in preparation for the determination. A plumb-line is essential for this.

A filling error of 1 mm to the fiducial mark of a type C viscometer results in a flow time change of about 0.05%. An aligning error of 1° from the vertical, in the plane dividing the viscometer arms, will change the flow time

by about 0.35%. Aligning errors in the plane of the arms have less effect upon flow times.

The solution should be sucked into the top viscometer bulb until the meniscus is above the top mark of the bulb. The time for the meniscus to drop from the top mark to the bottom mark, underneath the bulb, is timed with a stopwatch. Duplicate measurements should not vary by more than 0.3 seconds. If the sample has a high bacterial or enzyme content, successive determinations will steadily decrease. In this event, the first measurement should be used to calculate the viscosity of the sample and a note made of the instability of the sample. Erratic replicate times arise from suspended matter in the capillary and indicate poor filtration or poor cleaning.

The kinematic viscosity, ν , of the sample, in centistokes, can be calculated from the formula

$$\nu = Kt - \frac{a}{t}$$

where K = the viscometer constant; t = the measured flow time in seconds; a = the kinetic energy constant (1.5 for the type C viscometer, 2.4 for the No. 2 viscometer).

The dynamic viscosity of the sample, η , in millipoises, is calculated from the kinematic viscosity by the equation $\nu = \eta/10\rho$ where ρ is the density of a 6.2% gelatin solution at 60°C (B.S. 757 states that $\rho = 1.01$, but the results of Eastoe *et al.* (1952) indicate that 1.00 is a more accurate figure). Thus, for most practical purposes a reading in centipoises (cP) is equivalent to the value in centistokes (cS).

On completion of the measurement the viscometer should be drained, rinsed with warm water and preferably cleaned with chromic acid. In instances where the viscometer has to be reused immediately it should be rinsed with warm water several times, then with distilled water, acetone and finally ether. Final traces of ether can be removed with careful warming.

Although the viscosities of gelatin samples can be accurately determined by this method it is time consuming and thus not completely satisfactory for routine analytical work. This drawback can in part be overcome by making several determinations together, but this requires a stock of viscometers and stopwatches and much operator care if test accuracy is not to be lost.

B. The G.M.I.A. Viscosity Test

The apparatus recommended in this test is a calibrated viscosity pipette, a viscometer corresponding to and resembling the capillary arm of an Ostwald viscometer, except that the capillary is relatively short (9.45 ± 0.15 cm) and

the capacity 100 ml. This viscometer is jacketed by a small water bath maintained at $60 \pm 0.05^\circ\text{C}$.

As with the B.S. method, determinations can be made with freshly prepared 6 $\frac{2}{3}$ % gelatin solutions or remelted Bloom test gels.

The test recommends that the gel or solution is brought to 61°C then transferred to the viscometer, which is filled to about 1 cm above the upper mark. When the solution in the viscometer has equilibrated at 60°C , as shown by a calibrated thermometer, the meniscus of the solution is adjusted to the top mark of the viscometer. At this point, the solution should be free of bubbles. The end of the viscometer is unplugged and the time required for the meniscus to fall to the bottom mark of the viscometer is measured to 0.1 second.

As with the Ostwald viscometer, the formula relating flow time to the viscosity of the sample, η , in millipoises is $\eta = (At - B/t)$ where A and B are the appropriate viscometer and kinetic energy constants respectively, t is the measured flow time in seconds and ρ is the density of a 6 $\frac{2}{3}$ % gelatin solution at 60°C (quoted in the standard methods as 1.001).

This test method is less precise than the B.S. method because the pipette viscometer capillary is shorter and wider than those of the recommended U-tube viscometers. As a result the kinetic energy of the solution emerging from the capillary is greater. In an example given in the G.M.I.A. standard methods, the kinetic energy constant is quoted at 482.85. This of course includes a factor of 10 for the conversion of centipoises to millipoises, but it can be seen that the constant is far greater than those quoted for U-tube viscometers in the previous section. Because of this, and the smaller degree of accuracy with which the shorter flow times of this viscometer can be measured, this constant need not be quoted to such precise limits.

The test, as prescribed, is poorly suited to routine testing. With the large viscometer capacity, equilibration of the gelatin solution to the test temperature is slow. Also, the method is not ideal for duplicate viscosity determinations, which are essential for minimizing timing errors.

For routine testing it is common practice to melt several Bloom test gels, or prepare several solutions at once, in a water bath controlled to $60 \pm 0.1^\circ\text{C}$. When the solutions have equilibrated they are separately transferred to the viscometer, which is overfilled, and the time for the solution meniscus to fall from the top mark to the bottom mark measured. At the end of each determination the viscometer is filled with water at 60 – 62°C then drained thoroughly before refilling. This method is sufficiently accurate for normal commercial testing and is reasonably fast.

C. Factors Affecting Viscosity Determinations

- (i) Timing Errors—Kragh (1961) indicates that a careful operator should

be able to measure Ostwald viscometer flow times to within ± 0.05 sec. This is well within the accuracy required for normal commercial tests.

(ii) Temperature Requirements—Changes of temperature affect the viscosities of gelatin solutions considerably, because of the high temperature coefficient of the water in the solution. A temperature control of at least $\pm 0.02^\circ\text{C}$ is essential for routine viscosity determinations.

(iii) Concentration Requirements—Because the viscosities of gelatin samples can be determined from remelted Bloom test gels it is obvious that solutions for viscosity determinations should be prepared with the accuracy limits required for Bloom testing, i.e. 7.5 ± 0.01 g of gelatin and 105 ± 0.1 ml of distilled water. As with the Bloom test, gelatin viscosity will vary inversely with the moisture content of the sample. Kragh and Langston (1959) report that an approximate formula for calculating the change of gelatin viscosity with moisture content is:

$$\Delta\eta = -\frac{2\eta \Delta m}{100}$$

where η is the viscosity at a given moisture content. Therefore, it is essential to measure and quote the moisture content of a gelatin sample at the time that its viscosity is determined.

(iv) pH and Salt Concentration—Gelatin solution viscosity is strongly affected by pH, reaching a minimum at the iso-ionic pH. Minimum pH effect has been noted in the range pH 6–8 (Stainsby, 1952); therefore, for comparative viscosity testing the pH of gelatin solutions should be adjusted to a point in this range, or, for specific applications to the pH level of the application. The effect of pH is reduced by the presence of salts.

V. GELATIN VISCOSITY DEGRADATION TEST

Most normal gelatin samples have a bacterial content, and some an appreciable enzyme content not connected with living bacteria. This possibly arises from the use of bacterially degraded raw material. These contaminants if proteolytic will cause gelatin solutions to degrade, and the rate of this degradation depends upon the levels of these contaminants and the pH and temperature of the solution. Such degradation is manifested by a decrease in solution viscosity, which for many applications is undesirable.

Kragh and Langston (1959a) have evolved a simple test by which overall degradation can be measured and the relative proportions of enzyme and bacterial degradation assessed. The test is as follows.

Two 6 $\frac{2}{3}$ % solutions of the sample under test are prepared and adjusted to pH 7. Approximately 2% of a 0.2% solution of phenyl mercuric acetate

(PMA) solution is added to one gelatin solution, then both are transferred to U-tube viscometers held in a $40 \pm 0.1^\circ\text{C}$ water bath. The viscosities of the solutions are determined immediately after temperature equilibration and again after a period of 16–18 hours. From these results the percent viscosity loss per hour is calculated for each solution. A positive result for the solution without PMA indicates the overall degradation, containing thermal, bacterial and enzymic factors. A positive result for the solution containing the mercurial bacteriocide is a measure of thermal and bacterial degradation only. Thus a difference registered between the two values is the degradation attributable to the bacterial content of the sample. Because of the temperature at which the test is made, thermal degradation will be very small and may be neglected.

The initial viscosities of the two gelatin solutions will be different because of the dilution of one with PMA solution. This should not affect the result as the proportions of contaminants to gelatin remains unchanged, if the sample is homogenous.

A simple method of adjusting the pH of the solutions to 7 is to add a few drops of phenol red solution and adjust with N-HCl or N-NaOH until the solution colour changes to orange.

To reduce evaporation losses from the solutions, during the test, the arms of the viscometers should be closed with rubber bungs. Even with this precaution some moisture will evaporate from the surface of the solutions. For this reason the solutions should be re-equilibrated by sucking them into the top viscometer bulbs and allowing them to flow back before making the final viscosity measurements.

Pipette viscometers are less suitable for this test. Their use entails storage of the solutions in a separate 40°C bath and special viscometer cleaning precautions if more than one gelatin sample is being examined, to prevent cross-contamination.

The findings of Kragh and Langston (1959a) indicate that the viscosities of some 6 $\frac{2}{3}$ % gelatin solutions can degrade at rates in excess of 2% per hour, but that the viscosities of solutions of gelatins made from sound raw material, with good manufacturing techniques, degrade approximately 0.1–0.2% per hour, under the conditions of this test.

Because single enzyme reaction rates each follow an exponential relation with time (Dixon and Webb, 1960) the calculation of the enzymic degradation of gelatin as an average figure does not accurately depict the early, rapid degradation rate. Also, the test period of 16–18 hours may not be sufficient for the development of the maximum bacterial degradation rate. Nevertheless, the test period should cover the majority of gelatin uses, and the test is satisfactory for the differentiation of stable and unstable gelatin samples.

The conditions of this test, pH 7 and 40°C , were selected to accentuate

bacterial and enzymic effects while minimizing thermal degradation. At higher temperatures thermal effects will become more pronounced; with more acidic pH conditions both bacterial and enzymic degradation may be less. Therefore, for specific applications the conditions of this test should be modified to those of the applications.

Saunders (1956) has indicated that the enzymic degradation of gelatins causes structural changes similar to those of acidic degradation, where viscosity initially falls much more rapidly than gel strength, for high grade samples. Therefore, it is reasonable to expect that the Bloom strengths of good quality gelatins will initially decrease more slowly than the viscosity degradation rates determined by this test.

VI. METHODS FOR DETERMINING GELATIN pH

Although the pH values of commercial gelatins normally range from 4 to 7, the majority are between pH 5 and 6. The reasons for this are that gelatin manufacturers prefer to extract alkali pretreated raw material at a slightly acidic pH to inhibit bacterial growth, and to neutralize acid processed gelatins to this same range, after extraction, to prevent further degradation during subsequent processing.

In general, indicators are unsuitable for measuring the pH of gelatin samples. The colour changes of the ordinary indicators cover too wide a pH range and the colours of many gelatin samples interfere with the colour changes of the narrow-range indicators.

Both B.S. 757 and the Standard Methods of the G.M.I.A. recommend the use of a pH meter equipped with a glass electrode, but the conditions of the tests vary.

A. B.S. 757 pH Test

1 g of the gelatin is dissolved in a small quantity of recently boiled distilled water, diluted to 100 ml, cooled to room temperature and the pH measured with a glass electrode. Care in avoiding the absorption of atmospheric carbon dioxide during the test, is recommended.

B. G.M.I.A. pH Test

A 1½% solution of the gelatin sample in distilled water is prepared, cooled to 25°C and the pH determined with a pH meter standardized with pH 4 and 7 buffers, at 25°C.

It is doubtful whether the avoidance of carbon dioxide absorption, as recommended in the B.S. method, is necessary for routine testing. Gelatin has

a powerful buffering action, so that the pH of gelatin solutions is likely to be little affected by carbon dioxide. Similar arguments apply to the concentration differences of the two tests.

The recommended test temperatures are both below that at which gelation commences, therefore it is essential that temperature adjustment and pH measurement are quickly completed. Any glass electrode used for testing gelatin solutions should be frequently cleaned by soaking in 0.1 N hydrochloric acid and subsequent washing with warm distilled water.

For routine determinations of pH many laboratories find it convenient to use the remelted Bloom test gels, with suitable dilutions.

C. 40°C Method

For more precise work the pH of gelatin solutions should be determined at 40°C, using a pH meter whose electrodes have been standardized with buffer solutions at 40°C. A convenient method for this is to maintain the electrodes, buffer solutions and distilled water in a 40°C bath. After preparation, the gelatin solution is equilibrated to 40°C in the bath and its pH determined. After the test the electrodes should be thoroughly rinsed with the 40°C distilled water, then stored in fresh 40°C water. Excess solid potassium chloride should be added to the calomel electrode, to ensure that its solution remains saturated at 40°C.

VII. METHODS FOR DETERMINING THE GELATIN ISOIONIC POINT

Certain commercial blends of acid and alkali processed gelatins, Type A and Type B, give hazy solutions and gels, although solutions of the separate gelatins would be perfectly clear. This is thought to be due to the combination of the two oppositely charged gelatin molecules (Veis and Aranyi, 1960). These blends normally contain at least 80% of alkali processed gelatin. For many purposes such hazes are undesirable and thus gelatin producers and users require a test to determine or confirm gelatin type. Acid processed gelatins can be differentiated from alkali processed materials by their isoionic points. Those of acid processed gelatins lie in the range pH 6.0–9.5, those of alkali processed gelatins lie in the range 4.8–5.2, unless the raw material has been given an unusually short and mild pretreatment.

A. Rapid Method

An imprecise but rapid method of determining the gelatin isoionic point is to prepare a 1–2% solution of the sample and divide this into several 25 ml

fractions. The pH of each fraction is adjusted so that together they cover the pH range of 4.8–9.5 in, say, 0.5 pH unit steps. As cooled gelatin solutions at, or close to, the isoionic point appear hazy, a comparison of the clarities of the fractions will indicate the approximate isoionic point of the sample and thus type it. Having located the approximate isoionic point a further test can be made, with fractions covering the approximate point in smaller pH increments, say 0.2 of a unit. However, the accuracy with which the isoionic point can be located by this method is markedly dependent upon the ionizable salt content of the sample (Eastoe and Courts, 1963).

B. Ion Exchange Method

A precise method for determining gelatin isoionic points has been devised by Janus *et al.* (1951). In this method a 5% gelatin solution, at 35°C, is de-ionized by passing it through a mixed resin bed. The solution emerges from the bed at its isoionic point, which is determined by pH meter. The resins recommended by the authors are Amberlite IR120 and IRA 400. Eastoe and Courts (1963) recommended mixing the resins in the proportions of 5 volumes IRA 400 to 2 volumes of IR120, and describe a suitable apparatus.

VIII. DETERMINATION OF GEL MELTING POINTS

A. B.S. 757: 1975 Method

This method is based on the determination of the temperature at which gelatin gels soften sufficiently to allow carbon tetrachloride drops to sink through them. In the method, 5 ml of a solution of 7.5 g of gelatin in 105 ml of distilled water are pipetted into a thin walled soda glass test-tube, dimensions 150 × 16 mm, weight 10 g. This test-tube is closed with a bung and inserted in a test-tube rack which holds it at 45°C from the vertical. The rack is placed in a $10 \pm 0.1^\circ\text{C}$ bath where the solution is gelled and matured for 16–18 hours. After maturing, the test-tube in the rack is rotated through 180° along its axis, so that the gel surface is vertical, and 0.2 ml of carbon tetrachloride, containing 2% of a non-ionic wetting agent and coloured with a suitable dye is placed in the angle between the vertical gel surface and the test-tube wall. Lubrol W and Waxoline Red O.S. are recommended as suitable. The rack is then placed in a 15°C water bath and the bath heated to give a rate of rise of temperature of $0.25 \pm 0.05^\circ\text{C}$ per minute. The temperature at which the carbon tetrachloride moves freely down through the gel is taken as the melting point.

B.S. 757 indicates that adherence to the quoted limits of rate of heating ensures that errors in the melting point from this source are less than 0.25°C.

This is best achieved with a water bath fitted with a variac-controlled heater adjusted to give the required heat input.

The rack for holding the test-tubes should be of metal construction as it is immersed in water, and can be designed to hold several test-tubes for a series of melting point determinations.

In practice the change from gel to sol is gradual and the first phenomenon noted during the test will be the gradual sliding of the gel surface from the vertical to the horizontal plane. Before the gel surface is horizontal the carbon tetrachloride begins to descend. This point may not be detected easily, and Taylor and Stainsby (1958) recommend recording the temperature at which the carbon tetrachloride is half way down the gel.

B. Factors Affecting the Method

(i) Effect of Maturing Time on Melting Point—Stainsby and Taylor (1958) examined the dependence of melting point on maturing time. Their results indicated that the melting points of 6 $\frac{2}{3}$ % gels of high grade gelatins are dependent principally upon the first hour of maturing. An extension of the maturing time from 1 to 48 hours increased the melting point by only 0.15°C. Thus, for high grade gelatins the maturing time in the B.S. test can be appreciably shortened with small loss of accuracy.

(ii) The Concentration Dependence of Gel Melting Points—Ferry (1948b) has examined the dependence of gelatin gel melting point on concentration, and shown a straight line relationship between the reciprocal of the melting point, in degrees Kelvin, and the log concentration, for gels with concentrations above 2%.

