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Structural Changes in Collagen

2. ENHANCED SOLUBILITY OF BOVINE COLLAGEN: REACTIONS WITH HYDROGEN PEROXIDE AND SOME PROPERTIES OF SOLUBLE EUCOLLAGEN*

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Evidence by Fitton-Jackson (1953) and others shows that the generation of collagen *in vivo* takes place intercellularly to the stage of a soluble precursor and becomes insoluble extracellularly by some type of aging process likely to involve cross-linking. A substantial fraction of embryonic bovine collagen is soluble in citrate buffer (Gebhardt, 1960) and accounts for more than 13% of the total collagen at birth. The small fraction of adult human or bovine collagen which is solubilized in citrate buffer generally represents less than 1% of the total (Plotnikova, 1947, 1949) or about 4% in calf skin (Bowes, Elliott & Moss, 1955). The fraction similarly dispersed from the tail tendon of the rat or from teleostean collagen is considerably greater. Dumitru & Garrett (1957) obtained nearly quantitative solution of rat-tail tendon in distilled water at pH 6 after treatment with 0.5M-sodium dihydrogen phosphate, whereas bovine tendon did not dissolve in this way. Clearly, these highly dispersible collagens are different from bovine or human collagen. One common feature of acid-soluble collagens from a wide variety of connective tissues and species appears to be the retention of the highly ordered collagen structure as indicated by well-orientated X-ray-diffraction patterns and typical collagen 640Å spacings, confirmed by electron microscopy (Cowan, North & Randall, 1955; Randall, Booth, Burge, Jackson & Kelly, 1955; Schmitt, Gross & Highberger, 1955) and by high intrinsic viscosity in dilute solution (Boedtker & Doty, 1956; Gallop, 1955; Doty & Nishihara, 1958). Methods that lead to extensive solubiliza-

tion of bovine collagen, such as autoclaving in water above 100°, heating in ethylenediamine (Solomons, 1956) or potassium phosphate (Bate-Smith, 1938), also result in loss of collagen structure.

Courts (1960) has described the conversion of collagen into eucollagen in which substantial disorganization of covalently bonded structure is likely, but regions of hydrogen bonding maintain a stable collagen-type structure. The present study suggests that, during the extensive dispersion of eucollagen into acid buffer solutions, the retention of the collagen fold is more likely than conversion into gelatin; the material thus obtained has therefore been termed 'soluble eucollagen'. It seems probable that the conversion of collagen into eucollagen is a chemical reversal of the aging process. The reactions described in the present work have made possible a large-scale study of a solubilized collagen from a single batch of bovine connective tissue. The method has an added advantage of freeing the tissue from much of the non-collagen impurity. The present account of the fibre-forming properties is a more detailed study of this new form of collagen than that published by Courts & Stainsby (1959). The X-ray diffraction and electron-microscopy work on the fibres reconstituted from soluble eucollagen is reported elsewhere (Little, Kelly & Courts, 1961).

The alternative available method of non-thermal solubilization of native bovine collagen is by the action of lyotropic agents (Gustavson, 1955). Because of the extensive breaking of hydrogen bonds this type of reaction invariably gives gelatin (Courts, 1958), and the rigid rod-like molecular structure associated with native collagen (Boedtker

* Part 1: Courts (1960).

& Doty, 1956) is no longer discernible. The molecular weight of the gelatins, however, may still be substantial.

Recent interest in the function of polysaccharides in the collagen environment has promoted studies on the reaction of collagen with sodium periodate (Grassmann & Kuhn, 1955; Hörmann & Fries, 1958), and with hydroxylamine (Gallop, Seifter & Meilman, 1959). Gustavson (1957) describes the lyotropic effects of sodium periodate and perchlorate on collagen. It would appear that no previous study has been made with hydrogen peroxide on intact collagen. With gelatin at 40°, 1.7 M-hydrogen peroxide has the interesting property of impairing the viscosity without significant effect on the rigidity factor (P. R. Saunders, personal communication). In view of the implication in this observation that hydrogen peroxide can catalyse controlled peptide-bond hydrolysis, some investigations have been made on the reaction of collagen with 1.7 M-hydrogen peroxide, from which it is clear that the collagen will disperse with transformation into gelatin, provided that a mechanism that will break hydrogen bonds acts concomitantly.

