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The Preparation of an Alkali-Soluble Collagen from Demineralized Bone

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Ossein was solubilized by the action of alkali and a resulting high-molecular-weight fraction isolated. The chemical and physical properties of this fraction were studied and compared with those of an acid-soluble collagen prepared from calf skin by conventional techniques. From the results it is concluded that the alkali-soluble protein exhibits only minor differences from acid-soluble collagen, and that these differences can be ascribed for the most part to a decrease in the inter- and intra-molecular cross-linking.

The effect of alkali on collagen has been extensively studied by Bowes & Kenten (1948, 1950*a*,*b*) and by Bowes (1950). They reported that the swelling in alkali increased progressively with increase in pH from neutrality, but that there is no decrease at higher pH values corresponding to that which occurs in acid solution below pH 2 and which would be expected according to the Donnan theory. This anomalous swelling effect, they concluded, is due to loss of cohesion attributable, not to any hydrolysis of the major peptide chains, but to the breakdown of intermolecular bonds.

Courts (1960) described the alkaline pretreatment of insoluble collagen resulting in the production of a material soluble under acid conditions, which he calls 'eucollagen'. He suggests that collagen that has been subjected to alkaline treatment in the presence of a swelling restrainer [in this case 20% (w/v) sodium sulphate] is considerably different in many of its properties from native collagen. He states that the conversion of collagen into eucollagen involves the progressive hydrolysis of especially labile peptide bonds, resulting in a number-average molecular weight of 60000–65000, and that solution in the reagent occurs if this falls below 60000.

Kuhn, Zimmer, Waykole & Fietzek (1963) have studied the effect of alkaline pretreatment [5% (w/v) sodium hydroxide] on soluble collagen in the presence of saturated sodium sulphate. They reported an acid-soluble product with viscosity and optical rotation values similar to those of the parent soluble collagen. This material will form segment-long-spacing aggregates indistinguishable from those of the parent soluble collagen. Fibres are also formed, but they lack the characteristic banded appearance. On denaturation only one component is detectable by ultracentrifugation, and this has a sedimentation coefficient identical with that of the α-chains of the parent soluble collagen. They conclude that the fundamental effects of alkaline treatment are the loss of the amide groups of glutamine and asparagine and the splitting of inter- and intra-molecular bonds. Kuhn *et al.* (1963) also report that identical treatment of insoluble collagen results in an acid-soluble product with properties very similar to those of the alkali-treated soluble collagen.

Work described in the literature has been concerned with collagen that remains insoluble during alkaline treatment, and precautions have been taken to minimize solubilization by the alkali, either by keeping the pH below 13 or by using a suitable electrolyte, such as sodium sulphate, to restrain the swelling. Little has been said about any fraction soluble in alkali, apart from the statement by Courts (1960) that solution by the reagent results when the number-average molecular weight falls below 62000, and the observation by Bowes & Kenten (1948) that under their conditions about 5% did dissolve and, since some of this was precipitated on neutralization, it was presumably in the form of large polypeptides.

This present paper describes a method resulting in the complete solubilization of bone collagen in alkali, and the isolation from solution of a high-molecular-weight fraction. The properties of this fraction have been examined, and for several properties a direct comparison was made with an acid-soluble collagen from calf skin, since a full

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examination of the properties of the alkali-solubilized ossein meant extending the range of pH normally employed for collagen studies.

METHODS

Preparation of the ossein. Diaphyses from freshly obtained thigh bones of cows were broken with a hammer into as small pieces as possible. The pieces were defatted in chloroform–methanol (3:1, v/v) for 48h, dried and extracted in 0.2M-sodium phosphate buffer, pH7, for a further 48h. The bone pieces were then rinsed and decalcified in HCl at pH2.5 in a pH-stat. After 5 days the partially decalcified bone was further disintegrated in a blender. After a total period of 10 days, the ossein was thoroughly rinsed, freeze-dried and powdered in a ball-mill.

Alkaline extraction of ossein. For this procedure, ossein which passed through a no. 40 mesh sieve was used. The ossein was shaken for 3 days with 5% (w/v) NaOH made 0.1M with respect to NaCl. The pH was then adjusted to 4.0 and the resulting precipitate collected by centrifugation. This precipitate was dissolved in and dialysed against 0.5M-acetic acid, and then further purified by precipitation from solution by the addition of NaCl to a final concentration of 4% (w/v). This precipitation step was repeated, and followed by another dialysis as before. The yield of alkali-soluble material at this stage, as judged by total nitrogen determinations (Chibnall, Rees & Williams, 1943), was 28% of the original ossein.