(iii) The effect of pH and Salt Content on Gel Melting Point—Stainsby and Taylor (1958) examined the effect of pH and salt content upon the gelatin gel melting point. Their results showed that the melting points of both acid and alkali processed gelatin gels are pH dependent below pH 5, the melting point decreasing with increasing acidity. Between pH 5 and 9 there is virtually no change in the melting points of alkali-processed gelatin gels, whereas the melting points of acid-processed gelatin gels continue to rise slightly.

The presence of 6% sodium chloride decreased the melting points of alkali-processed gelatin gels at all pH levels. Data for the effects upon acid processed gelatin gels are not presented, but the authors indicate that the effects are similar.

Thus, for comparative testing it is necessary to ensure that the pH of the gelatin solutions is in the range pH 5–9, where the effects of pH on melting point are minimal. Where gelatin applications involve particular conditions of pH or salt content, melting point determinations should be made under those conditions.

The technique described above is a satisfactory method for the comparative testing of gelatin samples and products. Good reproducibility is obtainable with careful adherence to the stipulated test conditions. The equipment required is simple and inexpensive, apart from the 10°C maturing bath, which is standard for any laboratory concerned with gelatin testing.

IX. GELATION TIME TESTS

For many applications the speed with which a gelatin solution gels is extremely important. However, neither B.S. 757 nor the Standard Methods of the G.I.M.A. include a method for determining this property. A variety of methods exist, each applying to a particular gelatin use or product and each method has its own relevant conditions.

Details of two setting time tests are included here, one of which was evolved for testing edible gelatins, the other for testing photographic gelatins.

A. Edible Gelatin Setting Rate Test

A 6 $\frac{2}{3}$ % solution of the sample is prepared and equilibrated at 50°C. 30 ml of this sample are then poured into a large test-tube which is inserted into a gelation bath maintained at a temperature approximating to, or related to, the temperature at which the gelatin containing product is prepared or used, say 25°C. After a suitable period the test-tube is withdrawn and examined at one minute intervals for signs of gelation. It is returned to the bath between withdrawals. The elapsed time, from inserting the sample in the gelation bath until gelation is noted, is recorded.

This test lacks precision, because of the one minute intervals at which the sample is examined and because of the possible operator error in the judgment of the commencement of gelation. However, it is simple and can be adapted for the simultaneous examination of several samples by staggering the times at which the samples are placed in the gelation bath. Suitable specifications for an application can be quickly developed by the examination of a selection of different quality gelatin samples with this test.

Setting times are pH dependent, therefore when comparing samples for an application involving a particular level of pH the test should be made at that pH.

B. Photographic Gelatin Setting Rate Test

20 g of the gelatin sample are mixed with 380 ml of distilled water, allowed to swell for two hours then heated in a water bath until dissolved. The final solution temperature should be 45°C. 10 cc of a chrome alum solution (4.6 in

200 cc of distilled water) are added and the pH of the solution adjusted to 6.0 with 8% sodium hydroxide solution or 33% sulphuric acid. The solution is cooled to 38°C in a bath maintained at 19.5°C, then cooled to the following schedule. 1st minute, 33°C; 2nd minute, 29°C; 3rd minute 26°C; 4th minute, 23.6°C.

The solution is transferred to a 23.8°C bath and the solution "stirred with a stop" until, on the stop, bubbles in the solution swing back slightly before rising. The time interval from placing the solution in the 23.8°C bath until the onset of visco-elasticity is noted, is the setting time.

Wainewright (1966) has indicated that reproducible results can be obtained with this method, by careful control of gelatin concentration, but that it is time consuming. The time involved in the cooling cycle, from 38°C to completion of test, can be almost 20 minutes for samples with slow setting characteristics.

C. Factors Affecting Gelation Rates

Hopp (1964) has shown that setting time is pH dependent with minima in the pH/gelation time curves at pH 4.5–5.5 for alkali processed gelatins and at pH 6 for acid processed gelatins. The setting time decreased, as the Bloom strength increased to 200–220 g, to a constant value (for the particular conditions of his test). The viscosity/gelation time curves of acid and alkali processed gelatins were nearly identical, the gelation time decreasing as the viscosity increased from 65 to 140 mP.

D. The Tecam Gelation Timer

Basically, this instrument consists of a plunger which is raised by a synchronous motor but allowed to fall under its own weight. When the material under test has gelled or crosslinked sufficiently to support the weight of the plunger, a switch is closed which stops the instrument. The number of oscillations, and hence the gelation time, are automatically recorded.

An investigation of the suitability of this instrument for the measurement of gelatin setting rates (Wainewright, 1966) indicated that it measured an advanced state of gelation, thus requiring extended test-times. Despite the use of large diameter, light-weight plungers and concentrated gelatin solutions, the test could not be satisfactorily shortened. Also, there was poor differentiation between gelatins with widely differing setting rates, as determined by the photographic setting time test detailed above. Therefore it was concluded that this instrument is not suitable for the measurement of gelatin setting times.

REFERENCES

- Angus, J. and Culshaw, A. J. (1966). *Gelatin and Glue Research Association (GGRA) Bulletin* 17, No. 2, 8-14.
- Angus, J. and Wainwright, F. W. (1962). *GGRA Bulletin* 13, No. 2, 17-22.
- Angus, J., Pratt, A. N. and Wainwright, F. W. (1961a). *GGRA Bulletin* 12, No. 3, 13-15.
- Angus, J., Wainwright, F. W. and Wootton, J. (1961b). *GGRA Bulletin* 12, No. 2, 15-17.
- Association of Official Agricultural Chemists, Official Methods of Analysis (1965). Ed. Horwitz, W., AOAC, Washington, D.C.
- Bloom, O. T. (1925). U.S. Patent 1,540,979.
- Borker, E., Stefanucci, A. and Lewis, A. A. (1966). *J. Assoc. Off. Ag. Chem.* 49, 528-33.
- British Standard 757 : 1975, Sampling and Testing Gelatins, 6.
- Courts, A. (1972). British Food Manufacturing Industries Research Association Technical Circular No. 512.
- Dixon, M. and Webb, E. C. (1960). "Enzymes", 8, Longmans, England.
- Eastoe, J. E. and Courts, A. (1963). "Practical Analytical Methods for Connective Proteins", Chap. 6, Spon, London.
- Eastoe, J. W., Fysh, D. and Ward, A. G. (1952). *GGRA Bulletin* 3, No. 4, 10-13.
- Ferry, J. D. (1948a). *J. Am. Chem. Soc.* 70, 2244-9.
- Ferry, J. D. (1948b). "Advances in Protein Chemistry", 4, 24, Edit. by Anson, M. L. and Edsall, J. T., Academic Press, N.Y.
- Fysh, D. and Ward, A. G. (1953). GGRA Research Report B7.
- Gibbs, J. A., Langston, W. B. and Wootton, J. (1961). *GGRA Bulletin* 12, No. 4, 17-22.
- Hopp, V. (1964). *Leder.* 15, 59-63.
- Janus, J. W., Kenchington, A. W. and Ward, A. G. (1951). *Research, Lond.* 4, 247-8.
- Kinkel, E. and Sauer, E. (1925). *Ziets. Angew. Chem.* 38, 413-21.
- Koprowski, W. S. (1951). *Analyst* 76, 732-4.
- Kragh, A. M. (1961). "Analytical Methods of Protein Chemistry", 3, Chapter 5, Edit. by Alexander, P. and Block, R. J., Pergamon, Lond.
- Kragh, A. M. and Langston, W. B. (1957). *GGRA Bulletin* 8, No. 2, 17-19.
- Kragh, A. M. and Langston, W. B. (1959a). *GGRA Bulletin* 10, No. 4, 5-9.
- Kragh, A. M. and Langston, W. B. (1959b). GGRA Research Report C17, Part 3, 7.
- Kramer, F. and Rosenthal, H. (1965). *Food Technol.* 19, No. 9, 1417-20.
- Levi, S. M. (1960). *Kolloid. Zhur.* 22, 599-605.
- Sheppard, S. E. and Sweet, S. (1923). *Ind. Eng. Chem.* 15, 571-6.
- Saunders, P. R. (1954). GGRA Research Report All, 26.
- Saunders, P. R. (1956). Supplement to GGRA Research Report All.
- Saunders, P. R. and Ward, A. G. (1954). Proc. 2nd Int. Congr. Rheology, 284, Butterworth, London.
- Stainsby, G. (1952). *Nature, Lond.* 169, 662-5.
- Stainsby, G. and Taylor, J. T. (1958). GGRA Research Report A20.
- Timson, W. J. and Kelly, W. D. (1966). *Phot. Sci. Eng.* 10 (5), 278-80.
- Veis, A. and Aranyi, C. (1960). *J. Phys. Chem.* 64, 1203-10.
- Wainwright, F. W. (1961). *GGRA Bulletin* 12, No. 3, 16-18.

Wainewright, F. W. (1966). *GGRA Bulletin* 17, No. 3, 10-16.

Wootton, J. and Gibbs, J. A. (1960). *GGRA Bulletin* 11, No. 1, 13-15.

Wootton, J. and Kenchington, A. W. (1959). *GGRA Bulletin* 10, No. 3, 12-14.

EQUIPMENT AND APPARATUS SUPPLIERS

BRITISH:

<i>Bloom gelometer</i>	{ Formerly supplied by Griffin and George Limited. Gelometer production has now been discontinued.
<i>Gelation Bath (Model L238)</i>	{ Supplied by C. Stevens and Son Limited, Goswell Road, London E.C.1.
<i>The Boucher Electronic Jelly Tester</i>	{ Supplied by C. Stevens and Son Limited, Goswell Road, London E.C.1.
<i>Dummy Bloom Device</i>	{ Supplied by G. Farley and Sons Limited, Ponders End, Middlesex.
<i>Ostwald Viscometers and Saunders and Ward Jelly Tester</i>	{ Supplied by Techne Limited, Duxford, Cambridge.
<i>Tecam Gelation Tester</i>	{ Supplied by Techne Limited, Duxford, Cambridge.

NORTH AMERICAN:

<i>Bloom Gelometer</i>	{ Supplied by Precision Scientific Company, 3737 Cortland Street, Chicago 47, Illinois.
<i>Gelometer Plungers</i>	
<i>Shot-Flow Reducer</i>	{ American Instrument Company, 8010 Georgia Avenue, Silver Springs, Maryland.
<i>Gelation Bath</i>	
<i>Bloom Bottles</i>	{ T. C. Wheaton Company, Millville, New Jersey.
<i>Pipette Viscometer</i>	{ Scientific Glass Apparatus Company, Inc., 100 Lakewood Terrace, Bloomfield, New Jersey.

Author Index

Numbers in *italics* refer to pages on which the full reference is given

A

- Aboerhaloen E., 487, 502
Abitz, W., 206
Abrams, R. E., 417, 437
Abribat, A., 251, 288, 292
Accary, A., 240, 246
Accary-Venet, A. M., 119, 136
Ackerman, T., 287, 288
Adams, J. M., 60, 72
Adelman, 43
Adelmann, B. C., 66
Adox Fotowerke, Dr. C. Schleussner GmbH, 233, 241
Agfa, A. G., 220, 241
Aguire, M., 18, 30
Akabori, S., 487, 502
Albaum, H. G., 492, 502
Aldinger, W., 287, 292
Aldrick, P. J., 377, 381, 394
Alekseyev, V. F., 264, 293
Alexander, A. E., 129, 131, 135, 264, 267, 268, 273, 289
Alexandrescu, G., 145, 173, 316 (10), 362
Alford, W. C., 492, 502
Ali, S. A., 175
Alikonis, J., 390, 393
Allé, Th., 344 (55), 363
Allen, C. F. H., 225, 226, 230, 232, 241
Allison, A. C., 274, 288
Almy, E. G., 428, 437
Altenschöpfer, Th., 163, 164, 174, 175
Allentoff, N., 237, 243
Ambrose, E. J., 6, 27
Amelina, K. S., 274, 288
American Cynamid Co., 241
Ames, W. M., 83, 87, 89, 105, 156, 158, 160, 162, 163, 164, 173, 269, 288, 310 (27-34), 313, 314, 344 (53), 363, 369, 372, 392, 497, 498, 502
Aminoff, D., 493, 502
Ammann-Brass, H., 462, 465, 471
Analytical Methods Committee, 481, 498, 502
Andel Colloid Corp., 257, 288
Anders, G., 97, 106
Anderson, P. A., 87, 106
Andreasen, J., 389, 394
Andreeva, N. S., 6, 28
Andrew, P. F., 239, 244
Andrews, A. T. de B., 48, 56, 57, 66
Andrews, F. A., 223, 241
Andrikides, A., 55, 68
Anesey, J., 12, 30, 42, 72, 118, 136, 138, 156, 157, 177, 259, 275, 276, 293
Angus, J., 513, 517, 518, 519, 533
Anokhin, V. V., 262, 288
Anon, 367, 370, 376, 377, 378, 380, 387, 391, 392
Anthistle, M. J., 382, 392
Arakawa, K., 266, 268, 288, 294
Aranyi, C., 238, 247, 528, 533
Araya, S., 53, 66
Arbuckle, W. S., 388, 389, 393
Arens, H., 462, 472
Aresey, J., 42, 72
Arlinghans, R. B., 77, 106
Armes, J. N., 32, 66
Armour, 166
Armour and Co., 149, 173
Arnett, W. M., 64, 69
Ashworth, D. R., 301 (16), 313
Association of Official Agricultural Chemists, Official Methods of Analysis, 533
Astbury, W. T., 97, 105
Avssel, P., 274, 288
Autry, R. F., 405, 412

B

- Babloyan, O. O., 162, 165, 173
 Bachra, B. N., 52, 60, 61, 66
 Bailey, A. J., 2, 10, 13, 15, 19, 27, 28, 30, 155, 161, 166, 172, 173, 174
 Bailey, J. L., 482, 487, 502
 Bailey, P. J., 61, 72
 Baker, E. B., 230, 241
 Bakerman, S., 20, 27, 28
 Baldwin, R. R., 280, 289
 Balian, G. A., 19, 27
 Balo, J., 62, 63, 66
 Bamford, G. S. T., 387, 392
 Banga, I., 62, 63, 66
 Bangle, R., 492, 502
 Banfield, F. H., 367, 368, 371, 372, 373, 376, 378, 392
 Bank, O., 373, 393
 Baramboim, N. K., 142, 154, 175
 Barat, S. K., 64, 71
 Barker, E. F., 422, 436
 Barker, S. A., 45, 46, 66
 Barr, J., 458, 472
 Barrett, J., 65, 69, 408, 409, 411, 412, 412
 Barron, E., 237, 241
 Bartlet, J. C., 100, 105
 Bartos, J., 499, 502
 Basset, J., 266, 293
 Bassett, R. A., 465, 472
 Battacharya, S. D., 66, 71
 Bavd, C. A., 59, 66
 Bavetta, L. A., 42, 53, 70, 71, 155, 176
 Bavington, J. H., 173
 Bayer, O., 219, 245
 Bayley, H. G., 250, 288
 Bear, R. S., 6, 30
 Beavan, G. H., 491, 502
 Becker, E., 97, 106
 Becker, R., 202, 206
 Becker, R. O., 51, 67
 Becker, V., 17, 30
 Behner, J., 440, 472
 Behringswerke, A-G, 236, 241
 Behrmann, V. G., 418, 437
 Beier, G., 135, 147, 148, 173, 175, 195, 206
 Belavtseva, V. M., 91, 107
 Belcher, R., 496, 502
 Bellis, D. C., 218, 243
 Bello, H. R., 173, 185, 191, 192, 206, 225, 241
 Bello, J., 24, 27, 98, 105, 141, 173, 174, 185, 191, 192, 198, 199, 206, 225, 241, 284, 288, 379, 392, 495, 502
 Bendall, J. R., 65, 70
 Bendien, W. M., 458, 473
 Benesch, R., 236, 241
 Benesch, R. E., 236, 241
 Bennett, W., 279, 289, 290
 Bennion, E. B., 383, 387, 392
 Bensusan, H. B., 17, 27
 Berendsen, R., 456, 472
 Berg, E. W., 51, 67
 Berg, W. F., 458, 472
 Berger, A., 213, 241, 293
 Bermane, D., 256, 291, 445, 472
 Berrens, L., 40, 67
 Berube, L., 19, 30
 Bettelheim-Jones, F. R., 239, 241
 Beyer, G. L., 123, 124, 135, 279, 289
 Bhatnagar, R. S., 177, 177
 Biomead, O. S., 482, 502
 Bielen, R. J., 64, 67
 Bigwood, E. J., 482, 502
 Biotest-Serum-Institut GmbH, 236, 241
 Biotest Serum Institute, 419, 436
 Birr, E. J., 228, 230, 239, 241
 Bisaz, S., 61, 68
 Biserte, G., 487, 502
 Bitcover, E. H., 48, 67
 Bjorksten, J., 223, 224, 241
 Black, J. W., 367, 392
 Blackburn, S., 482, 483, 487, 495, 502
 Bladen, H. A., 11, 14, 16, 29
 Blain, G., 417, 436
 Blair, J. A., 404, 412
 Blake, J. N., 32, 67, 255, 293, 447, 473, 485, 502
 Blass, U., 218, 241
 Blatt, W. G., 371, 394
 Blazej, A., 142, 168, 173, 177
 Bloom, O. T., 509, 533
 Blout, E. R., 66, 71
 Blumenfeld, O., 13, 27
 Blumenfeld, O. O., 18, 30, 93, 98, 99, 100, 105, 169, 173
 Blumenthal, S., 384, 390, 392, 393