In the absence of any known enzymic reaction facilitating the dispersion of uterine collagen after parturition (Brown, Consden & Glynn, 1958) it is possible that a catalysed hydrogen-bond-breaking mechanism may be involved. The present work on bone collagen is interim to a study on uterine collagen.

MATERIALS AND METHODS

Collagen. The material used was the ox-bone collagen described by Courts (1960).

Soluble eu collagen. Collagen was converted into eu collagen by the procedure of Courts (1960), by the action of NaOH in the presence of Na₂SO₄ for 10 days at 20°. The eu collagen was freed from fluid by straining, washed in running water for 30 min. and then allowed to go through isoelectric shrinkage (as indicated by the translucent pieces' turning opaque) by suspension in 0.02 M-acetic acid for 4 hr. Shrinking occurs in the range pH 4-4.3. The pieces were then washed in running water for 1 day. The swollen eu collagen (50 g.) was removed on a nylon mesh and kept in one of the buffer solutions (1 l.) described below for 1 day at 20° with occasional stirring.

Buffer solutions. The addition of 0.05 mole of sodium citrate/l. to citric acid (0.1 M; pH 2.2) gave the pH 3.7 buffer used by Gallop (1955) and Boedtker & Doty (1956). Acetate buffer (pH 3.6) was obtained by adding 5 ml. of N-NaOH to 1 l. of 0.15 N-acetate acid. Lactate buffers in the range pH 2.2-3.8 were prepared by adding N-NaOH to 0.15 N-lactic acid. Mandelic acid (0.15 N) was used at pH 3.2.

Hydrogen peroxide. AnalaR-grade (British Drug Houses Ltd.) 30% (w/v) (8.6 M) was diluted to the indicated strengths.

Mucoprotein. A crude preparation of osseomucoid was

made by the propan-2-ol fractionation of an acid-processed ossein gelatin. This procedure concentrated the bulk of the mucoid into fraction 6 (Courts, 1959).

Amino acid analysis. Protein hydrolysates of dried, low-ash-content materials were assayed for amino acid composition by the Moore & Stein (1951) method as described by Eastoe (1955). The experiments were carried out by Dr A. A. Leach, Miss J. E. Long and Mr A. P. Williams.

N-Terminal amino acid residues. The method of Sanger (1945) was used. The procedure for gelatin was as described by Courts (1954) and for collagens by Courts (1960).

(a) Reaction of soluble eu collagen with fluorodinitrobenzene. Soluble eu collagen (0.5 g.) in citrate buffer was adjusted to pH 8 with N-NaOH and shaken with 1-fluoro-2,4-dinitrobenzene (1.4 g.) at 20° for 1 day. The yellow dinitrophenyl derivative separated out. This was suspended in water, acidified with 0.01 N-HCl, washed in water and dried *in vacuo* over P₂O₅. The dried derivative was then washed in several changes of acetone until no further yellow colour was discharged.

(b) Hydrolysis. Dinitrophenyl-protein (500 mg.) was heated with 5.5 N-HCl (10 ml.) for 16 hr. in sealed tubes at 100°. The subsequent extraction of dinitrophenyl-amino acids and their identification and quantitative determinations closely followed the procedure of Courts (1954).

Dialysis. Solutions were dialysed in cellulose tubing against running tap water. The complete precipitation of fibres from acid-soluble eu collagen required 3 days. The complete removal of hydrogen-bond-breaking reagents from gelatin solutions was more prolonged, but in 1-2 days (depending upon the protein concentration) a gel formed, which was purified by washing in running water for 1 day. With collagen from dura mater, Brown *et al.* (1958) recommend 10 days at 37° in 0.9% NaHCO₃ to remove sodium salicylate completely.

