PRACTICAL ANALYTICAL METHODS FOR CONNECTIVE TISSUE PROTEINS

J. E. EASTOE

and

A. COURTS

PRACTICAL ANALYTICAL METHODS FOR CONNECTIVE TISSUE PROTEINS

J. E. EASTOE
B.Sc., Ph.D., A.R.C.S., D.I.C.
Royal College of Surgeons of England

and

A. COURTS
B.Sc., Ph.D., F.R.I.C.
Unilever Research Laboratory, Colworth

with a Foreword by

Professor ALAN G. WARD
O.B.E., M.A., F.Inst.P.
Procter Professor of Food and Leather Science
University of Leeds

LONDON
E. & F. N. SPON LTD.
22 Henrietta Street, W.C.2
1963

First published 1963

Printed in Great Britain by
Spottiswoode, Ballantyne and Co. Ltd., London and Colchester

FOREWORD

In 1950 the authors of this book became part of a newly-formed team whose purpose was to study the scientific basis of gelatin and animal glue manufacture and use. It was an opportune moment. New techniques of protein analysis, and of physicochemical investigation, were waiting to be applied to collagen, to other macromolecular constituents of connective tissue, and to their derived products. While the two authors have now transferred their interest to other fields (dental research and food research), they have continued to exploit and extend the techniques they and their colleagues had developed at the British Gelatine and Glue Research Association.

The usual scientific paper rarely gives the many details of technique needed to permit a research worker, inexperienced in a particular field, to apply a new method. The present book makes available in ample detail the basic methods utilised by the authors for connective tissue proteins and other constituents. While this is useful in setting up the more complex analytical procedures, it is especially valuable in securing precision in apparently simple determinations.

Careful and critical use of the book will enable workers entering the field to apply the methods described to their own problems, and there is also much of value for experienced workers on connective tissue.

February, 1963

ALAN G. WARD
Procter Professor of Food and Leather Science
University of Leeds

AUTHORS' PREFACE

The earliest chemical studies of the connective tissues were largely confined to those technologies which use them for raw materials. The tanner has carried out much research in an effort to make better leather more cheaply, while somewhat more recently many gelatine manufacturers have become interested in research concerning the structure and behaviour of connective tissue proteins, following the pioneer investigations of Loeb and Ames in this corner of colloid science. The name collagen (Gr. κολλα=glue [producing]), the principal and definitive fibrous protein of connective tissues, reflects not only its adhesive potentialities but the difficulties facing early investigators in characterising constituents of animal tissues.

For many years, work on biological aspects of connective tissues was largely confined to the examination of stained histological sections. However, during the last two decades there has been a remarkable growth of interest in these tissues for their own sake. Near the beginning of this period, Chibnall and his co-workers carried out precise quantitative investigations on the composition of the protein constituents which made it possible to characterise them by analysis. More recently, increased interest in the field has largely resulted from two sorts of stimulus:

(i) a realisation of the importance and ubiquity of the connective tissues in the animal body, of their structural variety built on a unified basis of collagen and latterly, of the low but significant metabolic turnover of this protein, and

(ii) the independent development of a number of highly specialised techniques which have greatly increased our knowledge of the structure of biological macromolecules.

Connective tissues possess an unusually high proportion of extracellular material with collagen as the main fibrous element. They are essentially associated with the mesodermal layer and participate in the make-up of a wide variety of organs in a primarily mechanical capacity. Collagen is a major constituent of the corium of skin, of tendon, cartilage, bone, the dentine and cementum of teeth and the sclera of the eye; it also occurs in the loose connective

tissue between organs, basement membranes, lung, muscle, the walls of hollow viscera, brain, liver and kidney. Much information regarding the composition and structure of collagen has been obtained by means of chromatography, x-ray diffraction and electron microscopy.

As a result of these recent discoveries, investigators having a variety of interests have turned their attention to the connective tissues and their researches sometimes involve the use of techniques from other branches of science, for purposes of characterisation and measurement. In the past the Authors have sometimes experienced difficulty in finding the required kind of practical information in original papers, which, in this field, are published in a particularly wide range of journals. Over a period of some twelve years, they and their colleagues have tried a number of techniques and selected the most reliable for use in their research work. The present volume is largely a compilation of these methods.

In a book designed expressly for the laboratory bench, full practical details are given so that each technique may be carried out successfully, even by inexperienced workers, without the need for further reference. Wherever necessary, detailed information is provided in the light of personal experience. The methods have been selected so that they may be carried out with very simple equipment of a kind basic to the requirements of a modern biochemical laboratory. Sometimes problems are encountered which necessitate quantitative analysis of minute amounts of biological material. For this reason, modifications necessary for work on a micro scale are given where appropriate.