- Bodenhagen, H. B., 422, 437
 Bodor, 408, 412
 Boedtker, H., 7, 15, 27, 67, 114, 120, 123, 124, 135, 141, 173, 190, 206, 275, 289
 Boettger, R. M., 386, 392
 Bogatyrev, A. N., 165, 173
 Bogdanov, L. M., 219, 245
 Bogomolova, L. G., 215, 241
 Bogue, R. H., 129, 135
 Bolaffi, A., 280, 289
 Bolam, T. R., 442, 472
 Bolognani, L., 56, 64, 67, 72
 Bonferroni, B., 64, 72
 Bon, W. F., 253, 290
 Booth, R. B., 459, 473
 Borginon, H., 456, 466, 472
 Borker, E., 520, 533
 Bornstein, P., 11, 13, 14, 15, 16, 17, 18, 22, 27, 29, 88, 98, 99, 106, 169, 170, 173
 Bose, S. M., 47, 48, 49, 67, 70, 95, 98, 106
 Bosmann, H. B., 65, 67, 152, 173
 Bottazzi, F., 287, 289
 Bottoms, E., 171, 172, 173, 176
 Bourgoin, D., 187, 206, 278, 289
 Bourillon, R., 44, 67, 69
 Bouthilet, R. J., 241, 241
 Bovey, F. A., 281, 289
 Bowes, J., 397, 412
 Bowes, J. H., 19, 20, 25, 27, 37, 38, 40, 42, 44, 45, 67, 82, 83, 84, 93, 95, 101, 102, 104, 105
 Bowman, R. E., 437
 Bozzini, C. E., 46, 69
 Bradbury, E., 253, 261, 289
 Bradbury, E. M., 7, 27
 Bradshaw, N., 402, 410
 Brady, L. E., 467, 472
 Branot, K. D., 64, 67
 Braswell, E., 180, 206, 257, 290, 368, 370, 378, 390, 393
 Braudo, E. E., 288, 289
 Braunsdorf, K., 481, 502
 Brewer, T. H., 64, 72
 Briefer, M., 378, 385, 392
 Briggs, D. R., 239, 241
 British Pharmacopoeia, 416, 417, 436
 British Standard 757, 476, 477, 497, 501, 502
 Broady, K., 72, 177
 Brockmann, H., 487, 502
 Bronson, W. F., 373, 382, 384, 392
 Brown, A., 269, 293
 Brown, K. O., 492, 503
 Brown, L., 169, 173
 Brown, W. E., 52, 67
 Brunken, J., 233, 241
 Budurina, N. N., 230, 245
 Bühler, K., 110, 136
 Bump, S., 15, 22, 28
 Bundenberg De Jong, H. G., 117, 135, 268, 289
 Burckard, 57, 58, 67
 Burde, R. de La., 169, 173
 Burdygina, G. I., 262, 284, 289
 Burdzhanadze, T. V., 283, 289
 Burge, R., 7, 27
 Burge, R. E., 7, 25, 27
 Burness, D. M., 228, 229, 230, 232, 233, 234, 241, 242, 244
 Burnett, R. S., 241, 242
 Burton, E. F., 442, 473
 Bushmakin, I. N., 287, 294
 Butler, T., 170, 173
 Butler, W. T., 4, 11, 13, 14, 15, 16, 17, 18, 22, 27, 29
 Butskene, A. I., 218, 219, 242
 Butskus, P. F., 218, 219, 242
 Butzow, J. J., 62, 67
 Byčichin, A., 498, 502

C

- Cabanis, J. C., 498, 502
 Cahn, R. I., 381, 392
 Caldwell, J. R., 219, 242, 258, 289
 Callow, E. H., 257, 289, 388, 392
 Campbell, D. H., 236, 242
 Cambell, H., 418, 436
 Campo, R. D., 50, 63, 64, 67
 Caneghem, P. Van, 172, 173
 Cannon, R. K., 484, 505
 Carillo, A. L., 176
 Carless, J. E., 268, 274, 289, 292
 Carlin, G. T., 386, 392
 Carlström, 52
 Carlstrom, D., 67

- Carpenter, D. C., 276, 289, 379, 388, 389, 392
 Carroll, B. H., 225, 226, 241
 Cars, N., 160, 175
 Cartwright, H. M., 274, 289
 Cassel, J. M., 10, 27, 141, 173, 485, 502
 Cassella Farbwerke Mainkur, A-G, 218, 242
 Castellani, A. A., 56, 67
 Castello, R. A., 263, 289
 Cater, C. W., 19, 25, 27, 172, 173, 232, 242
 Cavanaugh, E. F., 151, 175
 Ceacareanu, D., 268, 291
 Cescon, I., 64, 72
 Cessi, C., 484, 505
 Cetini, G., 274, 289
 Chalmers, D. G., 417, 437
 Chambers, D. C., 486, 490, 505
 Chanal, J. L., 274, 288
 Chandrasekhanan, R., 7, 29
 Chapman, G. E., 7, 28
 Charles B. Knox Gelatin Co. Inc., 419, 436
 Chastel, R., 274, 294
 Chateau, H., 240, 246, 498, 504
 Chayen, I. H., 301 (16), 313
 Chayen, R., 301 (17), 313
 Chernon, N. V., 142, 154, 175
 Chibisov, K. V., 465, 472
 Chibnall, A. C., 83, 105, 477, 502
 Chipperfield, A. R., 57, 67
 Chochlowa, S., 299 (5), 313
 Christensen, R. G., 141, 173
 Christopher, E. F., 219, 231, 247
 Chung, H. Y., 239, 242
 Ciba Ltd., 218, 227, 228, 239, 242
 Ciferri, A., 143, 176
 Cifonelli, J. A., 47, 67
 Ciocalteo, V., 479, 503
 Cioni, P., 64, 72
 Ciricelli, J. S., 119, 136
 Clark, 235
 Clark, G. R., 232, 242
 Clark, R. C., 214, 216, 217, 218, 226, 227, 237, 238, 242, 420, 436, 492, 502
 Clarke, H. T., 217, 244
 Clayton, W., 371, 378, 390, 392
 Clegg, F. G., 305 (18) 313, 323 (40) 363
 Clyne, E. J., 368, 373, 392
 Cobbett, W. G., 216, 217, 242, 268, 287, 488, 492, 493, 500
 Cobbett, 85
 Cohen, J., 39, 42, 72, 112, 118, 127, 128, 136, 138, 157, 177, 247, 275, 293, 420, 437
 Cohen, H. L., 226, 228, 232, 245
 Cohen, J. H., 378, 385, 392
 Cohen, S., 420, 437
 Coleman, B., 263, 289, 294
 Combet, S., 489, 505
 Combret, S., 222, 242
 Comte, P., 35, 45, 71
 Cooper, D. A., 172, 173
 Cooper, D. R., 42, 43, 44, 67, 147, 174, 174, 176
 Coopes, I. H., 224, 242, 276, 277, 289
 Corben, L. D., 240, 242, 376, 392
 Cordier, S., 274, 289
 Coretti, K., 366, 392
 Corey, R. B., 5, 29
 Coulson, A. S., 417, 437
 Coulson, C. A., 91, 105
 Coupet, M., 498, 502
 Courts, A., 32, 67, 93, 95, 96, 105, 118, 119, 124, 135, 140, 148, 153, 155, 162, 163, 164, 167, 168, 173, 174, 175, 211, 218, 232, 239, 242, 244, 270, 289, 308 (21), 313, 396, 397, 403, 404, 405, 408, 409, 412, 478, 480, 482, 486, 487, 490, 502, 503, 517, 529, 533
 Cowan, P. M., 5, 25, 27, 28
 Cowan, S. W., 228, 242
 Crick, F. H. C., 5, 30
 Croome, R. J., 128, 135, 269, 273, 289, 305 (18), 313, 323 (40), 363, 372, 392
 Crosby, N. T., 142, 154, 174
 Cross, J., 14, 29
 Crouzat-Reynes, B., 274, 291
 Cruess, R. L., 59, 71
 Cruikshank, C. N. D., 46, 66
 Crumpton, M. J., 492, 502
 Culshaw, A. J., 517, 533
 Cumper, C. W. N., 129, 131, 135, 264, 267, 268, 273, 289
 Cunningham, G. E., 239, 246
 Cunningham, L. W., 4, 15, 17, 18, 27, 28, 29, 93, 100, 105
 Curme, H. G., 451, 452, 472
 Czajkowska, C., 482, 502

D

- Dabbous, M., 17, 28
 Dahl, O., 33, 34, 67
 Dahlberg, A. C., 379, 388, 389, 392
 Dailly, S. D., 54, 70, 399, 403
 Dakin, H. D., 98, 105
 Dallemagne, M. J., 51, 67
 Dalton, A. S., 217, 242, 422, 437
 Damschroeder, R. E., 212, 242
 Danilzenko, A., 56, 68
 Danilov, L. T., 239, 244
 Dann, J. R., 219, 242, 244
 Darlow, B. L. R., 188, 189, 192, 206
 Darlow, R. L., 457, 459, 472
 Darlow, R. L. R., 146, 150, 164, 175
 Das, S., 66, 71, 255, 293
 Dautrevaux, M., 57, 58, 67
 Davidson, E., 34, 35, 63, 70, 497, 498, 502
 Davidson, E. A., 41, 71
 Davidson, P. B., 422, 437
 Davidson, R. J., 42, 44, 67, 172, 174
 Davis, C. E., 129, 135, 186, 206
 Davis, H. F., 71, 101, 102, 106, 486, 504
 Davis, P., 213, 231, 242, 480, 489, 502
 Davison, P. F., 14, 15, 22, 28
 Deasy, C., 39, 67
 Deasy, C. L., 167, 168, 174
 De Cugnac-Pailliotet, A., 466, 473
 De Gloabec, V. C. E., 373, 393
 Dehio, H., 462, 472
 De Jong, H. G. B., 373, 393
 Denis, G. I., 218, 219, 242
 Denisova, A. A., 219, 242
 Derksen, J. L., 253, 290
 Deshmukh, K., 155, 166, 174-176
 Deutsch Gelatine-Fabriken, 174
 Deutsche Gelatin Fabriken, 160
- Devyatov, Ya B., 32, 167, 169, 72, 177
 Dew, G. D., 434, 437
 Dick, Y. P., 141, 170, 174, 176
 Dickerson, J. W. T., 39, 50, 68
 Dickinson, H. O., 459, 465, 472
 Digby, P. S. B., 61, 68
 Diorio, A. F., 206
 Dimarzio, E. A., 448, 449, 472
 Dische, P. S. B., 67
 Dische, Z., 56, 68, 492, 493, 502
 Dittmar, C., 91, 107
 Dixon, M., 526, 533
 Doghotti, L. M., 388, 394
 Dorfmann, A., 46, 71
 Doty, P., 7, 15, 27, 65, 67, 114, 120, 123, 124, 135, 141, 173, 174, 190, 206, 275, 289
 Doty, S. B., 59, 68
 Dowell, L. G., 257, 289
 Downey, T. B., 369, 373, 378, 384, 385, 390, 393
 Doyle, B. B., 66, 71
 Doyle, R. J., 141, 174, 239, 243
 Drake, M. P., 14, 15, 22, 24, 27, 30, 128, 136
 Drew, R. B., 226, 243
 Dreywood, R., 492, 502
 Duck, W. N., 381, 393
 Duecker, W. W., 388, 393
 Dugan, L. R., 143, 175
 Dumitru, E. T., 62, 68, 140, 149, 174
 Du Pont Nemurs, 229
 Durante, M., 99, 106
 Dusheiko, D. A., 230, 245
 Dustin, J. P., 482, 502
 Dutton, K. R., 226, 243

E

- Eanes, E. D., 52, 68, 144, 174
 Eastman Kodak Co., 228, 230, 232, 243
 Eastman Kodak Inc., 261, 289
 Eastoe, B., 49, 56, 59, 68, 101, 102, 103, 105, 477, 497, 503
 Eastoe, J. E., 3, 28, 40, 48, 49, 51, 53, 56, 58, 59, 68, 70, 72, 76, 77, 78, 79, 80, 81, 82, 83, 84, 86, 87, 88, 92, 93, 100, 101, 102, 103, 105, 107, 211, 243, 409, 412, 476, 477, 478, 479, 480, 482, 483, 485, 486, 491, 492, 495, 497, 499, 503, 529, 533
 Eastoe, J. W., 289, 523, 533
 Edeling, C., 363
 Edman, G., 388, 394
 Edman, P., 487, 503
 Edsall, J. T., 231, 243
 Edwards, P. R., 367, 368, 380, 393
 Eggenberger, D. N., 112, 136
 Ehrlich, R. M., 238, 244
 E. I. du Pont De Nemours & Co., 228, 243
 Eichhorn, G. L., 62, 67
 Eidinger, D., 56, 68

- Eirich, F. R., 263, 290, 447, 448, 449, 472, 473
 Ekhammar, J. A., 401, 412
 Elden, H. R., 26, 28
 Eldridge, J. E., 192, 199, 201, 206, 266, 267, 269, 275, 290, 291, 371, 378, 393
 Elfbaum, S. G., 236, 237, 245
 Eliassaf, J., 263, 290
 Ellenberger, W., 239, 243
 Ellerstein, S., 449, 472
 Ellins, H. J., 218, 243
 Elliott, A., 6, 27, 150, 174, 290
 Elliott, R. G., 37, 38, 44, 45, 67, 82, 83, 84, 95, 101, 102, 104, 105
 Ellis, S. C., 489, 503
 Elovsson, G., 345 (57), 363
 Elson, L. A., 492, 503
 Endres, H., 28
 Engel, J., 23, 28, 123, 127, 135, 141, 147, 173, 174, 195, 206
 Engström, A., 52, 67, 68
 Epifanov, G. I., 262, 292
 Esipova, N. G., 6, 28
 Evans, W. W., 264, 293
 Evva, F., 462, 472
- Faelens, P. A., 465, 466, 472
 Fairweather, B., 19, 30
 Fanis, B., 18, 19, 29
 Fanselow, J. R., 275, 291
 Fanta, P. A., 229, 243
 Farbenfabriken Bayer, A-G, 228, 243
 Faris, B., 166, 175
 Farr, A. L., 479, 504
 Ferguson, S., 481, 503
 Ferrania Societa per Azioni, 234, 239, 243
 Ferrel, R. E., 223, 246
 Ferri, G., 56, 67
 Ferry, J. D., 180, 182, 191, 192, 199, 201, 203, 206, 264, 265, 266, 267, 269, 275, 290, 291, 293, 378, 393, 505, 512, 530, 533
 Fessler, J. H., 155, 174
 Fiene, F., 392, 393
 Fietzek, P., 17, 22, 29, 30
 Fildes, J. E., 496, 502
 Filler, R., 235, 243
 Finean, J. B., 52, 67, 68
 Fink, P., 274, 290
 Fishel, C.-W., 239, 243, 247
 Fishman, L., 82, 105, 171, 174
 Fiske, C. H., 499, 503
 Fitton Jackson, S., 10, 28
 Flawe, R. W., 238, 244
 Fleisch, H., 61, 68
 Fleischmajer, R., 82, 105, 144, 174
 Flory, P. J., 24, 25, 28, 118, 119, 120, 126, 135, 140, 174, 201, 206, 258, 259, 290, 440, 472
 Folin, O., 479, 503
 Folk, J. E., 488, 503
 Fontana, B. J., 450, 472
 Ford, J. A., 228, 242
 Ford, J. D., 4, 18, 28, 93, 100, 105
 Fowler, L. J., 15, 19, 27, 161, 166, 173
 Fraenkel-Conrat, H., 209, 223, 245, 246
 Frame, G. F., 215, 216, 217, 245, 247
 Francis, A. C., 499, 503
 France, W. G., 283, 291
 Francois, C. J., 54, 55, 61, 68, 69
 Frandsen, J. H., 388, 389, 393
 Franzblau, C., 18, 19, 29, 30, 99, 105, 166, 175
 Fraser, R. D. B., 7, 28
 Frazier, A. W., 52, 67
 Freeman, I. L., 152, 174
 Freeman, M. C., 240, 246
 French, D., 231, 243
 Fridman, I. M., 262, 284, 289
 Frieser, H., 447, 467, 472
 Frisch, H. L., 447, 448, 449, 472, 473
 Froda, R. H., 400, 412
 Froeschlin, W., 419, 437
 Fu, H. C., 48, 71
 Fuchs, E., 223, 239, 243
 Fuji Photo Film Co. Ltd., 221, 226, 229, 237, 243
 Fujii, T., 152, 153, 154, 174, 184, 206, 311 (41), 312 (41), 314
 Fujimori, E., 171, 174
 Fujita, K., 240, 246
 Fukushi, S., 65, 68
 Fullerton, H., 426, 437
 Funakoshi, H., 3, 28
 Furrer, E., 274, 290
 Fuschs, E., 231
 Fysh, D., 91, 105, 286, 289, 510, 513, 523, 533