Washing is preferably carried out on strips of gel, 2 in. × ½ in. × ⅛ in. approx. The removal of H₂O₂ from gels was usually achieved in a few hours in this way and was probably facilitated by the hypochlorites present in tap water.

Soluble eu collagen concentration. (a) Where sufficient quantities were available, the most convenient method was to dialyse about 100 ml. of solution and centrifuge the fibre suspension at 8000g for 30 min. This gave a fibre mass containing 3-5% of protein, which was dried *in vacuo* over P₂O₅ and weighed. (b) Alternatively the hydroxyproline content of the protein solution was determined by the method of Neuman & Logan (1950) as modified by Leach (1960). In view of the hydroxyproline content of soluble eu collagen given in Table 1, the factor taken for converting hydroxyproline into collagen content was 100/14.9. Methods (a) and (b) were in good agreement.

Isoionic pH (pI). Gelatin was deionized by passing through mixed-bed resins (Janus, Kenchington & Ward, 1951), and the pH of the emerging solution measured.

Measurements of pH. Measurements were made in the Cambridge pH meter with a glass electrode and a saturated calomel electrode (Cambridge Instrument Co. Ltd., London).

Melting and shrinkage of soluble eu collagen. The measurement of shrinkage temperature (*T_s*) of eu collagen is described by Courts (1960). For reconstituted soluble eu collagen the dialysed solids were deposited by centri-

fusing at 8000g to increase the protein proportion to about 4%. A small quantity (about 1 g.) was smeared as a thin film around the inside wall of a test tube by using a glass rod. The test tube was clamped in a boiling tube containing water so that the level of water appeared just above the film. The water temperature was then raised through 1°/min. The melting of the soluble eucollagen fibres, as indicated by the film's collapsing, was readily observed.

Precipitation of soluble eucollagen fibres

Dialysis. If the initial concentration of eucollagen in solution is greater than 0.05%, the precipitate is likely to be gelatinous in appearance.

Salting out. Solutions of soluble eucollagen at pH 2.2, 3.7 and 5.0 were treated with *m*-NaCl, *m*-Na₂SO₄, chondroitin sulphate (L. Light and Co. Ltd.) and mucoprotein preparation.

Breaking of hydrogen bonds. In experiments on the conversion of soluble eucollagen into gelatin at 20° with hydrogen-bond-breaking reagents (Courts, 1958), it was observed that small quantities of these reagents induced fibre formation. Several 10 ml. portions of soluble eucollagen (0.1%, w/v) at pH 2.2 were treated with 0.1–1.5 ml. of (1) 4*M*-sodium salicylate, (2) 4*M*-KSCN, with stirring and the mixtures allowed to equilibrate for 1 day. The turbidity developed was measured at 540 mμ (EEL portable colorimeter; green filter) and the pH of each system was measured.

Turbidity–pH relationship. Soluble eucollagen in acid solution shows turbidity when adjusted to pH 4–5. This is a reversible reaction and shows a maximum turbidity effect. A similar behaviour is shown by gelatin in water, in which instance the pH of maximum turbidity indicates the isoelectric point of the gelatin (Briefer, 1929). With salt-free material this is also the isoionic point (Janus *et al.* 1951).

A solution of soluble eucollagen (0.1%, w/v) was divided into several 10 ml. portions, each of which was adjusted with *N*-NaOH to a pH between 4 and 5. The precipitates were allowed to equilibrate for 1 day at 20°, after which turbidity and pH values were measured. Wood & Keech (1960), in experiments on the precipitation of acetic acid-soluble calf-skin collagen, found that 1.5 hr. was required for equilibrium at 25°, with similar effects at 0°. However, the partial reversal, which these authors found after 16 hr. at 0°, indicates that this may be the time required for true equilibrium. The emphasis which Wood & Keech (1960) give to a strict experimental routine in collagen precipitation work is confirmed in the present work.

The effect of varying the molarity of buffer salts, and any specific acid radical effect due to acetate, citrate, lactate, mandelate and chloride, has been examined with solutions of eucollagen prepared by the following methods: (1) a single batch of eucollagen was divided for treatment with different buffer systems or with buffers at different concentrations; (2) soluble eucollagen in 0.15 *M*-citrate was dialysed against citrate buffers of lower molar concentrations; (3) soluble eucollagen, obtained by extraction in 0.1 *M*-citric acid, was dialysed against tap water, and the dialysis residue redispersed in buffers of various concentrations.