No excuse is offered for the resulting potpourri of techniques on the theme of connective tissues. These range from the preparation of relatively pure materials from animal tissues and bacterial cultures, through methods of chemical analysis, which form the body of the book, to simple physical measurements of special interest in this field. Attention has not been entirely restricted to proteins as the mention of the complex tissue polysaccharides and mucosubstances was considered essential owing to their interest and importance.

It is hoped that this volume will be useful to research workers in the fields of biological chemistry and physics, zoology and medicine. The problems of ageing, wound healing, uterine resorption, rheumatism and other diseases of connective tissues are now being widely investigated and require the application of appropriate analytical

methods. Technologists and chemists, associated with the leather, gelatine and adhesives industries, with food processing and photographic materials, should also be interested in the contents of this book. Finally it is suggested that both elementary and advanced students of biochemistry and their teachers will find it a suitable practical introduction to a stimulating field, which can provide valuable experience and in which much remains to be uncovered.

February, 1963

A. COURTS,
Unilever Research Laboratory,
Colworth House,
Sharnbrook,
Bedford.

J. E. EASTOE,
Leverhulme Fellow in Biochemistry,
Department of Dental Science
Royal College of Surgeons of England,
Lincoln's Inn Fields,
London, W.C.2.

ACKNOWLEDGEMENTS

The authors wish to thank all their colleagues at the British Gelatine and Glue Research Association who helped them by providing information. We are particularly indebted to Dr. A. W. Kenchington for making available the results of unpublished work on the refractive index of gelatin solutions and to Professor A. G. Ward for details of the sodium sulphate–sodium hydroxide method of pretreatment in the preparation of soluble eucollagen. Our thanks are also due to Dr. A. A. Leach for his help in seeking out various documents, to Dr. D. A. Sutton for his co-operation and to Mr. J. N. Barrett for data on thin layer chromatography. We are especially grateful to Professor A. G. Ward for writing the introduction and would like to express our appreciation of the encouragement he has given over many years.

We should like to thank both Professor B. Cohen of the Royal College of Surgeons of England and also our publishers for their advice and understanding throughout the preparation of this book. We are also much indebted to Dr. C. Long for reading the manuscript. Finally we should like to thank Mrs. Beryl Eastoe and Mrs. Joan Courts for their help in typing, proof reading and indexing, which has made early publication possible.

A.C.
J.E.E.

CONTENTS

ONE

METHODS OF PREPARATION

1.1 COLLAGEN

(*a*) Native collagen from soft tissues

Collagen is a structure protein and, as such, performs many functions in the body, most of which require a tough, durable material. This protein is the principal organic component of skin, tendon, bone and dentine, and it also occurs in quantity in lung and in the cornea. Collagen accounts for about 30 per cent of all body protein. Because of its wide distribution and of the great variety of extraneous materials which may be associated with it, consideration needs to be given to the choice of a favourable source of collagen which may lead to the preparation of the protein in a highly purified form. All methods of purification are a compromise between removing as much impurity as possible without denaturing the collagen, particularly by heat, from its native form.

Skin collagen

The following method is recommended by Bowes and Kenten (1948).

Strips of freshly removed ox-hide (calf is preferred) are washed in a rotating drum (1) with water (2) with 10% NaCl for 30 min. each (3) in 10% NaCl overnight (4) in several changes of distilled water (5) with acetone to dehydrate. This facilitates splitting off the grain layer with the hair roots and the inner flesh layer. The remaining corium is cut into small cubes (1 cm.), degreased with light petroleum at room temperature for several days, washed with several changes of distilled water and again dehydrated with acetone.

In this way, the fat content should be reduced to less than 0·1 per cent and ash to less than 0·05 per cent.

Note. Standard hide powder, which is sometimes recommended as a useful source of collagen, should be avoided in general research work as it is often very degraded.

Tendon

An alternative and readily available source of collagen occurs in chicken leg tendon. The knee joint of a freshly killed bird is cut with a scalpel and the lower limb (metatarsus) subjected to a short, sharp tug. The tendon bundles removed in this way are usually attached to muscle which is easily scraped off. Degreasing and washing may be carried out as with skin.

These tendons are of particular interest in protein swelling demonstrations at acid pH values and in many ways follow the characteristics of the more widely used rat-tail tendon.

(*b*) HARD TISSUES AND THEIR DEMINERALISATION

Bone

Bone tissue is most easily prepared in a state free from other tissues and suitable for chemical studies, if the starting material is carefully selected. Cancellous or spongy bone requires a great deal of painstaking and tedious work to remove all the marrow and other soft tissues by dissection, leaving only clean trabeculae, consisting entirely of bone. This can, however, be achieved for a strictly limited quantity. Compact bone is more easily prepared, since it contains a much larger volume of solid bone tissue. The shafts of the long bones of the larger mammals are particularly suitable.