G

- Gadet, M. C., 466, 473
 Gaevoi, E. V., 165, 173
 Galatik, A., 142, 173
 Gallop, P. M., 12, 13, 17, 18, 19, 27, 28, 30, 93, 98, 99, 100, 105, 167, 168, 169, 171, 173, 174, 176
 Galloway, J. W., 15, 29
 Garrett, R. R., 24, 28, 62, 68, 140, 149, 174, 259, 290
 Garrison, W. M., 279, 289, 290
 Gat, I., 52, 68
 Gates, J. W., 212, 215, 218, 219, 228, 237, 242, 243, 244, 245
 Gatovskaia, T. V., 6, 28
 Gedalia, I., 52, 68
 Gehenio, P. M., 257, 290
 Geisler, J., 60, 72
 General Aniline and Film Corp., 221, 243
 General Foods Corp., 238, 243, 257, 290
 Genin, J., 274, 294
 Gerngross, O., 313
 Georgakopoulos, P. P., 268, 274, 292
 Gerisch, G., 66, 71
 Gerke, K., 348 (61), 363
 Gerngross, O., 49, 68, 180, 206, 268, 290, 342 (49), 363, 491, 503
 Gerrard, F., 379, 380, 390, 393
 Gevaert-Agfa, N.V., 218, 234, 243, 244
 Gevaert Photo-Producten, N.V., 217, 218, 226, 227, 228, 229, 233, 243, 261, 290
 Gibbs, J. A., 214, 215, 216, 217, 238, 242, 244, 420, 436, 494, 495, 502, 514, 518, 519, 533, 534
 Gies, W. J., 55, 69
 Giles, B., 239, 242, 408, 412
 Giles, W. M., 62, 69
 Gillessen, I., 52, 68
 Gilligan, D. R., 33, 34, 70
 Gillios-Tos, M., 462, 472
 Glabau, C. A., 387, 393
 Glabe, E., 390, 393
 Gladner, J. A., 488, 503
 Glasgow, S., 63, 64, 70
 Glegg, R. E., 68
 Glimcher, J., 54, 55, 68
 Glimcher, M., 145, 166, 174, 177
 Glimcher, M. J., 53, 54, 55, 60, 61, 68, 69
 Gloxhuber, C., 219, 245
 Goebel, E., 49, 68, 313
 Goldblith, S. A., 280, 291
 Golovteeva, A. A., 154, 176, 177
 Goltz, R. W., 41, 69
 Golub, L., 166, 177
 Goodall, H., 375, 393
 Goodwin, T. W., 490, 491, 503
 Gordon, A. H., 482, 483, 485, 503
 Gorfinkle, W. I., 386, 393
 Gorodetzkaia, R., 305 (19), 313
 Gorsuch, T. T., 496, 497, 498, 503
 Got, R., 44, 67, 69
 Gottschalk, A., 485, 504
 Govinlock, E. V., 118, 119, 120, 135
 Goyan, J. E., 263, 289
 Grabar, P., 291
 Graham, J. J., 404, 412
 Graham, J. L., 233, 234, 244, 247
 Graham, T., 250, 290
 Grainger, F., 500, 503
 Grand, R. J. A., 119, 135
 Grant, M. E., 152, 174
 Grassmann, W., 3, 13, 28, 82, 85, 93, 105, 106, 127, 135, 147, 163, 174, 484, 503
 Gray, V. R., 266, 290
 Gray, W. R., 217, 244
 Graziano, V., 56, 67
 Green, B. K., 419, 420, 437
 Green, F. C., 97, 107, 487, 503
 Greenberg, D. M., 258, 291
 Greenberg, J., 171, 174
 Greenspan, M., 273, 293
 Grettie, D. B., 385, 393
 Grettie, D. P., 145, 159, 174, 238, 244
 Griffin, K. P., 216, 245
 Griffin, W. C., 428, 437
 Grigor'eva, N. V., 262, 290
 Grinberg, V. Ya., 264, 293
 Gron, P., 55, 72
 Gross, J., 7, 11, 12, 13, 14, 16, 19, 24, 28, 29, 30, 66, 71, 80, 81, 106, 148, 149, 174
 Gross, L. G., 281, 283, 290
 Groves, M. L., 276, 291
 Guest, G. H., 484, 504
 Guia, E. de, 42, 71
 Gunthardt, H., 376, 393
 Gupta, R. C., 263, 290

- Gurin, S., 217, 244
 Gurule, F. T., 240, 246
 Gustavson, G. H., 24, 25, 28
 Gustavson, K. H., 35, 69, 168, 174, 209, 224, 239, 244
 Gutienez, A. M., 18, 30
 Gutmacher, R. G., 449, 472
 Guttoff, F. B., 462, 472

H

- Haas, H. C., 146, 174
 Habermann, E., 40, 69
 Haefele, C. R., 218, 245
 Hagen, R. F., 238, 244
 Halámek, C., 498, 502
 Hall, C. E., 30
 Hall, L. A., 373, 380, 393
 Halpin, J. C., 206
 Hamilton, J. F., 467, 472
 Hamilton, L. D. G., 489, 490, 503
 Hamilton, P. B., 87, 106
 Hamm, F. A., 467, 472
 Hannig, K., 3, 28, 97, 106, 152, 175
 Hanson, H. L., 381, 393, 423, 437
 Hanus, A., 141, 175
 Hanzlick, J., 65, 69
 Happey, F., 35, 71
 Harborne, M. R., 434, 437
 Harding, J. J., 2, 13, 17, 28, 62, 69, 82, 98, 106, 140, 174, 490, 503
 Hardy, W. B., 393
 Harkness, M. L. R., 19, 28, 155, 169, 173, 174
 Harkness, R. D., 19, 28, 41, 42, 69, 155, 169, 173
 Harkness, S. R. D., 174
 Harper, H. A., 484, 505
 Harper, R. A., 52, 68
 Harrap, B. S., 141, 175, 177
 Harriman, B. R., 232, 245
 Harrington, W., 80, 106
 Harrington, W. F., 2, 11, 23, 28, 66, 70, 121, 122, 126, 135, 136, 146, 177, 204, 206, 276, 290, 293
 Harriott, R. M., 209, 244
 Harris, E. H., 143, 176
 Harrow, R. A. J., 227, 229, 244
 Hart, P. J., 497, 505
 Hartles, R. L., 53, 58, 69, 70
 Hartman, B. K., 20, 27, 28
 Hartman, F. W., 418, 437
 Hashimoto, S., 42, 69
 Hatschek, E., 264, 265, 266, 268, 290
 Hautot, A., 462, 472
 Havez, R., 57, 58, 67
 Hawk, P. B., 55, 69
 Hayouk, 66, 71
 Hayek, E., 51, 52, 69, 71
 Healy, M. T., 472
 Healy, T. W., 459, 472
 Heidemann, E., 143, 160, 169, 175, 317 (11), 362
 Hein, R. W., 219, 245
 Heiney, R. E., 215, 245
 Heinrich, W., 169, 175
 Helbert, J. R., 492, 503
 Helling, J. O., 454, 472
 Hellman, N. Y., 448, 472
 Hening, J. C., 379, 388, 389, 392
 Henkel & Cie, GmbH, 226, 244
 Hennemann, J. P., 268, 289
 Henson, E., 105
 Herbage, D., 141, 175
 Herfeld, H., 175, 491, 503
 Herman, K., 180, 206
 Herman, L., 434, 437
 Herring, G. M., 48, 56, 57, 66, 69, 71, 139, 175
 Herz, A. H., 454, 472
 Hesselink, F. Th., 450, 472
 Hey, C. D., 154, 175
 Heyns, K., 95, 97, 106, 153, 175, 487, 503
 Higgs, D. G., 154, 174, 175
 Highberger, J. H., 7, 30, 148, 174
 Hikaku, N., 152, 175
 Hill, R. L., 41, 71
 Hilt, W. T., 64, 69
 Himmelman, W., 229, 244
 Hinterwaldner, R., 319 (35), 325 (44), 325 (45), 327 (46), 327 (47), 345 (57), 363
 Hipp, N. J., 276, 291
 Hirai, N., 268, 290
 Hirota, G., 281, 290
 Hirs, C. H. W., 209, 244

- Hisamura, H., 56, 69, 106
 Hitch, S. F., 489, 503
 Hithcock, D. I., 87, 92, 94, 106, 213, 244, 477, 503
 Hlavaček, K., 498, 502
 Hoare, D. G., 222, 244
 Hochstadt, H. R., 403, 412
 Hodge, A-J., 8, 9, 10, 13, 15, 20, 28, 29, 61, 68
 Hoerman, K. C., 77, 106
 Hoeve, C. A. J., 448, 449, 472
 Hoffman, P., 34, 35, 47, 62, 63, 70
 Holliday, E. R., 491, 502
 Holm, S., 345 (59), 363
 Homan, P. J., 232, 244
 Honnen, L., 97, 107
 Hoover, S. R., 38, 69
 Hopp, V., 280, 290, 532, 533
 Hörmann, H., 13, 21, 28, 82, 93, 95, 105, 106, 152, 169, 175
 Hörmann, V. H., 164, 175
 Hornsby, K. M., 223, 244
 Horvath, A. A., 239, 244
 Houck, J. C., 42, 69, 91, 101, 107
 Houck, R. C., 129, 136, 500, 505
 Housley, T., 19, 30
 Hulmes, D. J. S., 15, 29
 Hudson, J. H., 101, 107, 500, 505
 Hult, A-M., 41, 69
 Humphrey, J. H., 43, 44, 69, 222, 244
 Humphrey, J. H., 274, 288
 Huntoon, R. B., 238, 244
 Hutchin, M. E., 484, 505

I

- I.C.I. Ltd., 433, 437
 Idson, B., 180, 206, 257, 290, 368, 370, 378, 385, 390, 393
 Igarashi, S., 12, 30
 I.G. Farbenindustrie, 232, 244
 Ikeda, S., 62, 71
 Iler, R. K., 240, 244
 Ilford, Ltd., 232, 233, 244
 Illingsworth, B. D., 219, 242, 244
 Inokuchi, K., 240, 246
 Ingleton, J. F., 369, 371, 378, 393
 International Estate & Co., 299 (8), 313
 International Polaroid Corporation, 217, 235, 244
 Irving, J. T., 55, 72
 Isemura, T., 62, 71
 Istranov, L. P., 142, 154, 165, 173, 175
 Ivanova, A., 380, 393
 Ivanova-Chumakova, L. V., 264, 290
 Izmailova, V. N., 146, 175, 276, 292
 Izyumov, D. B., 264, 293

J

- Jackson, D. S., 72, 78, 106, 151, 152, 163, 173, 174, 175, 177
 Jackson, J. J., 65, 67
 Jacob, H., 401, 412
 Jacob, R. A., 42, 69
 Jacobs, H. G., 4, 29
 Jacobs, M. B., 481, 503
 Jacobs, S., 78, 106
 Jaffe, L., 481, 503
 Jahn, A. S., 141, 176, 283, 290
 Jakus, M. A., 30
 Jane, R. S., 264, 290
 Janus, J. W., 457, 459, 472, 489, 496, 500, 503, 504
 Japan Leather Co., 152, 154, 175
 Janus, J. W., 101, 106, 146, 150, 164, 175, 185, 188, 189, 192, 206, 222, 244, 275, 290, 324 (43), 363, 529, 533
 Jayko, M. E., 279, 290
 Jefferson, H., 71
 Jeffree, G. M., 59, 69
 Jeffreys, R. A., 232, 233, 244
 Jenkel, E., 449, 472
 Jermolenko, N., 287, 290
 Jirgensons, B., 122, 135
 Jobling, A., 271, 272
 Joel, E., 450, 474
 Johlin, J. M., 287, 290
 John, P., 173
 John, P. M. V., 39, 68
 Johns, P., 155, 167, 168, 175, 270, 290, 405, 412
 Johnson, M. S., 241, 241
 Johnson, P., 43, 44, 67, 202, 206
 Johnson, and Johnson Inc., 403, 412
 Joly, M., 187, 206, 278, 289, 290
 Jones, N. R., 381, 393

- Jones, R. T., 216, 217, 242, 494, 502
 Jopling, D. W., 224, 244, 254, 255, 256, 271, 290, 445, 446, 447, 472
- Jordon Lloyd, D. J., 442, 443, 444, 472
 Joseph, K. T., 95, 98, 106
 Josse, J., 121, 135, 204, 206
- K**
- Kahn, A., 449, 472
 Kahn, L. D., 42, 69
 Kainz, G., 490, 504
 Kaisha, K., 152, 175
 Kakiyama, H., 240, 246
 Kalafatas, N. J., 238, 244
 Kamei, S., 350 (63), 364
 Kamiya, I., 281, 283, 290
 Kaneko, M., 264, 268, 273, 291
 Kang, A. H., 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 27, 29, 30, 88, 98, 99, 106, 166, 173, 175
 Kao K.-Y., 64, 69
 Karacsony, D., 388, 393
 Kargin, V. A., 252, 262, 268, 284, 289, 294
 Karjala, S. A., 241, 244
 Karler, A., 241, 241, 244
 Kartha, G., 5, 30
 Kaspar'yants, S. A., 165, 173
 Katchalski, E., 293
 Katchalsky, A., 144, 177
 Katersky, E. M., 33, 34, 70
 Katti, P. K., 279, 290
 Katz, E. P., 53, 68, 145, 174
 Katz, J. R., 253, 290
 Kauffman, M. E., 259, 289
 Kawahara, K., 123, 136
 Kawai, S., 42, 69
 Kawamura, A., 165, 176
 Kawamura, F., 281, 290
 Kawanishi, Y., 53, 66
 Kay, L. M., 97, 107, 487, 503
 Kazanskii, V. M., 283, 291
 Keenan, A. L., 452, 472
 Kefalides, N. A., 3, 29, 64, 69
 Keil, H. L., 151, 175, 238, 244
 Keller, H. E., 467, 468, 472
 Kelly, W., 33, 72
 Kelly, W. D., 264, 293, 515, 533
 Kenchington, A. W., 87, 93, 94, 101, 105, 106, 213, 214, 221, 222, 223, 225, 226, 228, 244, 275, 277, 290, 291, 324 (43), 363, 480, 488, 489, 490, 494, 495, 496, 502, 503, 504, 516, 529, 533, 534
- Kennedy, J. F., 45, 46, 66
 Kent, P. W., 40, 48, 56, 57, 66, 69, 72, 82, 107, 139, 175
 Kenten, R. H., 20, 27, 84, 105
 Kenyon, W. O., 230, 244
 Keopff and Soehne, GmbH, 244
 Kershaw, F. G., 498, 505
 Keshishyan, G. O., 239, 244
 Keyes, G. H., 232, 246
 Khenokh, M. A., 273, 291, 294
 Khisimatullina, L. A., 219, 220, 245
 Killick, M. W., 232, 246
 Kimmel, J. R., 484, 505
 Kimura, S., 65, 69, 172, 175, 281, 291
 King, A. O., 394
 King, G., 281, 291
 King, J. A., 387, 388, 393
 King, R. W., 202, 206
 Kinkel, E., 264, 267, 291, 308 (22), 313, 515, 533
 Kirk, D., 149, 174
 Kishimoto, S., 268, 290
 Kitagorodskii, A. I., 91, 107
 Klein, E., 454, 455, 462, 472
 Klenk, M., 164, 175
 Klose, A. A., 381, 393
 Klevens, H. B., 403, 412
 Klimas, G., 401, 412
 Klotz, I. M., 215, 236, 237, 245
 Klyuchevitch, V., 454, 474
 Knott, E. B., 232, 245
 Kodak Ltd., 219, 221, 232, 233, 234, 245, 261, 291
 Koepff and Soehne, GmbH, 239
 Koepfli, J. B., 236, 242
 Kofoed, J. A., 46, 69
 Kohn, J., 436, 437
 Kolthoff, I. M., 449, 472
 Konigsberg, M., 495, 505
 Konno, A., 264, 268, 291
 Kaprowski, W. S., 518, 533
 Koral, J., 449, 472
 Korn, A. H., 38, 41, 69, 70
 Korneva, E. D., 230, 245
 Kosar, J., 426, 437