A similar control experiment was carried out with a gelatin (0.3%, w/v) of pI 4.9 in 0.1 *M*-citrate buffers over the range pH 3–6.

Solubilization of collagen with hydrogen peroxide

At 21° in the presence of reagents breaking hydrogen bonds. Collagen (100 g.) was treated with the reagent (500 ml.) and 30% H₂O₂ (125 ml.; final concn. 1.7 *M*). The reagents used were 4*M*-sodium salicylate, 4*M*-LiBr, 8*M*-CaCl₂, expressed as final concentrations. The same reagents (625 ml.) were also used to treat the collagen in the absence of H₂O₂. In addition the effect of 1.7 *M*-H₂O₂ alone was examined. (The addition of H₂O₂ to KSCN produced a delayed but violent reaction in which S was deposited. No experiments were made therefore with this reagent.)

Experiments were carried out in closed jars at 21° for 4 weeks with occasional stirring. After straining off the solids on to nylon mesh, the solutions were filtered and dialysed.

Treatment at 60°. Collagen (100 g.) was allowed to equilibrate overnight in 1.7 *M*-H₂O₂ (500 ml.) and the mixture heated at 60° for 2½, 3 and 3½ hr. The solutions were quickly cooled to form gels, which were cut into strips and washed in running water. The residues were washed free from H₂O₂ and either heated in water at 60° or immersed in 4*M*-sodium salicylate for 1 day at 18° and dialysed. Further gels were obtained in this way.

Effect of sodium sulphate on the peroxide reaction. The stability of the collagen–Na₂SO₄ system against hydrolysis by 0.14 *N*-H₂SO₄ at 80° has been demonstrated by Deutches Gelatine Fabriken (1952) and some chemical aspects of the reaction described by Courts (1960). The effect of Na₂SO₄ on the conversion of collagen into gelatin at 60° by 1.7 *M*-H₂O₂ was examined by soaking the collagen in 20% Na₂SO₄ overnight, then adding 30% (w/v) H₂O₂ to the stated strength. The mixture was then heated at 60° for (1) 3½ hr., and (2) 4½ hr. The solutions were dialysed.

Effects of peroxide on certain amino acids. Potential alterations in the structure of certain amino acids, which are constituents of collagen and gelatin, were separately investigated by heating them in 1.7 *M*-H₂O₂ at 60° for 3½ hr. After evaporating to dryness *in vacuo*, the residue was taken up in water and examined along with controls by paper chromatography, by the descending method with butanol–acetic acid–water (4:1:5, by vol.). Serine, aspartic acid, proline and hydroxyproline, methionine, histidine and also glycylglycine were treated in this way.

Catalytic effect of peroxide in gel liquefaction. Sodium bromide and urea are listed by Courts (1958) as 'weakly active' in their relative efficiency in liquefying a gelatin gel. These reagents have now been re-examined in the presence of H₂O₂. Gelatin granules (0.5 g.) of 600±100 μ in a stoppered test tube were allowed to swell in water (5 ml.) for 30 min. at 18°. The reagent (5 ml.) at twice the indicated strength was added and the swollen mass agitated with a glass rod for a few seconds. The time taken for the gelatin gel to liquefy completely was measured.

Gel strength. This was measured at 10° on gelatin gels (5.65%, w/v) which had matured at 10° for 18 hr. according to British Standards Institution (1944) 757. A Bloom gelometer (Griffin and George Ltd., London) was used and the results were expressed in 'grams Bloom'.

Viscosity. The viscosity of gelatin was measured on sols at 5.65% (w/v) at 40° in an Ostwald-type viscometer, no. 2 [British Standards Institution (1937) 188].