Preparation of acid-soluble collagen. Acid-soluble collagen was prepared from calf skin by the method described by Steven & Tristram (1962).

Amino acid analyses. Amino acid analyses were carried out on a Phoenix automatic amino acid analyser, with the system devised by Piez & Morris (1960). Samples were hydrolysed under N_2 for 24h at 105°C. Hydroxyproline was included in the amino acid calibration mixture, and was also determined by the method of Stegemann (1958).

N-Terminal analysis. The method used was that described by Signor, Biondi, Terbojevich & Pajetta (1964), with 2-chloro-3,5-dinitropyridine.

Amide nitrogen. Samples of protein (100–200mg) were hydrolysed in 10ml of 2M-HCl at 100°C for 1h. The hydrolysate was transferred to a Kjeldahl distillation apparatus along with 5 drops of 1% thymolphthalein in 50% (v/v) ethanol and liquid paraffin to prevent excessive frothing. Then 30% (w/v) NaOH was added until the contents were alkaline to the indicator; the NH_3 was distilled out, collected in 2% (w/v) boric acid and determined by titration.

Optical rotation. Optical rotation was measured in a Hilger-Watts standard polarimeter. Samples were allowed to equilibrate at the stated temperature for 30–60min before the reading was taken. To determine the extent of renaturation, samples were left for 24h at 25°C before the optical rotation was measured.

Electron microscopy. Electron microscopy was carried out in an AEI EM6B electron microscope. (i) Segment-long-spacing form. Collagen solutions were dialysed exhaustively against 5mM-acetic acid, the pH was adjusted to 2.5 and the segment-long-spacing form precipitated by the addition of 0.5% ATP solution in 0.5M-acetic acid. After application to the grids and drying, the material was stained with 1% phosphotungstic acid at pH2. (ii) Fibrous form. The fibrous form was prepared by precipitation from 0.05M-acetic acid by adjusting the pH to 4.0, or by dialysis against distilled water. Staining was accomplished with 1% phosphotungstic acid at pH2.

Viscosity. Viscosity was determined in 0.5M-acetic acid in an Ostwald capillary viscometer with a flow time for water of 61.6s at 20°C.

Ultracentrifugation. Ultracentrifuge runs were carried out in 0.5M-acetic acid in a Beckman–Spinco model E ultracentrifuge at 20°C and a speed of 59780rev./min. For the calculation of the $s_{20,w}$ value, the partial specific volume was taken as 0.645ml/g (Heaps, Johnson & Stainsby, 1966).

Polyacrylamide-gel electrophoresis. The system used was essentially that described by Clark & Veis (1968), with the electrode buffer being 6mM-sodium acetate buffer, pH4.7. Staining was achieved by immersing the gels for 1h in 1% Amido Black, and removal of the excess of stain was accomplished by the application of an electric field across the short axis of the gel. The apparatus used for this was a simplification of that described by Schwabe (1966). For the direct comparison of two samples they were run side by side in the same gel, by using the method described by Clarke (1964).

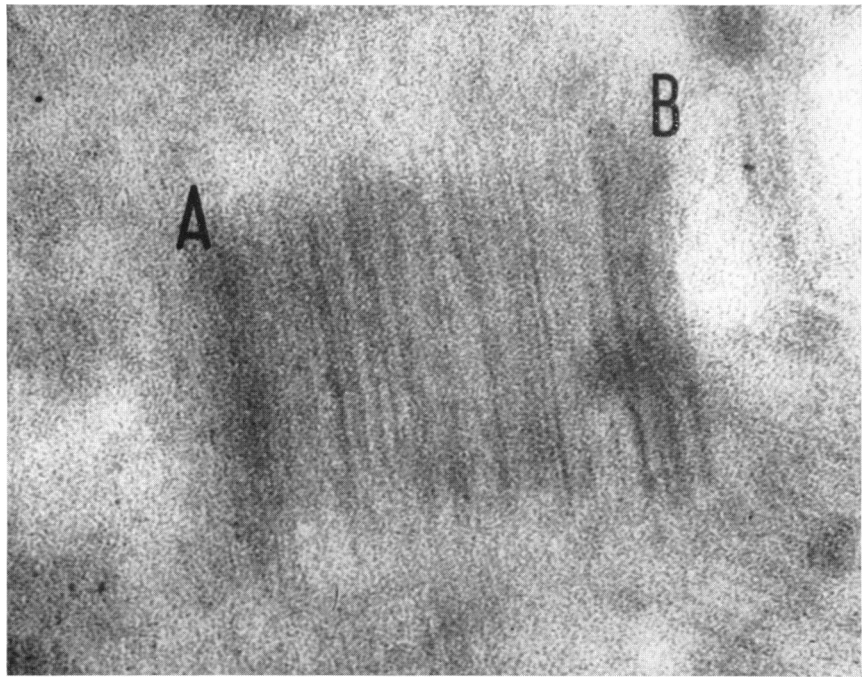
RESULTS

The results of the amino acid analyses are given in Table 1, and these show no major deviations from the characteristic collagen composition. There are rather higher contents of imino acids and a small amount of ornithine was also detected, resulting

Table 1. *Amino acid analysis of the alkali-soluble protein*

These results are an average from three determinations and are not corrected for hydrolytic losses.