- Koshland, D. E., 222, 244
 Koslov, P. V., 262, 284, 287
 Kossiva, D., 55, 68
 Kotina, V. E., 262, 291
 Kotov, M. P., 262, 288
 Kovacic, P., 219, 245
 Kovalenko, L. V., 165, 173
 Kovalichev, F. F., 274
 Kowal, W., 299 (7), 313, 319 (31), 363
 Kozlov, P. V., 147, 175
 Kraemer, E. O., 275, 291
 Kragh, A. M., 105, 131, 135, 231, 245, 256, 287, 288, 291, 293, 425, 437, 447, 450, 452, 453, 454, 455, 458, 459, 472, 478, 502, 513, 522, 525, 526, 533
 Krajewski, J. J., 219, 220, 245
 Kramer, F., 368, 369, 371, 372, 373, 378, 379, 381, 393, 513, 533
 Krane, J. G., 480, 504
 Krane, S. M., 54, 55, 60, 61, 68, 69
 Kress, B. H., 231, 245
 Krischer-Kroll, 350 (64), 351 (64), 364
 Krol, S., 144, 174
 Krvml, J., 65, 72
 Krus, G. I., 264, 290
 Kruyt, H. R., 117, 135
 Kubota, A., 311 (38), 313
 Kubota, K., 65, 69
 Kubota, M., 65, 69, 172, 175, 281, 291
 Kubato, A., 311 (40), 314
 Kučera, 280
 Kudryavtsev, B. B., 291
 Kuhlman, R. A., 287, 292
 Kuhn, A., 378, 393
 Kühn, J., 22, 29
 Kühn, K., 8, 17, 22, 29, 30, 66, 82, 106, 135, 136, 147, 148, 152, 163, 164, 175, 177
 Kuhn, L. P., 437
 Kukhtin, V. A., 219, 220, 245
 Kumai, A., 239, 245
 Kunitz, M., 267, 292
 Küntzel, A., 317 (11), 362
 Küntzel, Von A., 160, 175
 Kunz, E., 412
 Kurata, M., 240, 246
 Kurtz, J., 213, 241
 Kyle, E. J., 427, 437

L

- La Bella, F. S., 17, 29
 Laird, R. M., 225, 245
 Lambert, R. H., 462, 473
 La Mer, V. K., 459, 472, 473
 Lampitt, L. H., 378, 393
 Landucci, J. M., 32, 72, 99, 100, 106, 107, 492, 505
 Lane, J. M., 11, 14, 16, 29, 175
 Lane, L. B., 423, 424, 437
 Langston, W. B., 131, 135, 240, 245, 425, 437, 450, 458, 459, 472, 477, 504, 513, 518, 525, 526, 533
 Lanza, P., 454, 472
 Lapanje, S., 123, 136
 Lapiere, C. M., 172, 173
 Lapin, P. M., 252, 291
 Lapinskaya, E. M., 273, 291
 Larsen, B., 168, 175
 Larsen, S., 60, 69
 Larson, C. E., 258, 291
 Lauder, W. E., 226, 228, 244
 Lavrova, L., 380, 393
 Laws, W. D., 283, 291
 Lawson, N. W., 62, 69
 Leach, A. A., 32, 59, 65, 69, 76, 78, 79, 80, 83, 84, 88, 89, 90, 92, 101, 102, 103, 104, 105, 106, 216, 217, 219, 226, 238, 242, 245, 408, 409, 411, 412, 412, 420, 436, 477, 484, 492, 493, 494, 495, 500, 501, 502, 504
 Leach, S. J., 485, 504
 Leaver, A. G., 53, 58, 59, 69
 Ledward, D. A., 185, 195, 198, 203, 206
 Lees, R., 385, 391, 393
 Le Gette, J., 97, 107
 Legler, G., 95, 97, 106, 153, 175, 487, 503
 Leheimann, H. L., 432, 437
 Leich, A., 264, 266, 291
 Lehr, J. R., 52, 67
 Leistner, L., 368, 393
 Lemmerling, J. T., 233, 245
 Lent, L. W., 18, 30
 Lentz, C. P., 286, 291
 Leoni, A., 462, 473
 Lepilkina, L. A., 263, 294
 Lerner, H. W., 232, 245

- Letsky, B. H., 429, 437
 Levene, C. I., 18, 29
 Leventhal, H., 52, 68
 Levi, S. M., 146, 176, 219, 220, 230, 245, 271, 282, 291, 292, 510, 533
 Levy, A. L., 487, 504
 Levy, M., 17, 29, 171, 174
 Levy, M., 495, 505
 Lewis, A. A., 520, 533
 Lewis, E. J., 254, 291
 Lewis, M. S., 12, 29, 65, 70, 142, 176, 482, 504
 Ley, F. J., 482, 502
 Libicky, A., 256, 291, 445
 Lichtenstein, I., 213, 245
 Lieberman, E., 403, 412
 Likins, R. C., 82, 106
 Limberg, H., 450, 473
 Lindgren, B., 345 (58), 363
 Linhart, J., 65, 72
 Linke, W. G., 459, 473
 Linker, A., 34, 35, 47, 62, 63, 70
 Lipatov, S. M., 252, 291
 Lippolis, M. T., 454, 472
 Lister, D., 19, 27
 Little, K., 148, 173
 Llory, J., 281, 291
 Loening, E. E., 467, 473
 Loeven, W. A., 62, 70, 164, 175, 316 (1), 361
 Loewi, G., 46, 70
 Logan, M. A., 33, 34, 70, 77, 106, 484, 504
 Long, J. E., 86, 105, 485, 503
 Lovett, W. G., 218, 243
 Lowe, B., 373, 393
 Lowe, W. G., 216, 245
 Lowry, J. R., 172, 175, 280, 289
 Lowry, O. H., 33, 34, 70, 479, 504
 Lowther, A. G., 487, 502
 Ludoweig, L., 71
 Lukin, M., 17, 28, 99, 105
 Lundberg, J. L., 448, 472
 Lurgi, M., 362
 Luyet, B. J., 257, 290, 292
 Luyet, L., 257, 292
 Lyaskovskaya, Y., 380, 393
 Lykow, M., 362

M

- MacAllister, R. V., 267, 293
 McBride, O. W., 66, 70
 McClain, 70
 McConaughty, B. L., 215, 237, 245
 McConnell, D., 52, 70
 McFarlane, W. D., 484, 504
 McGavack, T. H., 64, 69
 McGavin, S., 5, 9, 25, 27, 28, 29
 McGraw, J., 262, 292
 McGregor, J., 60, 70
 McKenna, E., 485, 502
 McKernan, W. M., 54, 70, 399, 412
 McClain, P. E., 143, 175
 McLaughlan, K. A., 7, 28
 McLaughlin, G. D., 35, 45, 70
 McLean, F. C., 51, 70
 MacMasters, M. M., 239, 242
 McMeekin, T. L., 276, 291
 MacRae, T. P., 7, 28
 McRoberts, L. H., 481, 505
 Macek, T. J., 232, 247
 Mach, M. H., 146, 174
 Macheboeuf, M., 266, 293
 Maclagan, N. F., 480, 505
 Mager, A., 236, 246
 Maklakov, A. G., 32, 72, 167, 169, 177
 Malinowski, J., 462, 473
 Mal'Tseva, I. I., 91, 107
 Manahan, J., 171, 175
 Mancewitz, S. A., 77, 106
 Mandelkern, L., 149, 173, 186, 206
 Mandl, I., 23, 29, 171, 175
 Mandlkern, L., 10, 27
 Manning, A. B., 156, 175
 Manning, M. J., 146, 174
 Marignan, R., 274, 288, 291
 Marini, M., 462, 472
 Markes, E. M., 215, 247
 Markham, R., 477, 504
 Marko, A. M., 41, 42, 69
 Maron, N., 102, 103, 104, 106, 497, 498, 501, 504
 Marquarot, H., 66
 Marrs, W. M., 270, 291
 Marsden, A. W., 497, 504
 Marshall, R. D., 485, 493, 504
 Martin, A. J. P., 485, 503

- Martin, C., 253, 261, 289
Martin, G. R., 12, 22, 29, 54, 60, 70,
144, 145, 166, 173, 175, 176
Martin, Y. C., 215, 237, 245
Marzlov, V. P., 147, 175
Maser, M. D., 66, 70
Mason, C. M., 91, 106
Masski, G., 282, 291
Masuzawa, M., 282, 291, 293
Mateles, R. I., 280, 291
Maternaghan, T., 457, 473
Matheson, N. A., 487, 504
Mathewson, H. D., 500, 503
Matschiner, H., 504
Matsuda, A., 145, 176
Matthews, M. B., 63, 64, 70, 71
Matukas, V. J., 12, 29
May and Baker Ltd., 232, 245
Mecca, C. E., 22, 29
Mecchi, E. P., 381, 393
Mechanic, G., 19, 30, 495, 505
Mechanic, G. L., 17, 29
Medley, J. A., 281, 291
Meerson, S. I., 284, 291
Meilman, E., 13, 17, 28, 98, 99, 105,
167, 168, 171, 174, 176
Melcher, A. H., 40, 70
Mellon, E. F., 38, 39, 40, 41, 48, 67, 69,
70
Melnick, S., 396, 412
Mergenhagen, S. E., 60, 70
Merory, J., 382, 393
Merrill, R. E., 220, 245, 433, 437
Merton, T., 434, 437
Merzlov, V. P., 276, 291, 292
Meyer, K., 34, 35, 46, 47, 62, 63, 70, 492,
504
Meyer, K. H., 180, 206
Mezzino, J. F., 280, 289
Michaels, A. S., 238, 245, 459, 473
Michel, G., 497, 498, 504
Michele, S. C., 168, 174
Mickevici, M., 390, 393
Mikhailov, I. G., 273, 291
Milch, R. A., 232, 245
Miller, A., 15, 29
Miller, E. J., 11, 12, 14, 16, 20, 29, 54,
63, 70, 144, 145, 174, 175
Miller, E. M., 143, 175
Miller, G. A., 377, 381, 394
Miller, L. F., 232, 242
Miller, M., 266, 267, 291
Miller, P. E., 215, 218, 243, 245
Mills, G., 53, 70
Mills, G. L., 487, 504
Mindru, I., 268, 291
Minkin, E. V., 154, 175, 176
Minsk, L. M., 226, 228, 232, 245
Mitchell, W. A., 376, 393
Mitsuda, H., 145, 176
Miyada, D. S., 484, 504
Miyata, T., 42, 69, 176, 311 (39), 314
Mobbs, D. R. A., 55, 70, 155, 176
Moeller, J., 419, 437
Moeller, W. M., 406, 412
Moisar, E., 454, 455, 462, 472
"Mogul", 374, 375, 393
Mohanaradharrishnan, V., 172, 176
Mohr, V., 65, 70
Moline, S. W., 257, 289
Moncrieff, R. W., 389, 393
Money, R. W., 378, 393
Monier, J. B., 215, 245
Monroe, D., 19, 30
Monsanto, Co., 382, 393
Moore, A. W., 260, 283, 293
Moore, S., 479, 482, 483, 484, 502, 504,
505
Moran, T., 257, 291, 393
Morel, J., 291
Morelos, O., 459, 473
Morgan, J., 228, 245
Morgan, P. H., 4, 29
Morgan, W. T. J., 492, 503
Morozov, V. M., 165, 173
Morris, L., 483, 504
Morton, R. A., 490, 491, 503
Moss, J. A., 37, 38, 44, 45, 67, 82, 83,
84, 93, 95, 101, 102, 104, 105
Mueller, F. W. H., 232, 245, 465, 466,
473
Muggenberg, H., 366, 392
Muir, H., 64, 67
Muir, H. M., 41, 42, 69
Muir, R. J., 394
Mullen, T. C., 227, 245
Mussel, S., 144, 177
Musselwhite, P. R., 389, 393
Myers, P. A., 86, 107

N

- Nagai, Y., 11, 13, 14, 28, 29
 Naghski, J., 39, 40, 70, 143, 176
 Nakanish, I. S., 53, 66
 Narahashi, Y., 311 (42), 314
 Narath, A., 466, 473
 Narita, K., 487, 502
 Nash, N. H., 389, 390, 394
 Nassau, M., 215, 246
 Natale, C. C., 451, 452, 472
 Natarajan, M., 47, 70
 National Association of Glue Manufacturers, 430, 433, 435, 437
 National Cash Register Co., 226, 245, 420, 421, 422, 437
 Navrátil, V., 100, 106
 Nawn, G., 33, 72
 Nawn, G. H., 464, 473
 Naylor, A., 35, 71
 Nayudamma, Y., 172, 176
 Neale, A. E. T., 434, 437
 Needles, H. L., 235, 245
 Neiman, R. E., 284, 291, 292
 Nellist, D. R., 500, 504
 Neuberger, A., 41, 42, 43, 44, 69, 163, 175, 504
- Neuman, R. E., 33, 34, 70, 83, 84, 87, 106, 484, 504
 Neuman, W. F., 55, 61, 68, 72
 Newesely, H., 51, 52, 70, 71
 Newly, E. J., 143, 144, 177
 Nichols, J., 151, 176, 403, 412
 Nicolaidis, N., 48, 71
 Nikl, J., 481, 504
 Nimni, M. E., 19, 29, 42, 71, 155, 174, 176
 Nishihara, T., 42, 69, 141, 152, 153, 174, 176, 311 (37), 314
 Nishizowa, M., 311 (40), 314
 Niven, C. D., 281, 292
 Nixon, J. R., 268, 274, 289, 292
 Nobel, P. C., 258, 292
 Noda, H., 3, 28
 Noguchi, J., 213, 241
 Nomoto, M., 311 (42), 314
 Nordin, B. E. C., 60, 70
 Norowig, A., 82, 97, 106, 141, 170, 174, 176
 Norris, T. O., 262, 292
 North, A. C. T., 5, 28
 Northrop, J. H., 267, 292
 Nozaki, Y., 123, 136
 Nutten, A. J., 496, 502

O

- Oakes, E. T., 129, 135, 186, 206
 Odin, L., 493, 505
 Oehr, O. E., 449, 473
 Ogle, J. D., 77, 106
 Ohashi, T., 172, 175, 281, 291
 Ohba, S., 239, 245
 Ohno, K., 487, 502
 Okamoto, Y., 282, 292
 Okamura, H., 151, 165, 176
 Olcott, H. S., 209, 223, 245, 246
 Oldroyd, D., 48, 57, 71
 Oliff, D. L., 33, 71, 100, 107, 464, 473, 493, 505
 Olsen, A. G., 188, 189, 206
 Olsen, B. R., 9, 22, 29
- Oncley, J. L., 269, 293
 Oneson, I. B., 151, 176
 Ono, I., 263, 293
 Ore, A., 282, 283, 292
 Orink, M. T., 232, 245
 Orkenyi, J., 282, 291
 Ornes, C. L., 41, 71
 Orekhovich, K. D., 41, 71
 Orekhovich, V. N., 41, 71
 Osteux, R., 487, 502
 Ostwald, W., 378, 394
 Ottewill, R. H., 454, 473
 Oury, S. J., 369, 394
 Overbeek, J. Th. G., 442, 443, 450, 473

P

- Palmer, J. W., 492, 504
 Palmer, M. M., 389, 394
 Pankhurst, K. G. A., 112, 136, 489, 503
 Paradissis, G. N., 416, 437
- Parkhill, E. M. J., 485, 504
 Paronik, S. I., 103, 107, 403, 412
 Paronik, S. L., 500
 Parrott, E. L., 437

- Parry, D. A. D., 15, 29
 Partridge, S. M., 20, 29, 41, 71, 101, 102, 106, 486, 504
 Pasynskii, A. G., 281, 292
 Paul, G., 17, 29
 Pauling, L., 5, 29, 236, 242
 Pavlovskaya, T. E., 281, 292
 Pchelin, V. A., 175, 262, 276, 287, 290, 291, 292
 Peach, C. M., 15, 19, 27, 161, 166, 173
 Peacock, R., 452, 453, 454, 455, 472
 Peacocke, A. R., 56, 57, 60, 71, 72
 Pearce, R. H., 46, 71
 Pearse, A. G. E., 59, 71
 Pearson, A. M., 143, 175
 Pearson, C. H., 35, 71
 Peckham, L., 169, 173
 Pedrini, 64, 71
 Peerson, 282, 283, 292
 Pektor, V., 100, 106
 Pentz, L., 388, 393
 Pereverzev, N. A., 165, 173
 Perkel, R., 449, 473
 Perkins, D. J., 43, 44, 69
 Perron, R. R., 281, 292
 Perry, A., 54, 72
 Perry, E. S., 462, 473
 Persidsky, M. D., 257, 292
 Persson, K., 33, 34, 67
 Perutz Photowerke, GmbH, 228, 245
 Petrie, S. E. B., 202, 206
 Petruska, J. A., 8, 9, 10, 13, 20, 28, 29
 Peyser, P., 448, 449, 472
 Phillips, H., 495, 502
 Pieper, G., 219, 245
 Pierce, J. A., 62, 69
 Piez, K. A., 2, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 27, 29, 30, 53, 54, 65, 66, 70, 71, 77, 80, 81, 82, 88, 98, 99, 106, 119, 136, 142, 144, 145, 147, 170, 173, 175, 176, 482, 483, 490, 504
 Pikkarainen, J., 65, 71
 Pilgrim, A. J., 499, 503
 Pillersdorf, A., 437
 Pinoir, R., 254, 292
 Piolat, G., 240, 245
 Plaster, F. H., 32, 67, 485, 502
 Pleass, W. B., 258, 290
 Plotinkova, N. E., 41, 71
 Pollak, F., 232, 245
 Polla-Mattiot, G., 462, 472, 473
 Pollard, A. G., 477, 499, 506
 Polson, A., 240, 246
 Poole, H. J., 264, 265, 266, 292
 Porter, R. R., 487, 504
 Posner, A. S., 52, 68, 72, 206
 Pospisilova, J., 168, 176
 Pottel, R., 282, 292
 Pouradier, J., 32, 33, 71, 72, 83, 99, 100, 106, 107, 112, 118, 119, 136, 150, 176, 192, 201, 206, 240, 246, 251, 252, 254, 267, 270, 285, 287, 288, 292, 441, 451, 452, 456, 464, 466, 473, 492, 498, 504, 505
 Powers, M. J., 54, 70, 145, 175
 Pratt, A. N., 216, 217, 238, 242, 420, 436, 494, 502, 519, 533
 Prerovsky, I., 65, 72
 Pristoupil, T. I., 481, 504
 Privalov, P. L., 141, 176, 283, 289
 Prockop, D. J., 484, 505
 Procter, H. R., 25, 29
 Proctor, H. R., 442, 473
 Przybylowicz, E. P., 500, 505
 Puchkova, N. N., 284, 291
 Puchtler, H., 40, 71
 Puett, D., 143, 176
 Purcell, A. W., 141, 176
 Putnam, F. W., 209, 246
 Putman, R. C., 228, 246