RESULTS

Soluble eucollagen

Amino acid composition. The complete amino acid assay for a citric acid-soluble eucollagen preparation is given in Table 1 and compared with a typical analysis given by Eastoe (1955) for a gelatin obtained from ox bone by an alkaline pretreatment. The results represent a single analysis except for hydroxyproline, which was carried out in quadruplicate. Probable errors for the analysis are as given by Eastoe (1955). Hydroxyproline values were reproduced within 2% of the mean. In general, the results are in close agreement with analyses for bovine collagens and gelatins (Eastoe, 1955; Eastoe & Leach, 1958). Two significant differences are discussed below.

N-Terminal amino acid analysis. Values obtained for citrate-soluble eucollagen in residues/100 kg. of protein gave a total of 1.62 N-terminal amino acid residues. Assuming one N-terminal residue for each protein chain, this total is equivalent to a number-average chain weight (C_n) of 62 000 (defined in Courts & Stainsby, 1958). Similar values were obtained for the eucollagen (C_n , 62 000) before citrate-buffer treatment, and for the gelatin (C_n , 60 000) derived from the same eucollagen sample by hydrothermal shrinkage at 50°.

Melting and shrinkage temperatures. With a reconstituted form of soluble eucollagen, pH 6, the smeared film had an opaque appearance. On rais-

ing the temperature the opacity was retained. The fibres melted sharply at 43° to form an opaque liquid. A sharp-melting phenomenon is contrary to usual observations on collagen or eucollagen. The melt did not set to a gel on cooling, indicating that, at this stage, transition to gelatin had not occurred. This was confirmed by redispersing (1 g.) in 0.1M-citric acid and measuring the viscosity at 20°. The unheated fibres were similarly redispersed and measured as a control. The 43° melt gave a relative viscosity ($\eta_{rel.}$) of 8.8 approximating to the control $\eta_{rel.}$ 7.5. Both of these dispersions, when heated to 44°, gave $\eta_{rel.}$ 1.2, measured at 20°. The results clearly show the 43° melt to have retained a collagen-type structure.

On raising the melt to 44° or above, it clarified and set to a gel on cooling. As a result of the transition to gelatin the viscosity in 0.1M-citric acid was considerably reduced ($\eta_{rel.}$ 1.2).

The isoionic pH. The mixed-bed deionization technique of Janus *et al.* (1951) is unsuitable for determining the isoionic pH (pI) of a protein solution, such as soluble eucollagen, which relies upon the environmental ions for solvation. It is reasonable to assume, however, that the transformation of soluble eucollagen into gelatin at neutrality would not involve alterations to charged groups. The derived gelatin was deionized and found to be isoionic at pH 4.8, a value in accord with the low amide-nitrogen value for soluble eucollagen given in Table 1. A similar procedure was adopted by Jackson & Neuberger (1957) for determining the pI of rabbit-skin soluble collagen.

Turbidity studies

Relationship with pH. The development of turbidity in dilute solutions of gelatin in water is a function of pH and is maximal at the isoionic pH (Briefer, 1929; Janus *et al.* 1951). A similar turbidity-pH relationship has been noted with soluble eucollagen dispersed in certain buffer solutions and over a range of buffer concentrations. The results showing the pH of maximum turbidity are summarized in Table 2.

Citrate. The development of turbidity in soluble eucollagen-citrate is confined to the range pH 4-4.6 for 0.15M-buffer, with a well-defined maximum at pH 4.34. This is illustrated in curve A, Fig. 1. Of the solvents examined here, citrate is unique in showing a single narrow peak. In addition, curves prepared from results with 0.05M-, 0.075M- and 0.125M-citrate were indistinguishable from curve A, Fig. 1. The alkali-processed gelatin in 0.1M-citrate showed no turbidity from pH 3 to 6. Thus the citrate has inhibited the isoelectric behaviour in gelatin.

Acetate. Soluble eucollagen in 0.15M-acetate gave the broader turbidity-pH range of the type

Table 1. *Amino acid composition and pI of soluble eucollagen and peroxide precursor gelatin*

Values are given as g. of amino acid/100 g. of dry ash-free protein.