Separation of compact bone from ox femur. Fresh ox or pig femora are obtained from butcher's shops, soon after removal from the carcases. The knuckle ends are removed with a butcher's saw, leaving the centre of the diaphysis some 10 cm. in length. The fatty marrow is easily scooped out completely from the hollow tube of bone, using a metal spatula. Small areas of spongy bone which may remain on the inside of the shaft are scraped away with a scalpel. The soft periosteum on the outside is similarly removed, the bone being kept moist during the operation. Finally the portion of softer, dark-coloured tissue near the tendon insertion is sawn off. The remaining bone tissue should be completely white, dense and hard.

Finely-powdered bone tissue. The technique used for powdering bone should be selected according to the purpose for which the tissue is required:

(1) Perhaps the most convenient method is to clamp one end of the tube of bone in the four-jaw independent chuck of a lathe. After centring it accurately, the bone is turned, using a fairly light facing cut on the end remote from the chuck. The turnings are

collected on a clean cloth and transferred in 10-g. lots to a high-speed disintegrator (Magimix, Waring Blendor, etc.). The motor is run at full speed for 1 min., a rubber pad being pressed down on the goblet to prevent loss of powder. The disintegrated bone is transferred to a 100-mesh sieve, the powder passing this being retained, while the remainder is returned to the disintegrator and further broken up. The powdered bone may be spread out in a thin layer exposed to the air, to allow its moisture content to reach equilibrium.

(2) Where large quantities of bone powder are required, the tube of bone is sawn into slices about 3 mm. thick with a bone saw. These are broken with pincers into pieces sufficiently small to enter the gate of a beater-cross grinding mill, fitted with a fine-mesh sieve plate at the exit. The mill is started and the pieces dropped in one at a time, sufficient time being allowed for each piece to be disintegrated before adding the next. Overloading results in momentary jamming of the mill, which throws off the driving belt.

(3) The above methods, when carefully carried out, necessitate only a slight temperature rise in the bone, for a short period. Where it is essential that the bone should be kept cold, the following technique is suitable. The tube of bone is cooled in powdered solid carbon dioxide and broken into pieces with a large hammer. The pieces are placed one at a time into a diamond mortar, which is similarly cooled to low temperature before powdering the bone by hammering the piston.

Bone Collagen. Powdered bone (50 g.) is suspended in 1 l. of water and the suspension is stirred mechanically. Sulphur dioxide gas is passed in until pH 2·5 is reached. The mixture is then stirred for a further 30 min., when the solid is allowed to settle and the supernatant liquid is removed by suction. A further portion of water is added and sulphur dioxide is bubbled through, with continuous stirring, for another hour. The solid is allowed to settle and washed twice with further 1 l. portions of water.

The decalcified bone is stirred for 4 hr. with 1 l. of 10% (w/v) sodium chloride solution to remove albumins and the suspension adjusted to pH 6–7. After allowing the suspension to settle and washing twice with water, 1 l. of 10% (w/v) calcium chloride solution is added to dissolve muco-substances and the solution adjusted to pH 9–10. The solution is stirred for 1 hr., heated to 35°* for a few minutes and cooled. The suspension is allowed to stand overnight, stirred again, allowed to settle and the liquid is removed.

* Temperatures are given in degrees Centigrade throughout this book.

After washing the solid three times with water, a further 1 l. portion of water is added and the stirred suspension brought to pH 3 with N-hydrochloric acid. After three further washings with water, the stirred suspension is adjusted to pH 6–7. The bone collagen is then washed once more with water and finally with four lots of acetone. The solid is filtered by suction and the bulk of the acetone removed in a vacuum desiccator, connected to a water pump. The powder is spread out in a thin layer and its moisture content allowed to reach equilibrium with the laboratory air. The total nitrogen content on a dry basis should be approximately 18·3 per cent, the ash content 0·08 per cent and the moisture content 15 per cent.

Commercial demineralised bone (*ossein*). Large quantities of bone are demineralised on a commercial scale for the production of gelatin. The degreased bones are treated with approximately 2 N-hydrochloric acid for about 7 days, the product being known as 'ossein'. Since the raw material does not consist entirely of bone tissue, the product is to a certain extent contaminated with other tissues and is in part pigmented, probably from small amounts of blood. The pieces are of a suitable size to be selected and sorted by hand to give a more uniform batch.

Dental tissues

The tissues of teeth are more difficult than bone to isolate in a purified state. This results from the juxtaposition of three types of hard tissue, firmly bonded together. In the centre of the tooth is the pulp, a core of soft tissue composed mainly of loose connective tissue with blood vessels and nerves. The pulp is almost completely surrounded by *dentine*, the innermost zone of hard tissue, consisting of a collagen matrix with hydroxyapatite crystallites. The dentine is covered by a cap of *enamel* which forms the crown of the tooth and provides the biting surface. It is a tissue of epithelial origin and the organic matrix, which is not collagen, diminishes in amount during maturation, the mature enamel consisting almost entirely of hydroxyapatite. Around the roots of the tooth and reaching as far as the enamel is a rather thin layer of *cementum* which also covers the dentine and is anchored to surrounding bone by unmineralised collagen fibres. Cementum itself is mineralised and is similar to bone in structure and composition.