Q

Queen, G., 17, 29

R

- Racicot, P. A., 481, 503
 Radkevich, D., 159, 176
 Radkevich, D. P., 165, 173
 Raistrick, A. S., 40, 67
 Rajagh, L. V., 143, 176
 Ramachandran, G. N., 5, 6, 7, 29, 30, 66, 71
 Ramanathan, N., 172, 176

- Randall, J. T., 5, 7, 27, 28
 Randall, R. J., 479, 504
 Rankine, A. D., 266, 292
 Ranyl, C. A., 420, 437
 Ranz, E., 445, 467, 472
 Rapatz, G., 257, 292
 Rastelli, A., 274, 292
 Rastogi, M. C., 454, 473
 Rauterberg, J., 17, 30
 Rebinder, P. A., 262, 264, 290
 Reece, M. C., 391, 394
 Reed, R., 154, 174, 175
 Rees, M. W., 477, 482, 487, 502, 505
 Rehner, J. Jr., 258, 290
 Reich, G., 159, 160, 176
 Reicheneder, 229
 Reinder, W., 473
 Reiss, W., 42, 71, 151, 176
 Reitz, H. C., 223, 246
 Remenec, M., 497, 505
 Remington, R. E., 481, 505
 Rexrodt, F., 17, 30
 Reynolds, R. J. W., 220, 234, 246
 Rhi, M., 18, 30
 Ricca, F., 274, 289
 Rice, G. R., 48, 71
 Rice, R. V., 66, 70
 Rich, A., 5, 30
 Richards, L., 54, 68
 Richardson, E. G., 188, 206
 Riebel, A., 229, 244
 Riedel, A., 163, 164, 174, 175
 Riehl, N., 281, 292
 Riese, H. C., 24, 27
 Riese-Bello, H., 284, 288
 Rigby, B. J., 142, 143, 176
 Rinfret, A. P., 257, 289
 Riso, R. R., 217, 248
 Rizzo, A., 60, 70
 Robb-Smith, A. H. T., 40, 71
 Robert, L., 35, 45, 71
 Roberts, D. E., 10, 27, 149, 173
 Robertson, P. B., 20, 29
 Robins, S. P., 19, 30
 Robinson, C., 254, 275, 276, 292
 Robinson, I. D., 232, 233, 235, 246
 Roche, E., 445, 455, 462, 472
 Roddy, W. T., 41, 71
 Roden, L., 47, 67
 Roe, D. A., 38, 71
 Roe, R. J., 448, 473
 Rogers, C. J., 484, 505
 Rogers, H. J., 56, 71
 Rojkind, M., 18, 30, 169, 173
 Roman, J., 240, 246, 451, 452, 456, 473
 Romer, R., 455, 473
 Rondeau, A., 83, 106
 Rose, H. J., 232, 246
 Rosebrough, N. J., 479, 504
 Rosenthal, H., 370, 386, 394, 513, 533
 Rosik, J., 239, 247
 Rosowsky, A., 246
 Roth, P. H., 462, 472
 Rothwell, J., 389, 394
 Rougvie, M. A., 6, 30
 Rousselot, A., 89, 107, 257, 292
 Rousselot, S., 319 (27), 324 (41), 362, 363
 Rowles, S. L., 52, 71
 Royle, R. J., 239, 247
 Rozenberg, S. G., 147, 175
 Rudall, K. M., 38, 71
 Rudenko, S. V., 146, 176, 282, 292
 Rudin, A. D., 406, 408, 412
 Rumbach, B., 449, 472
 Russell, A. E., 44, 67, 147, 172, 173, 176
 Russell, F. J., 216, 247
 Russell, G., 33, 59, 71, 76, 100, 107, 139, 176, 464, 473, 493, 497, 505
 Russell, R. G. G., 61, 68
 Russell, T. J., 143, 176
 Russoff, I. J., 382, 394
 Rutsikij, N., 317 (14), 362
- S**
- Sácha, F., 280, 291
 Saha, A. K., 66, 71
 Saha, N. N., 66, 71
 Saiki, K., 282, 292
 Saini, G., 462, 472, 473
 Saito, S., 53, 66
 Sakai, T., 13, 30, 59, 62, 71
 Salvinien, J., 489, 505
 Samachson, J., 51, 71
 Samuelson, E. G., 345 (59), 363
 Sandell, E. B., 496, 505
 Sanger, F., 164, 176, 218, 246, 486, 487, 504, 505
 Sanders, H. L., 215, 246

- Sanzharovskii, T., 263, 292
Sasabe, H., 273, 294
Sasaki, T., 264, 294
Saver, E., 264, 267, 287, 291, 292, 313, 344 (51), 363, 364, 515, 533
Saunders, P. R., 112, 120, 131, 136, 150, 159, 176, 180, 184, 192, 197, 201, 207, 254, 264, 265, 267, 269, 270, 275, 292, 293, 294, 510, 515, 527, 533
Saunders, W. M., 119, 136
Sayce, L. A., 434, 437
Scaria, S. K., 64, 71
Scatchard, G., 269, 293
Schachnasarowa, M., 313
Scheraga, H. A., 118, 119, 120, 135
Scheremet, M., 305 (19), 313
Schiffmann, E., 166, 176
Schiller, S., 46, 71
Schleyer, M., 3, 21, 28, 30, 98, 107
Schlueter, R. J., 54, 71, 72
Schmidt-Thome, J., 236, 246
Schmitt, F. O., 7, 8, 15, 22, 28, 30, 61, 68, 148, 174
Schneider, A. L., 105
Schnell, J., 146, 177
Schoenauer, W., 218, 246
Schoene, H. H., 236, 246
Schofield, J. D., 152, 174
Schram, C. J., 404, 412
Schroeder, W. A., 97, 107
Schremp, F. W., 264, 266, 267, 291, 293
Schryver, S. B., 156, 175
Schubert, B., 164, 175
Schuster, S., 171, 172, 173, 176
Schvadchenko, L. P., 230, 245
Schwander, H. R., 219, 246
Schwarz, K., 492, 502
Schwarzkopf, M., 300 (10), 313
Schweikert, E., 239, 246
Science Journal, 434, 437
Scott, D. B., 60, 70
Scott, J. W., 264, 293
Scott-Dodd, A., 498, 505
Seddon, J. D., 220, 246
Segrest, J. P., 4, 29, 93, 100, 105
Seidel, W. C., 376, 393
Seifter, S., 13, 17, 28, 93, 98, 99, 100, 105, 167, 168, 171, 174, 176
Sela, M., 293
Selby, J. W., 193, 207, 367, 370, 371, 372, 373, 377, 378, 379, 380, 394
Sens, J., 117, 135
Serafini-Cessi, F., 484, 505
Sevcik, F., 282, 293
Seymour-Jones, F. L., 156, 176
Sezerat, A., 499, 502
Shentall, R. D., 35, 71
Sheppard, S. E., 91, 101, 107, 136, 188, 207, 264, 265, 267, 268, 287, 293, 381, 394, 462, 473, 500, 505, 509, 533
Shepperd, 129
Sheppy Glue & Chemical Works Ltd., 301 (11), 313
Shestakova, I. S., 154, 175, 176
Shimizu, M., 166, 177
Shimokomaki, M., 19, 30
Shreiner, S. A., 263, 293
Shuttleworth, C. A., 53, 58, 59, 69, 70, 144, 177
Shuttleworth, S. G., 239, 240, 246
Sidorowicz, A., 455, 473
Sieg, A. L., 232, 246
Siegrist, H., 218, 246
Siemroth, J., 499, 504
Silberberg, A., 448, 453, 458, 473
Silcox, H. E., 91, 106
Silvester, N. R., 81, 107
Simha, R., 447, 448, 449, 472, 473
Simms, E. M., 255, 293
Simms, W. M., 447, 473
Simon, S. L., 52, 66
Sinyakova, L. I., 219, 242
Sjöquist, J., 487, 503, 505
Skyudes, A., 419, 437
Slobodian, E., 495, 505
Slonimskii, G. L., 91, 107, 264, 288, 289, 293
Smejkal, P., 168, 177
Smellie, R. H., 459, 473
Smidsrod, O., 168, 175
Smirnov, O. K., 230, 245
Smith, D. A., 230, 244
Smith, G. R., 275, 276, 293
Smith, E., 425, 437
Smith, J. G., 41, 71
Smith, J. P., 52, 67
Smith, J. W., 9, 30

- Smith, R. C. M., 112, 136
 Smith, R. H., 53, 72
 Smyth, E. M., 492, 504
 Snellman, O., 40, 72
 Sobel, A. E., 61, 66, 72
 Società Generale per L'Endustria
 Mineraria E Chimica, 219, 246
 Sode, T., 42, 69
 Sokoloff, L., 70
 Solomons, C. C., 55, 61, 72
 Soltzberg, S., 495, 505
 Somers, P. J., 45, 46, 66
 Soper, A. K., 254, 291
 Souter, F., 480, 505
 Spackman, D. H., 483, 505
 Spadaro, J. A., 51, 67
 Spies, J. R., 486, 490, 505
 Spiro, R. G., 65, 68
 Spracklen, D. M., 466, 473
 Spühler, A., 110, 136
 Spurr, O. K., 25, 28
 Ssobolew, N., 489, 505
 Staab, H. A., 217, 246
 Staaf, O., 274, 293
 Stainsby, G., 76, 107, 112, 113, 115, 118,
 119, 120, 129, 132, 134, 135, 142, 154,
 155, 164, 174, 175, 177, 184, 186, 187,
 192, 194, 201, 203, 206, 207, 211, 242,
 270, 293, 525, 530, 533
 Stainsby, R. G., 136
 Stainsby, W. J., 382, 394
 Stalder, K., 484, 505
 Stanford, J. W., 66
 Stark, G. R., 219, 246
 Stark, M., 17, 30
 Starkey, A. J., 228, 245
 Stather, F., 159, 160, 176
 Staud, C. J., 232, 246
 Steber, A., 28
 Steer, D. C., 142, 177
 Štefan, V., 499, 500, 505
 Stefanucci, A., 520, 532
 Stegemann, H., 484, 505
 Steigmann, A., 33, 72, 100, 107, 268,
 293, 473, 492, 493, 497, 505
 Steigmann, A. E., 462, 464, 472
 Stein, W. H., 479, 483, 484, 504, 505
 Steinberg, I. Z., 293
 Steiner, R. F., 122, 136
 Sterling, C., 282, 291, 293
 Serman, M. D., 242
 Steshov, G. I., 154, 165, 173, 176, 177
 Steven, F. S., 64, 72, 140, 151, 152, 153,
 174, 177, 211, 246
 Stewart, J., 387, 392
 Stistrup, K., 389, 394
 Stockmayer, W. H., 200, 207
 Stonham, J. P., 463, 473
 Strafford, N., 498, 505
 Strates, B., 61, 72
 Straumann, F., 61, 68
 Strelba, 280, 291
 Strickland, R. D., 240, 246
 Stromberg, R. R. 447, 449, 457, 473
 Subba Rao, K., 255, 293
 Subbarow, Y., 499, 503
 Susich, G., 388, 394
 Sussman, M. V., 144, 177
 Süszer, A., 145, 173, 314 (10), 360
 Sutton, D. A., 216, 217, 231, 232, 237,
 238, 242, 246, 420, 436, 494, 502
 Svejcar, J., 65, 72
 Swartling, P., 345 (58), 363
 Sweet, S. S., 188, 207, 264, 265, 267,
 268, 287, 293, 381, 394, 509, 533
 Synge, R. L. M., 482, 485, 495, 503, 505
 Szczesniak, A. J., 267, 293
 Szot, Z., 60, 72

T

- Tabor, B. E., 120, 136, 146, 150, 164,
 175, 188, 189, 192, 206, 231, 233, 242,
 244
 Tachibana, T., 240, 246
 Talwar, G. P., 266, 293
 Tamura, M., 240, 246
 Tancous, J. J., 45, 72
 Tanford, C., 123, 136
 Tanzer, M. L., 12, 19, 30
 Tappel, A. L., 482, 502
 Tarkow, H., 263, 289, 294
 Tarutina, L. I., 273, 291
 Tavkhelidze, N. N., 289
 Taylor, A. M., 367, 368, 370, 371, 378,
 382, 385, 386, 394
 Taylor, B. E., 175

- Taylor, D. J., 256, 293, 445, 473
 Taylor, D. M., 57, 67
 Taylor, E. W., 230, 244
 Taylor, J. T., 530, 533
 Taylor, T. G., 52, 72
 Tendeloo, H. J. C., 497, 505
 Termine, J. D., 52, 72
 Theis, E. R., 35, 45, 70
 Thies, C., 239, 247
 Thomé, K. E., 345 (59) 363
 Tice, L. F., 260, 283, 293, 371, 394, 417, 437
 Tiemstra, P. J., 370, 372, 385, 386, 394
 Tiilikka, A., 466, 473
 Tiktopulo, E. I., 141, 176
 Timple, R. W., 45, 72
 Timson, W., 33, 72
 Timson, W. J., 515, 533
 Tinacci, F., 64, 72
 Tkocz, C., 147, 148, 175, 177
 Tobias, J., 388, 394
 Tobolsky, A. V., 225, 247, 263, 284, 285, 293, 294
 Todo, A., 123, 136, 145, 150, 177, 197, 207, 270, 293
 Tolstoguzov, V. B., 91, 107, 264, 288, 289
 Tomanová, V., 481, 504
 Tombs, M. P., 480, 505
 Tomka, I., 110, 136, 441, 473
 Tomoda, Y., 280, 293
 Tomono, M., 273, 294
 Tourtellotte, C. D., 50, 63, 64, 67
 Tourtellotte, D., 215, 247, 419, 437
 Traub, W., 14, 30
 Trautz, O. R., 52, 66
 Travis, D. F., 53, 68
 Trelstad, R. L., 12, 30
 Triffitt, J. T., 53, 58, 70
 Trigny, L., 270, 292
 Tristram, G. R., 53, 72, 85, 107, 140, 142, 177, 211, 246
 Troll, W., 484, 505
 Trott, G. F., 239, 247
 Tschekalin, M. A., 227, 247
 Tschiegg, C. E., 273, 293
 Tsuda, M., 280, 293
 Tsuk, A. G., 237, 241
 Tsuzuki, T., 405, 412
 Tu, S., 151, 177
 Tunson, W. J., 264, 293
 Turner, R. L., 35, 71
 Tustanovskii, A. A., 41, 71
 Tutt, R., 423, 424, 437
 Twiss, D. F., 434, 437
 Tyazhelova, T. P., 252, 293

U

- Udenfriend, S., 484, 505
 Ueno, W., 263, 266, 293
 Ullman, R., 449, 472, 473
 Ullman, R. J., 472
 Umberger, J. Q., 258, 259, 260, 293
 Umbreit, W. W., 492, 502
 Undzenas, A., 284, 289
 Undzenas, A. I., 147, 175
 Union Carbide Corp., 261, 293
 United Shoe Machinery Corp., 224, 247
 Upjohn Company, 239, 247
 Urist, M. R., 60, 72
 U.S.F.D.A., 385
 U.S.M. Corporation, 405, 412
 Utsy, 215