	Soluble eucollagen	H ₂ O ₂ extracted	Bone gelatin (Eastoe, 1955)
Alanine	11.6	10.9	11.3
Glycine	26.1	26.7	27.2
Valine	2.4	2.5	2.7
Leucine	3.4	3.5	3.4
Isoleucine	1.4	1.4	1.5
Proline	16.3	15.0	15.5
Phenylalanine	2.2	2.3	2.4
Tyrosine	0.15	0.5	0.2
Serine	3.9	5.0	3.7
Threonine	2.3	3.0	2.3
Methionine	0.6	0.2	0.6
Arginine	8.6	9.1	9.0
Histidine	0.5	0.4	0.70
Lysine	4.7	4.0	4.3
Aspartic acid	6.3	7.1	6.7
Glutamic acid	10.8	11.8	11.6
Hydroxyproline	14.9	13.8	13.3
Hydroxylysine	0.8	0.9	0.7
Total	116.9	118.1	117.6
Amide N	0.10	0.6	0.24
pI	4.8	5.7	4.9

shown in curve *B*, Fig. 1. Although a reasonably well-defined maximum occurred at pH 4.4, secondary influences are evident about pH 4.8.

Mandelate. Where 0.15M-mandelic acid was the solvent for soluble eucollagen, the curve resembled that for acetate.

Lactate. The results were similar to curve *B*,

Table 2. Summary of results for turbidity maxima

Solvent	Molarity	Method of solution	pH of max. turbidity
Citrate	0.05	1	4.30
	0.075	1	4.30
	0.10	1	4.35
	0.125	1	4.33
	0.15	1	4.36, 4.32, 4.34
	0.04	2	4.22
	0.06	2	4.36
Mandelate	0.15	1	4.36
HCl	0.01	1	4.38
Acetate	0.15	1	4.35
Lactate	0.05	3	4.25-4.45
	0.085	3	4.3-4.5
	0.10	3	4.33
	0.15	3	4.4 (approx.)

Fig. 1. Turbidity change with pH of soluble eucollagen. Curve *A* represents the changes obtained in 0.05M-, 0.075M-, 0.125M- and 0.15M-citrate buffers. Curve *B* represents the changes obtained in 0.15M-mandelic acid. Changes of systems in 0.2M-acetate, 0.15M-lactate buffers and in 0.01N-HCl (0.05M-NaCl present) were similar to curve *B*.

Fig. 1. The turbidity maximum was reasonably defined at pH 4.3 ± 0.1 where the lactate concentration was 0.15M or 0.1M. Below this concentration the turbidity maximum was more difficult to define.

Hydrochloric acid-sodium chloride. In this experiment soluble eucollagen had been extracted in 0.01N-HCl and NaCl equivalent to 0.05M added to facilitate pH adjustment. The turbidity-pH relationship was again similar to curve *B*, Fig. 1, in which the maximum at pH 4.38 resembled the curves obtained with eucollagen in organic acids. The secondary effects, however, cover a considerably broader pH range.

Hydrogen-bond-breaking reagents

Potassium thiocyanate. KSCN (4M) was added to soluble eucollagen in 0.1M-citric acid, pH 2.2, to give concentrations up to 0.8M. All the systems remained at pH 2.2. It was clear from the fibre formation that transition to gelatin had not taken place and there was no indication of such a change up to 7 days. The turbidity-KSCN concentration relationship is shown in Fig. 2. A steep increase in turbidity was observed from 0.2M- to 0.4M-KSCN followed by a gradual turbidity decrease between 0.4M- and 0.8M-KSCN.

It is probable that pH 2.2 represents a favourable condition for demonstrating KSCN-induced turbidity. The maximum turbidity effect at this pH value was obtained with 0.4M-KSCN and this concentration was used to demonstrate declining turbidity with increasing pH, obtained by additions of N-NaOH up to pH 5. The results are shown in Fig. 2. In this instance, a steep fall in turbidity occurs in the range pH 4-4.6, quite contrary to the effect when KSCN is absent. This turbidity change with pH is reversible in both cases.