Separation of dentine, enamel and cementum. Two types of method have been used for separating the hard dental tissues from one another.

These depend upon selection of particular portions of the tooth and density differences respectively. Perhaps the simplest use of mechanical separation is to break off the roots of suitably shaped teeth with pincers, an easy method for obtaining dentine which is free from enamel, though still associated with cementum. Ivory from elephants' tusks is a convenient source of large quantities of pure dentine. Where heating of the tissues is permissible, a more complete separation can be obtained by grinding the individual tissues away with a dental burr. To remove the greater part of each tissue in an uncontaminated state by this method requires care. A knowledge of dental anatomy is also useful but, without this, it is helpful to notice that dentine is yellowish and relatively soft, whereas enamel is glistening white and much harder. To minimise contamination, the neighbourhood of the junctions between the various hard tissues should be avoided.

For separation of the tissues on a density basis, the whole of the tooth, except the pulp, is initially powdered in a diamond percussion mortar. This may be carried out at a low temperature. The process is continued until all the powder passes through a 60-mesh sieve. For the initial separation of enamel (density 2·9–3·0 g./cu. cm.) from dentine (density 2·1–2·2) plus cementum (density 2·05) the tooth powder is centrifuged in a mixture of 91 vol. of bromoform and 9 vol. of acetone (density 2·70) for 2 min. at 2200 r.p.m. (Manly and Hodge, 1939). This is carried out in a 15 ml. conical centrifuge tube having an inner tube of somewhat smaller diameter suspended inside it by means of a rubber stopper. The inner tube is pushed through a hole in the stopper which rests on top of the outer tube. The inside tube is also tapered and cut off so that the lower end is of 2 mm. bore and 2 cm. above the bottom of the outer tube. The powdered sample is introduced through the inner tube and after centrifugation, a finger is placed over the top of the inner tube which is lifted out with the lighter fraction inside, in a similar way to a pipette.

Enamel of nearly 99% purity is obtainable in a single separation using this method. Slight further purification is possible by recentrifuging the enamel in a mixture of density 2·89 g./cu. cm. The dentine from the initial separation varies in purity from 97 to 99 per cent, but a second centrifugation in liquid of density 2·42 increases this value to 99·7 per cent. This fraction still contains cementum which can be separated by sedimentation in a mixture of density 2·07 in which the dentine sinks. The separated tissue powders are

finally washed three times with acetone with intermediate centrifuging, in order to remove bromoform.

A variation of the sedimentation method makes use of two separating funnels connected one above the other by means of a rubber bung (Battistone and Burnett, 1956). The powder to be separated is placed in the top funnel and its tap is opened so that the heavier particles fall into the lower funnel under gravity. A liquid of density 2·70 g./cu. cm. is obtained by mixing 87·8 parts of symtetrabromoethane with 12·2 parts of acetone.

The purity of samples is most readily checked by a particle count under the microscope (Manly and Hodge, 1939). It is possible to distinguish between enamel (refractive index 1·60) and dentine (1·56) using the Becke line method. The particles are placed in a liquid of refractive index 1·59; the microscope is focussed and the tube raised slightly. Enamel particles are seen to be surrounded by a dark line and dentine particles by a bright one. With even the purest enamel samples obtainable by sedimentation a small percentage of 'junction' particles, which contain some dentine, remain. These are particularly objectionable in protein studies of enamel, for which purpose it is perhaps better to prepare small selected blocks of enamel by chipping. Dentine is removed with a burr under a dissecting microscope, the enamel is powdered and checked for purity by the microscope method.

Demineralised tooth tissues. Dentine and cementum powders may be readily demineralised with sulphurous acid using the technique described for bone (p. 3). Mature enamel leaves only a very small residue after demineralisation so that use of mild reagents is advisable.

Demineralisation with chelating reagents. A number of reagents which form chelate complexes with calcium ions have become available recently, which enable demineralisation to be carried out at relatively high pH values. By far the most widely used of these is ethylenediaminetetra-acetic acid (EDTA) usually supplied as the readily-soluble disodium salt. A 10% (w/v) solution is prepared and sodium hydroxide is added to adjust the solution to a pH value usually within the range 7·0–7·6. Thus EDTA solutions have the advantage of optimum activity within the physiological range of pH. The main disadvantage is a very slow rate of action compared with even weak acids. For this reason a concentrated solution is employed, but nevertheless, the mineralised tissue should be in powdered form, since with larger pieces of tissue, demineral-