V

- Vail, M. S., 20, 29
 Valet, G., 141, 175
 Van Campen, J. H., 234, 242, 247
 Van Der Korst, J. K., 70
 Van Driel, L. M. J., 40, 67
 Van Slyke, D. D., 490, 505
 Vasalko, J., 239, 247
 V.E.B.-Filmfabrik Agfa Wolfen, 229, 230, 232, 234, 247
 V.E.B. Fotochemische Werke Berlin, 230, 247
 Veis, A., 4, 12, 21, 23, 24, 30, 39, 42, 54, 71, 72, 110, 112, 118, 119, 120, 121, 127, 128, 136, 138, 144, 146, 149, 156, 157, 169, 173, 177, 180, 183, 197, 203, 205, 207, 238, 247, 259, 275, 276, 293, 310 (26), 313, 344 (52), 363, 408, 412, 420, 437, 528, 533

- Veitinger, J., 364
 Venet, A. M., 32, 72, 100, 107, 112, 118, 119, 136, 150, 176, 192, 206, 240, 246, 464, 473, 492, 505
 Venkateswara, R. N., 80, 106
 Venner, H. A. L., 230, 247
 Verzar, F., 19, 30
 Vetterl, V., 282, 293
 Vigneron, L., 274, 294
 Vilenskii, Yu. B., 230, 245
 Vinograd, J. R., 24, 27, 185, 191, 192, 206, 379, 392, 495, 502
 Viola, S. J., 38, 39, 40, 69, 70
 Virnik, D., 317 (112), 362
 Visser, S. A., 239, 247
 Vlidick, 143, 144, 177
 Volker, H. H., 390, 394
 Von Arnim, K., 484, 503
 Von Hippel, P. H., 2, 11, 23, 24, 25, 26, 28, 30, 126, 127, 136, 141, 142, 146, 147, 188, 201, 209
 Voss, K., 491, 503
 Vranken, M. N., 233, 245
- W
- Wada, Y., 273, 294
 Wainwright, F. W., 513, 518, 519, 532, 533, 534
 Waley, S. G., 488, 505
 Walker, D. A., 389, 394
 Walther, D., 497, 499, 505
 Walther, S., 159, 160, 176
 Walther, W., 228, 230, 239, 241
 Wang, H., 397, 398, 380, 412
 Warburton, C. D., 500, 505
 Ward, A. G., 74, 87, 93, 94, 101, 105, 106, 112, 118, 119, 120, 131, 136, 150, 154, 155, 159, 161, 162, 164, 174, 176, 177, 180, 184, 192, 197, 201, 207, 222, 240, 251, 264, 265, 267, 269, 270, 275, 286, 289, 290, 292, 293, 294, 320 (36), 324 (43), 344 (50), 363, 366, 370, 394, 488, 489, 490, 496, 502, 503, 504
 Warren, C. A., 86, 107
 Warren, L., 493, 505
 Wasley, W. L., 209, 225, 247
 Watase, M., 266, 268, 294
 Watson, E. M., 46, 71
 Watson, H. E., 480, 504
 Watson, J., 488, 505
 Watson, M. R., 81, 107
 Waykole, P., 17, 29, 42, 72
 Weatherall, J. A., 61, 72
 Weatherwax, R. C., 263, 289, 294
 Weaver, E. S., 126, 135, 140, 174
 Webb, E. C., 526, 533
 Webers, V. J., 283, 294
 Webster, E. R., 230, 241
 Wegerle, D., 77, 107, 486, 506
 Wehrli, W., 218, 241
 Weiderhorn, N. M., 25, 30
 Weidmann, S. M., 61, 72
 Weir, C. E., 24, 30
 Weisier, M., 45, 72
 Weiss, E., 142, 176, 482, 504
 Weiss, M., 489, 505
 Wendel, K., 217, 246
 Werner, I., 493, 505
 Wesley, J. M., 82, 69, 106, 140, 174
 Whiteside, 220, 247
 Whitfield, R. E., 209, 225, 247
 Wiegand, J. H., 271, 294
 Wildenauer, A., 401, 412
 Wilkins, D. J., 86, 107
 Wilkinson, D. I., 48, 72
 Wilkinson, G. R., 7, 27
 Willan, A. L. D., 86, 105, 477, 485, 503, 504
 Williams, A. E., 382, 383, 387, 390, 394
 Williams, A. P., 32, 57, 60, 71, 72, 81, 89, 90, 101, 103, 107, 158, 177, 476, 500, 503, 505
 Williams, C. T., 372, 383, 387, 391, 394
 Williams, E. F., 477, 502
 Williams, H. E., 215, 247, 419, 437
 Williams, J. W., 119, 136, 269, 293
 Williams, P. A., 56, 72
 Williamson, B. S., 241, 244
 Willis, A. J., 492, 506
 Wilson, (1968), 233
 Wilson, B. D., 228, 233, 234, 235, 242, 247
 Wilson, J. A., 25, 29, 239, 247
 Wilson, W. H., 442, 473
 Winand, L., 52, 72
 Windrum, G. M., 40, 72, 82, 107
 Wingard, W. H., 376, 394

- Winkelmann, R. K., 176
Winther, C., 253, 294
Wirnik, A. D., 227, 247
Wisnewski, A., 143, 176
Witnauer, L. P., 42, 69, 141, 143, 176, 283, 290
Woessner, J. F., 64, 72
Wolfrom, M. L., 495, 505
Wolff, I., 72, 72
Wolff, R., 499, 504
Wong, K., 141, 142, 177
Wong, K.-Y., 11, 24, 30, 127, 136, 199, 207
Wood, G. C., 10, 30, 141, 177
Wood, H. W., 100, 107, 217, 247, 492, 499, 500, 501, 505, 506
- Wood, P. D., 133, 136, 270, 291
Woodall, D. L., 20, 29
Woodlock, A. F., 141, 177
Woodside, E. E., 239, 243, 247
Woodward, H. Q., 281, 294
Wootton, J., 217, 238, 240, 242, 245, 420, 436, 514, 516, 518, 519, 533, 534
Wootton, J. W., 118, 119, 136
Worrall, J., 142, 151, 177
Wright, B. A., 281, 292
Wright, D. N., 60, 70
Wuelfing, A., 282, 292
Wundrich, K., 280, 290
Wuthier, R. E., 55, 72
Wyatt, P. F., 498, 505
- Yannas, I. V., 225, 247, 263, 268, 294
Yannas, J. B., 284, 285, 294
Yano, T., 462, 463, 474
Yasumoto, K., 145, 176
Yasunaga, T., 264, 294
Yates, J. R., 42, 72, 152, 177
Yemm, E. W., 492, 506
- Yonezawa, T., 239, 245
Yoshida, N., 239, 247
Young, H. H., 219, 231, 247
Yuen, S. H., 477, 499, 506
Yuill, M. E., 222, 244
Yuttsy, H. C., 216, 217, 247
- Zabik, M. E., 394
Zahn, H., 77, 107, 486, 506
Zaides, A. L., 284, 294
Zambito, R. H., 232, 247
Zambotti, V., 64, 72
Zanker, 229
Zappert, R. H., 232, 247
Zeichner, M., 18, 30
Zelmenis, G., 56, 68
Zhukov, I. I., 273, 287, 294
Zhurkina, Z. N., 268, 294
Zimkin, E., 454, 474
- Zimkin, E. A., 32, 72, 103, 107, 167, 169, 177, 500, 506
Zimmer, E., 8, 29, 163, 164, 177
Zimmermann, B. K., 127, 135, 136, 147, 148, 175
Zipkin, I., 52, 68
Zitco, 239
Zitko, V., 247
Znamenskaya, T. V., 215, 241
Zsigmondy, R., 450, 462, 474
Zubov, P. I., 252, 263, 268, 293, 294
Zukerman, H., 52, 68
Zwicky, H., 232, 247

Subject Index

- A
- Abrasive coating, 429, 430
Accelerated methods, 308, 309, 316
Acetyl gelatin, 185, 214, 218, 223, 454, 495
Acid anhydrides, 225, 226
Acid halides, 225, 226
Acid hydrolysis, 89, 217
Activation energy, 282
Adsorption of gelatin, 425, 426, 447–460
Adsorption isotherms, 448, 458
 layers, 449, 450, 457, 458
 reversibility, 448, 452, 453, 455
Agar, 252, 282, 368, 373, 376, 377, 379, 381
Aggregates, 114, 123–125, 127, 129, 134, 135, 183, 188, 189, 192, 198, 202, 252, 277, 279
Air content, 375
Aldehyde groups, 99, 501
Alginate, 373, 376, 389, 401, 403, 408
Alkoxy-methylamines, 234
Alum, 422, 481
Amide groups, 83, 90, 159, 211, 308
 nitrogen, 86, 154, 163, 485
Amino acid contents, 33, 35, 39, 45, 59, 62, 63, 65, 77–83, 92, 94, 103, 121, 211, 409, 410, 454, 479–486
 sequences, 16, 109, 121
Amino groups, 213, 219, 220, 223, 454
Ammonia formation, 163
Animal feed, 325
A.O.A.C. test, 520
Aroyl substitution, 214, 217, 218, 220, 238
Arsenic content, 498
Artificial casings, 401–405
Aryl substitution, 214, 218
Ash content, 477
Audiofrequency method, 273
Aziridines, 229
- B
- Bacterial culture media, 436
 effects, 347, 366, 525–527
Baryter coating, 470
Beer finings, 65, 155, 374, 391, 405–412
Benzoyl gelatin, 216
Binders, 433, 439
Biodegradable detergents, 215
Biuret test, 240
Birefringence, 277–279
Bleaching, 324
Bloom strength, *see* Gel strength
Bone autoclaving, 158, 165
 citrate, 52, 53, 58
 chick, 53, 145, 170
 composition, 48, 59, 82, 297, 298
 crushing, 48, 151, 298–301
 degreasing, 299–304
 demineralization, 139, 140, 304, 305
 extraction, 317, 321, 322
 fresh, 296, 297
 Indian, 296
 mineral, 50–53, 55, 59, 60, 61, 74, 304
 solubilization, 53, 54, 59, 154, 167
 steaming, 158, 300
Book binding, 432
Borohydride reduction, 19
Breaking load, 270
Brightener capsules, 422
Bromelin, 373
- C
- Caecum, 397, 398
Calcium analysis, 497
Capsules, 414, 415, 416
Carbamoylated gelatin, 218, 219, 494, 495
Carbo-diimide reaction, 222, 234, 236
Carbonless paper, 419
Carboxyl-azide reaction, 222, 223
Cartilage, 63, 64
Casein, 215, 423, 436, 481

- Casings, *see* Sausage casings
- Cattle hide, age, 159, 296
- alkali, 32, 45, 139, 161, 165, 405
 - cooking, 156, 157
 - corium, 37, 39, 40
 - dried, 139
 - "impurities", 37, 39–41, 43–48, 84, 162
 - mechanical breakage, 151, 401, 405
 - pumping, 306
 - radiation, 172
 - solubilization, 23, 45, 167, 168
 - swelling, 139, 164, 310
 - tannery, 139, 161, 295, 306
 - unhairing, 37, 138, 139, 161, 306
- Cattle sinew, 160, 296, 297
- Cellulose, 40
- Chain, α -, 88, 98, 109, 120, 125, 127, 147, 154, 156, 157, 163, 176, 184
- β -, 109, 127, 147, 153, 157, 184
 - weight, 98, 119, 164, 211, 270, 321
- Charcoal adsorption, 501
- Chayen process, 301, 302, 303
- Chelation, 152
- Chemical modification, 55, 209, 210, 212, 376, 420, 432, 454, 463, 493, 496
- Chillers, Band, 352
- Drum, 353
 - Votator, 353
- Chilling, 193, 204, 352–354
- Chloride ions, 499
- solvents, 258, 277
- Chromium analysis, 498
- Clarity, 368, 376
- Coacervates, 111, 112, 216, 217, 218, 220, 239, 414, 420, 463
- Coatings, 380, 381, 420, 423, 424, 431, 467
- Collagen, amino acids, 2, 3, 84
- avian, 3, 83
 - cornea, 65
 - doughs, 402, 403, 405, 407
 - fibres, 152, 401, 402
 - films, 402, 403, 404
 - fish, 3, 65, 77, 396, 407
 - fold, 125, 205, 270, 408
 - gels, 60, 149, 166
 - ground, 319
 - helix, bonds, 141, 144
- Collagen helix, denaturation, 24, 128, 146, 153, 197
- formation, 24, 147, 239
 - stability, 26, 53, 66, 74, 125, 126, 164, 198, 204
- Collagen, invertebrate, 66, 81, 82
- isoionic pH, 156, 160, 163
 - mammalian, 3, 34, 39, 77
 - molecular weight, 157, 168, 169
 - pretreatment, 316, 317, 321
 - shrink temperature, 23, 24, 156, 168, 316, 403
 - solutions, 12, 259
 - see also* "Soluble Collagen"
 - swimbladder, 18, 167
- Collagenase, 18, 45, 48, 54, 171
- Colloid Mill, 318, 401
- Colour, 218, 368
- Confectionery, 371, 374, 383–387, 391
- Co-polymers, 219, 220, 261
- Copper ions, 199, 367, 436, 497
- Cork compositions, 435
- Cosmetics, 241, 414, 424
- "Cotton Effect", 199
- Creep, 263, 264, 266
- Crosslinking, 121, 127, 128, 200, 210, 223–241, 254, 255, 263, 267, 270, 271, 276, 284, 285, 308, 381, 414, 419
- auto, 238
- Crosslinks, Collagen, aldehyde, 18, 19, 22, 166
- breaking, 75, 155, 156, 164, 169, 308, 320
 - chemical, 143, 404
 - determination, 25
 - location, 17
 - phosphate, 400
 - polymerized, 145, 151, 152, 157, 171
- Crosslinks, Gels, aldehyde, 420, 469, 493
- chromium, 240, 427, 428, 431
 - drying, 224, 469
 - mechanism, 225
 - melting, 224
 - swelling, 445
 - viscosity, 223
- Cyanogen bromide, 14, 20, 22, 23, 170
- Cyclic substitution reagents, 221
- D
- Dairy products, 481
- Davis roller, 429
- Deamination, 213
- Degradation, 418, 419

- Degreasing, 299–304, 318
 Deguanidation, 213
 Density, 286
 Dentine, 54, 55, 61, 82, 83, 155
 Desserts, 387
 Dessert jellies, see Table jellies
 Detergents, 112, 278, 286, 463
 Deuterium oxide, 281
 “Devro” casings, 402, 403, 404
 Diacyl-gelatins, 215
 Dicalcium phosphate, 305
 Dichromate gelatin, 426, 427, 428, 434
 Dielectric constant, 282
 Differential scanning calorimetry, 202
 Differential thermal analysis, 143, 146, 283, 285
 Diffraction grating, 434
 Diffusion, 273, 274
 Di-isocyanates, 230
 Dioxalanes, 233
 Disinfectants, 241, 525, 526
 Dispersing agents, 257
 Disulphide bonds, 123, 155, 236
 Donnan equilibrium, 25, 308, 441
 Double bond reaction, 219, 228
 Dressings, 416
 Dryers, 348–358
 band, 356
 batch, 354
 fluidized bed, 345, 356–358
 Drying collagen, 404
 Drying gels, 213, 225, 319, 345, 347–358
 Drying temperatures, 445, 447
 Dummy Bloom, 508, 510, 512, 517, 518
 Dusting powder, 416
 Dyes, 274, 281, 415, 427, 451, 460
- E
- Edible gelatin, 324, 344, 531
 E.D.T.A., 48, 53, 58, 59, 139, 152
 Effluent, 360
 Elasticity, 261, 264, 270, 415, 428, 429, 511
 Elastin, 20, 33, 34, 140, 154, 397
 Elastoidin, 65
 Electrical conductivity, 281
 Electric double layer, 443, 444, 460
 Electron microscopy, 3, 7–10, 91, 127, 148, 458
 Electrolyte effects, 114–117, 131, 134, 185, 192, 460
- Electrophoretic mobility, 458, 459
 Electrostatic charge interaction, 442, 443, 452, 459
 Emulsion coatings, 223, 224, 287, 374
 Emulsions, 390, 414, 420, 421, 424, 425
 End group analysis, 14, 15, 21, 55, 89, 93, 95–98, 119, 162, 163, 171, 202, 211, 217, 270, 321, 486–488
 Enzyme digestion, 15, 21, 75, 126, 146, 152, 164, 165, 166, 171, 311, 373, 434, 454, 523, 525–527
 Epidermis, 36, 37, 38
 Epoxy gelatin, 220, 230
 Equilibrium gel, 182
 sedimentation, 119
 Ester bonds, 98, 164, 167, 168, 169, 225
 Esterification, 496
 Eucollagen, 21, 55, 153, 162, 405, 407
 Evaporation, 252, 283, 316, 332–343, 376
 Evaporators, Centritherm, 341
 Plate, 338
 Thin film, 339
 Tubular, 335
 Extraction, 142, 157, 184, 307–312, 316, 319, 320, 325
 continuous, 317, 325
 Extractors, 323
 Extrusion, 401, 402, 404, 405
- F
- Fats, 38, 48, 49, 50, 64, 74, 90, 103, 104, 113, 297, 299, 300, 304, 308, 319, 396, 501
 Fermented drinks, 405
 Fibre drawing, 262
 Fibrils, 42, 148, 149, 150, 163
 SLS, 4, 157
 Film, 253, 254, 261, 262, 275, 281, 283, 284, 288, 414, 426, 430, 431, 440, 445, 447, 469, 471
 Filter aid, 425
 Filters, 406, 409
 Filtration, 163, 275, 326–332, 522
 Fire extinguisher capsules, 421
 Fleshings, 295, 309
 Flocculant, 406, 407, 408, 414, 425, 426, 459, 460
 Foaming power, 370
 Fogging, 466
 Food acids, 378, 402, 406
 regulations, 344, 385, 390