Amino acid	Amino acid composition (residues/1000 residues)
Alanine	107.3
Glycine	310.3
Valine	22.9
Leucine	25.6
Isoleucine	11.9
Proline	144.8
Phenylalanine	10.7
Tyrosine	1.8
Serine	28.3
Threonine	15.2
Methionine	3.9
Arginine	46.5
Histidine	4.8
Lysine	27.7
Ornithine	4.7
Aspartic acid	44.1
Glutamic acid	75.4
Hydroxyproline	105.8
Hydroxylysine	8.3



EXPLANATION OF PLATE 1

Segment-long-spacing form (A–B) from the alkali-soluble protein. Details are given in the text. Magnification ×300000.

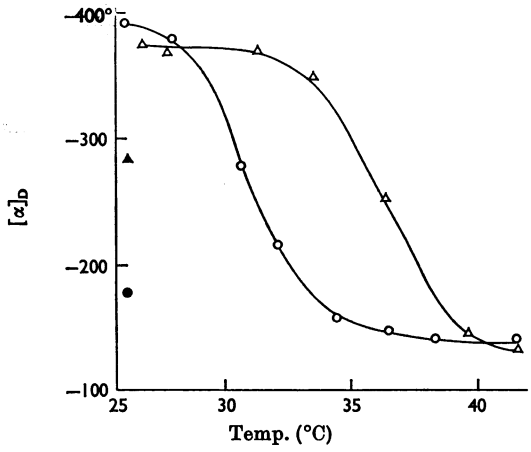


Fig. 1. Variation of optical rotation with temperature. ○, Alkali-soluble material; △, acid-soluble collagen; ● and ▲, respective values after 24h renaturation at 25°C.

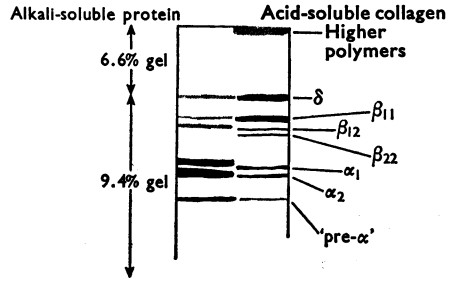


Fig. 2. Results from a typical 'split-gel' run of the alkali-soluble protein and the acid-soluble collagen. The bands are assigned as described by Clark & Veis (1968).

presumably from the deguanidation of arginine under alkaline conditions. The only *N*-terminal amino acid detected by this method was alanine; no *N*-terminal amino acids were detected in the acid-soluble collagen.

The amide nitrogen content is low (0.13%, expressed as a percentage of dry ash-free protein). This represents 8–9 amide residues/1000 amino acid residues, compared with the normal value for collagen of 40–44 amide residues/1000 amino acid residues (Eastoe, 1967).

Optical-rotation studies, summarized in Fig. 1, indicate a very high content of the characteristic collagen helix in both alkali-soluble and acid-soluble collagen. One difference that is apparent, however, is that on cooling the alkali-soluble collagen, its specific rotation, and thus its helical structure, is not regained to any significant extent compared with the acid-soluble collagen. In addition, the temperature of transition, i.e. the melting temperature, is lower for the alkali-soluble material.

Von Hippel & Wong (1963) defined the term ΔT as the rise in temperature occurring between the loss of one-quarter and three-quarters of the specific rotation. They describe this term as being a measure of the sharpness of the phase transition, and as such a measure of the co-operativity within the structure. These authors reported that the value found for collagen is remarkably constant at 2°C, whereas the value for the denaturation of a cooled gelatin is in the region of 7°C. The value of ΔT for the alkali-soluble material was found to be 3°C, compared with a value of 1.9°C for the acid-soluble collagen under identical conditions.

The alkali-treated preparation formed segment-long-spacing aggregates without difficulty (Plate 1), and these are essentially identical with the segment-long-spacing form produced from the acid-soluble collagen. Rubin, Pfahl, Speakman, Davison & Schmitt (1963) regard this as a very sensitive test of normal tropocollagen structure. Although fibres are formed these are generally featureless; however, in some isolated regions traces of cross-banding can be distinguished. Where banding does occur it has a periodicity of approx. 700 Å.

Ultracentrifugation at 20°C gives a single, very sharp peak with an $s_{20,w}$ value of 1.87S, compared with a value of 1.61S for the acid-soluble collagen under identical conditions. Viscosity determinations gave a value of 10.5dl/g for the alkali-soluble material, compared with a value of 16.3dl/g for the acid-soluble collagen under the same conditions.

The subunit composition was examined by polyacrylamide-gel electrophoresis, and the results obtained are summarized in Fig. 2. Clark & Veis (1968) have characterized the pattern obtained with acid-soluble collagen, and assigned the bands as indicated in Fig. 2. Comparison of the distribution resulting from the separation of the denatured alkali-soluble material shows a much larger proportion of α-subunits, and very little in the way of γ- or higher polymers. In addition, there is a band running in front of the α-region that has been termed 'pre-α' by Clark & Veis (1968), but they offer no explanation for its existence. This band is more prominent in the alkali-soluble material. The β-subunits have been identified as shown, and this suggests that the alkali-soluble material lacks β_{22}, the only dimeric subunit that must arise from intermolecular cross-linking. This assertion, however, should perhaps be treated with a certain amount of caution, as the effect of the decrease in the number of amide groups may affect the relative rates of migration of the β-chains. The relative migration of the α-chains, however, suggests that this decrease would not affect the order determined by Clark & Veis (1968).

DISCUSSION

These results show that, in the absence of swelling restrainers, ossein can be solubilized by alkali, and a fraction of high molecular weight isolated.

The high negative optical rotation exhibited in solution is indicative of the primary and secondary structure normally associated with collagen. The fact that it is not regained by the alkali-soluble collagen to any extent after denaturation can be attributed, at least in part, to a smaller extent of inter- and intra-molecular cross-linking, as indicated by the high proportion of α-chains revealed by gel electrophoresis. The results obtained by Altgelt, Hodge & Schmitt (1961) show that the higher the proportion of γ-subunits, the greater the degree to which the helical structure will re-form. Gelatin containing only α- and β-subunits will regain only 10–15% of the specific rotation value achieved on the renaturation of a gelatin containing γ-subunits. As mentioned by Kuhn *et al.* (1963), the effect of the change in the charge distribution caused by the loss of amide groups, although difficult to determine, cannot be ignored.

The value of 3°C for ΔT could be taken to indicate some small regions of random configuration within the structure of the alkali-soluble product, or that the lack of inter- and intra-molecular cross-links has some effect on the co-operativity. What it does indicate, however, is that, if this increase in ΔT over the value generally found for collagen is due to some regions of more random structure, then the incidence of these regions is very small compared with the incidence in gelatins.

The formation of the segment-long-spacing form, taken along with the sedimentation coefficient and the results from the viscosity determinations, indicates a quaternary structure closely resembling that of tropocollagen. The fact that the preparation does not form the cross-striated fibrous form to any significant extent may be related to the lack of intermolecularly cross-linked subunits that is suggested by polyacrylamide-gel electrophoresis, as these subunits would already have the necessary overlap and could conceivably act as 'formers' for the fibre structure.

It is evident from the results of polyacrylamide-gel electrophoresis, ultracentrifugation and the segment-long-spacing pattern that there has not been any extensive decrease in the size of the molecule. Finding alanine as the *N*-terminal residue, however, shows that some peptide-bond cleavage has occurred in this region. Bornstein & Piez (1966) showed that an intramolecular cross-link exists very close to the *N*-terminus and thus there is a very strong possibility that this cross-link may be present in the peptide(s) lost from this part of the molecule. According to Bornstein & Piez (1966), in rat skin collagen this cross-link is derived from lysine residues situated five residues from the *N*-terminus, and the first alanine is a further two residues into the molecule (Piez, 1967).

Apart from this decrease in the extent of some of the aggregation and reaggregation properties, probably as a result of the decrease in the intra- and, in particular, inter-molecular bonding, this preparation displays many of the properties of collagen to a degree consistent with the presence of a high proportion of the molecular organization associated with collagen.

Although the molecular properties and structure of collagen have been extensively studied, generally, these studies have been on that very small fraction which is readily soluble. The method described here results in the complete solubilization of bone collagen, and the subsequent studies show that the resulting material has suffered very little degradation in the form of peptide-bond cleavage, and merits the description of 'collagen'. Arising as it does from a starting material that is essentially insoluble, further studies of this preparation may well prove useful in determining the factors responsible for the insolubility and fibrillogenesis of collagen.

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