- Formol titration, 494
 Fractionation, 419
 Fractions, 88, 112, 113, 117, 118, 120, 157, 185, 189, 193, 197, 202, 269, 270
 Frappé, 387
 Freeze drying, 348, 401
 Fruit, 382, 387
 Functional groups, 20
 Functions of gelatin, 374, 388, 422
 Furniture, 431
- G**
- Gaskets, 435
 Gelatin types, "A" and "B", 307, 368, 369, 528
 carp, 80, 127
 chicken, 79
 cod, 80, 121
 crocodile, 79
 invertebrate, 81
 lungfish, 80
 ox bone, 78, 120, 124, 191, 422
 ox hide, 78, 84, 92, 122, 184, 191
 pigskin, 78, 131, 135, 191
 python, 79
 rabbit, 78
 shark, 80, 281
 toad, 79
 whale, 78
 Gelation, 123, 150, 159, 181, 203-205, 278, 281, 282, 463, 510, 532
 timer, 532
 Gel depressants, *see* Hydrogen bond breakers
 Gelometers, Bloom, 510, 511, 516, 520
 Boucher Electronic, 516, 517, 521
 Boucher Original, 517, 518, 521
 Japanese, 518, 519
 Gel strength, 184, 185, 192, 194, 196, 199, 264, 280, 312, 369, 508-520
 Glassy state, 263, 268, 285
 Glazes, 380, 384
 Glue, 90, 102, 103, 133, 158, 212, 271, 287, 297, 318, 414, 430-433
 γ -Glutamyl links, 98
 Glycerol (& Sorbitol), 261, 263, 268, 274, 282, 285, 380, 381, 416, 417, 428, 429, 432, 501
 G.M.I.A. pH test, 527
 G.M.I.A. viscosity test, 524
- Gold number, 450, 480
 Golf balls, 434
 Grain layer, 403
 Grinding, 358, 359
 Groundnut protein, 401
 Guanindylation, 185, 221, 494, 495
 Gum Arabic, 111, 278, 373, 377, 383, 391, 420
 Gummed paper tape, 431, 470
 Gums, 239, 373, 377, 383, 391, 424
- H**
- Haemacol, 419
 Haemostatic sponge, 417
 Halogeno substitution, 218, 227, 228
 Hardeners, *see* Crosslinking
 Haze in beer, 411, 412
 Heat of adsorption, 456
 Helix/random coil, 285
 Hexosamines, 4, 492
 Hexoses, 4, 17
 Holographic plate, 434
 Hofmeister series, 444
 Hot and cold dried films, 446
 Hot melt adhesives, 220, 432
 Hydration, 408, 444
 Hydrazine, 168, 169
 Hydrogen bond, 183, 187, 320, 446, 447, 458, 469
 breakers, 111, 114, 122, 144, 145, 146, 181, 186, 199, 268, 271, 276, 321, 432, 444
 Hydrogen/deuterium exchange, 146
 peroxide, 167, 168, 236, 419
 Hydrolysis methods, 21, 33, 479, 481, 482, 485, 486, 487, 527
 Hydroxylamine, 167, 168, 169
 Hydroxyproline method, 478, 483-485
 Hygiene, 361, 374
- I**
- Ice cream, 373, 379, 388-390
 penetration, 256
 Icings, 390
 Imino acids, 121, 122, 126, 199, 202, 205, 206, 409
 Indentation tester, 256
 Infra-red dichroism, 262
 Intestinal submucosa, 64
 Intestines, 395-401
 Invertebrate collagen, 66

- Ion exchange, 46, 101, 104, 319, 483, 501, 529
Iron determination, 497
Isinglass, 396, 405-412
Iso-alloxazines, 404
Isoelectric gels, 205, 254
Isoionic point, 83, 86, 114, 115, 116, 118, 129, 158, 163, 189, 211, 219, 273, 274, 275, 287, 308, 310, 312, 370, 371, 408, 419, 443, 528, 529
Isoxazolium salts, 234
- J
- Japanese Industrial Standard, 518, 519
Photographic, 519
Jellied products, 193, 367, 371, 377, 379, 380
Jellies, non-sugar, 377-381
sugar, 381-384
- K
- Keratin, 38, 77, 281
Keto cross-linking, 233
Keto-imide link, 210
Kidney membrane, 64
Kiln dryer, 351
- L
- Latent image, 467, 468, 471
Latex emulsion, 424
Lathrogens, 155
Lauryl gelatin, 215
Lead determination, 498
Leather, 35, 155, 224, 240
Light scattering, 118, 119, 120, 123, 124, 135, 141, 190, 279
Lipids, *see* Fats
Liquorice, 384, 391
Load/extension, 261, 262, 270
Lowry method, 479
Lozenges, 391
Lymphocytes, 417
Lyotropic salts, *see* Hydrogen bond breakers
- M
- Machine coating, 424
Maleyl gelatin, 215
Marshmallow, 370, 381, 384, 388
Match heads, 433
Maturing time, 530
Meat products, 374, 376, 377, 379, 380, 401, 404, 405
Mechanochemical engine, 144
Melting point, 24, 146, 180, 185, 189-193, 198, 200, 201, 204, 280, 282, 284, 312, 366, 371, 378, 386, 428, 529-531
Mesenteries, 396
Metals, 366, 367, 496-498
ion crosslinking, 240
refining, 414, 436
Metaphosphate reaction, 238, 239
Methane sulphonate reaction, 216, 234
Methylation, 222
Microencapsulation, 112, 217, 238, 419, 422
Microwave, 282
Middle casings, 396, 397
Milk products, 481
Milling, 358, 359
Mineral separation, 425
Moisture content, 86, 90-92, 401, 476, 512, 513
resistance, 424
Molecular diffusion, 421
sieves, 240, 274
size and shape, 10, 11, 119-121, 191, 406
weight, 75, 113, 117, 118, 119, 123, 164, 184, 192, 198, 211, 240, 251, 269, 311, 452, 469
Mucoproteinase, 62
Mucosa, 398, 399, 400
Multilayers, 458
Muscle, 398, 399, 400
- N
- "Naturin" casings, 402
Network structure, 182, 183, 185, 186, 190, 194, 196, 199, 203, 205
Ninhydrin method, 478, 484, 494, 495
Nitrogen content, 58, 76, 89, 101, 103, 396, 397, 477, 478, 482
Non-collagen components
carbohydrate, 17, 32, 35, 40, 45-48, 55-58, 61-64, 75, 98-102, 104, 113, 308, 482, 485, 492, 493
elastin, 33, 34, 41, 49, 61, 65, 140
"impurity", 84, 90, 319, 367, 376, 425, 440, 485, 486, 500, 501

- Non-collagen components (*cont.*)
 inorganic, 100, 101, 367, 396, 480, 496-500, 525, 530
 mucoprotein, 500, 501
 nucleic acids, 33, 40, 47, 75, 139, 140, 464, 466, 471, 493
 removal, 87, 138
 serum proteins, 43, 44, 49, 58, 75, 101, 113
 Non-covalent cross-linking, 238
 Nougat, 387
 Nucleation, 126
- O
- Optical birefringence, 266
 rotation, 121-123, 127, 145, 150, 196-199, 270, 275, 276
 Osmotic pressure, 119, 418, 442
 Ossein, *see* Bone sections
 Ostwald ripening, 455, 457
 Over-cut, finings, 406
 Over run 385, 388
 Oxidation, 235
 Oxy-poly gelatin, 236, 419
- P
- Packaging, 395, 397, 431
 Paint brush handles, 433
 Papain, 47, 269, 283
 Paper sizing, 422-424, 434
 Pastilles, 382, 383, 384, 414, 416
 Pectin, 404, 408
 Pepsin, 22, 40, 127, 146, 269, 283, 312
 Peptide bond cleavage, 167, 168, 170, 236
 Permeation chromatography, 117
 Peroxide treatment, 319, 325, 368
 Petrol capsules, 422
 pH control, 114, 212, 372, 525-527, 530
 Phagocytosis, 395
 Pharmaceutical gelatin, 415-419
 Phosphate ions, 499
 Photo-catalysis, 224
 Photographic developer, 468
 emulsion, 439, 460, 461-463, 470
 film, 466-470
 gelatin, 257, 262, 288, 324, 519, 531, 532
 restrainer, 464, 465
 Photosensitive paper, 423, 470
 Phthalyl gelatin, 215
- Pigskin, age, 159
 debristling, 139
 enzyme process, 47, 166
 frozen, 296, 306
 ion binding, 159
 non-collagen components, 160
 Plasma expander, 418, 419
 substitute, 414
 Plasticiser, 261, 263, 285, 414, 428, 432, 469
 Plate heat exchanger, 345
 Polydispersity, 110
 Polymer adsorption, 447-449
 degradation, 271
 Polyproline structures, 121, 122, 125, 126, 276
 Polysaccharide interactions, 239, 373
 Pot life, 221, 431
 Precipitation, collagen, 402, 404
 Precipitation, gelatin, 111, 186, 206, 257, 260, 371, 424, 463
 Pretreatment, acid, 88, 89, 113, 117, 118, 158, 159, 160, 295, 309, 310, 316, 317, 318, 320
 alkali, 21, 32, 45, 88, 89, 113, 117, 118, 139, 140, 161, 164, 165, 307, 316, 317, 322, 405
 dual soak, 90, 140
 Printers rollers, 414, 428, 429
 Printing, carbon, 426
 collotype, 427
 photogravure, 427
 silk screen, 427
 wash off, 428
 Process control, 308
 Procion dyes, 226
 Pronase, 22, 184, 311, 312
 Propylene oxide gelatin, 221
 Protective colloid, 414, 424, 425, 440, 450, 462, 480, 481
 Protein detergents, 215
 Pumping, 406, 409
- Q
- Quartz suspensions, 450, 458, 459
 Quick gel test, 514, 515
 Quinone, 252
- R
- Rabbit skin, 140

- Radiation, 171, 172, 224, 279–281, 317
 Radioactive tracers, 274
 Radon diffusion, 274
 Random coils, 120, 121, 122
 Reactive gelatin, 237, 238
 Reactive groups, 488
 ϵ -amino, 489
 carboxyl, 491
 guanidino, 489
 hydroxyl, 491
 imidazol, 489
 indolyl, 490
 phenyl, 491
 tyrosyl, 490
 Refractive index, 276, 479, 480
 Refractories, 425
 Relative humidity, 254, 255, 261, 281, 429, 430, 447
 Renaturation, 125, 126, 127, 146, 147, 204, 205
 Reticulin, 40
 Reversion, 125, 187, 198, 204
 Ribityl phosphate, 404
 Rigidity, 135, 180, 182, 185, 189, 190, 193–197, 202, 205, 264–271, 370, 378, 381, 408, 409, 469, 508
 factor, 123, 125, 185, 193, 198
 test, 515, 516
 Rotary dies, 415
 Rounds casings, 397, 398
 Rubber-like structure, 263, 268, 271, 440
- S
- Salt effects, 378
 Sand paper/cloth, 429, 430
 Sausage casings, 395–405
 Sedimentation, 417, 418
 Sensitization, gold, 465, 466
 reduction, 465
 sulphur, 463, 464, 467
 Sequence studies, 97, 487, 488
 Serosa, 398, 399, 400
 Setting phenomena, 185, 187, 188, 189, 193, 203, 280, 312, 371, 386, 440, 467, 471, 531, 532
 Shear, 117, 264, 273, 403, 459
 Sheet gelatin, 374
 Shrinkage, thermal, 142, 143, 144, 308, 399, 402, 409
 Sialic acids, 48, 50, 60, 103, 492
 Silver bromide, 451, 453, 454, 457, 458, 459, 461, 467
 SLS fibrils, 13, 22, 127, 157
 Sludge separator, 326
 Snake skin, 404
 Soap gels, 351
 Solids content, 509
 Solubility, 111, 251–258, 262, 375, 376, 510
 Soluble collagen, α -chains, 12, 13, 65, 99, 170
 β -chains, 170
 chromatography, 142
 chromium reaction, 240
 denaturation, 11, 12, 23–25, 62, 98, 126, 140, 141, 142, 145
 fibrils, 172
 hydrogen bond breakers, 53, 140
 imino acids, 141, 409
 partial specific volume, 141
 optical rotation, 11, 23, 141, 276
 preparation, 41, 42, 53, 62
 specific cleavage, 13–17
 U.V. adsorption, 141
 viscosity, 23, 62, 141, 409–411
 Solvents, for gelatin, 212, 258, 259, 260
 Sorbitol, *see* glycerol
 Specific ion effects, 444, 445
 Spectrophotometers, 494
 Splits, 295
 Spray drying, 348, 376
 Stabilizers, 388, 389
 Standards, 366, 383, 385, 390, 477, 499, 509, 510, 512, 512, 514, 518, 519, 520, 522, 524, 527, 529
 Steam pressure, 360
 Sterilization, 344–347
 Storage, 360
 Stress/relaxation, 266
 Sturgeon skin, 404
 Submucosa, 398, 399, 400
 Substitute reactions, 213–223
 Succinyl gelatin, 215, 217
 Sugar paste, 391
 Sulphate ester reaction, 239
 process, 162, 309
 Sulphite, 499
 Sulphonation, 216, 217, 220, 223, 494, 495
 Sulphur dioxide, 318, 324, 366, 407, 499
 Suppositories, 414, 417

- Surface action, 286, 287
 films, 288, 452
 tension, 370
 Suspending agent, 405, 406, 407
 Sutures, 402
 Swelling, 25, 54, 162, 251-258, 283, 350,
 397, 399, 402, 403, 440-447, 468,
 469, 471, 509
 pressure, 440-445
 rate, 445
 restrainer, 154, 161, 162, 444
 Swim bladder, 395, 406
 Syneresis, 203, 206, 378, 390
- T
- Table jelly, 382, 519-521
 Tablets, 416
 Tack, 431, 432
 Tanning, 143, 224, 239, 262, 268
 Taste, 368
 Teleopeptides, 15, 42, 121
 Tendon, 61-63, 82, 83, 140
 rat tail, 61, 140, 151, 170
 Tensile strength, 261
 Textiles, 427, 433
 Texture, 377, 385, 389
 Thermal conductivity, 286
 hydrolysis, 110, 114, 129, 269, 283,
 344, 369, 372, 383
 ThermascREW, 300
 Thickeners, 379, 390, 424
 Thiolation, 236
 Thiosulphate ions, 500
 Titration curves, 87, 93, 94, 111, 117,
 442, 488, 489, 494
 Triazines, 226, 227, 237, 238, 256, 494
 Triboelectric properties, 283
 Tropocollagen, 26, 41
 Trypsin, 22, 45, 120, 146, 236, 269, 454
 Tub sizing, 422
 Turbidity, 275, 325, 368, 481
- U
- UF Resin, 430, 432
 Ultracentrifugation, 127, 147, 191, 202,
 270
 Ultrasonic irradiation, 344
 Uterus, 64
 UV adsorption, 93, 199, 480
 irradiation, 273
- V
- Van der Waals forces, 253
 Vinyl grafts, 220
 Visco-elastic gels, 133, 223, 240, 250,
 271, 402, 403, 532
 Viscometer, capillary, 117, 134, 273,
 522, 523, 525, 526
 concentric cylinder, 273
 parallel plate, 254
 Viscosity, 113-119, 124, 128-135, 145,
 186, 187, 193, 240, 252, 271, 272,
 279, 281, 312, 370, 381, 385, 423,
 426, 432, 443, 521-527
 concentrated solution, 128-135
 degradation test, 525-527
 dilute solution, 113-119
 GMIA test, 524
 intrinsic, 117, 118, 120, 122, 123, 130,
 280, 409, 410, 411
 reduced, 113, 115, 116
- W
- Wallace Indentation Tester, 256
 Washing, gels, 463, 470, 471
 raw materials, 309, 310
 Water, free and bound, 282, 283
 usage, 360
 Weisenberg, effect, 223, 271, 402
 Whipping power, 378, 384, 385
 "White Foot", 367, 380
- X
- X-Ray diffraction, 4-7, 25, 38, 144, 148,
 179, 257, 262, 285
 film, 460
 radiation, 171, 172, 225, 281
- Y
- Yeast cells, 408
 treatment, 319
 Yoghourt, 390
 Youngs modulus, 265
- Z
- Zeta potential, 408, 458
 Zinc chromate capsules, 422
 ions, 498