Sodium salicylate. The addition of small quantities of 4M-sodium salicylate to soluble eucollagen in 0.1M-citric acid resulted in sharp turbidity changes. The turbidity value reached a maximum at 0.12M-salicylate, at which concentration the system was at pH 3.9. Further additions of salicylate caused the turbidity to diminish so that at 0.4M and pH 4.3 the solution had almost cleared (see Fig. 2). If a further small volume of 4M-sodium salicylate was added and the mixture kept for a few minutes, a felted mass of salicylic acid in characteristic needle-shaped crystals slowly deposited. This last stage demonstrated that, with the higher concentration of sodium salicylate, the soluble eucollagen had the property of behaving as a protective colloid so that the slow crystallization of salicylic acid resulted. The addition of small quantities of 4M-sodium salicylate to 0.1M-citric acid in the absence of soluble eucollagen resulted in the immediate precipitation of salicylic acid.

Fibre formation with sodium sulphate and chondroitin sulphate. Fibrous precipitates are formed from acid solutions of soluble eucollagen by NaCl and Na₂SO₄ when the solution is below pH 5. The difficulty experienced in washing a precipitate free from Na₂SO₄ suggests a degree of ionic complex formation. Fibrous precipitates were obtained with an osseomucoid preparation (Courts, 1959) and chondroitin sulphate below pH 5. The latter precipitate appeared to be a complex of approximately equal proportions by weight of soluble eucollagen and chondroitin sulphate.

Fig. 2. Turbidity changes of soluble eucollagen in 0.1M-citric acid with added reagents: ○, 4M-KSCN; ▲, 0.4M-KSCN, soluble eucollagen system altered with N-NaOH; ●, 4M-sodium salicylate. The numbers alongside the curves show the pH at the points indicated; with 4M-KSCN pH 2.2 was maintained throughout.

Solubilization of collagen with hydrogen peroxide

Treatment at 21°, with hydrogen-bond-breaking reagents. Only negligible amounts of collagenous protein were dissolved by 1.7M-H₂O₂ at 21°. The collagen-H₂O₂ system equilibrated at pH 5.5. The same treatment had very limited action in converting the collagen into eucollagen since only 9%, washed free from H₂O₂, was converted into gelatin at 60° in 3½ hr. Certain of the highly active hydrogen-bond-breaking reagents were more successful in solubilizing collagen, particularly 4M-LiBr (see Table 3), but, as already indicated by Courts (1958), 8M-urea is comparatively ineffective.

The presence of 1.7M-H₂O₂ with all the hydrogen-bond-breaking reagents examined had a marked effect on collagen solubilization and, with sodium salicylate, almost complete conversion into gelatin was obtained within 4 weeks (Table 3). The pH value of 1.7M-H₂O₂ was altered by the addition of 8M-urea or 4M-sodium salicylate, becoming pH 8 in both instances.

Treatment at 60°. When the reaction with 1.7M-H₂O₂ was performed at 60°, collagen was converted into gelatin, the amount converted being proportional to the time of heating. The gelatin, once formed, was then subject to degradation at 60° by the H₂O₂. The collagenous residue washed free from H₂O₂ yielded further quantities of gelatin, either in water at 60° or in 2M-sodium salicylate at 18°. The gelatins obtained during the second extraction generally showed superior physical properties to those obtained in the presence of H₂O₂. The yield and properties are given in Table 4. The presence of Na₂SO₄ completely inhibited the transformation of collagen into gelatin in 1.7M-H₂O₂ at 60°. This was shown in both the 3½ and 4½ hr.

Table 3. Effect of 1.7M-hydrogen peroxide on the solution of collagen in lyotropic salts at 21°

Reagent	Collagen dissolved by the reagent (%)		Gelatin viscosity obtained via reagent in H ₂ O ₂ (cP)
	In water	In H ₂ O ₂	
4M-LiBr	17	48	3.5
4M-Sodium salicylate	3	80	4.1
8M-CaCl ₂	3	53	4.2
8M-Urea	1	58	—
1.7M-H ₂ O ₂	1	—	—
8M-Urea + M-citric acid	4	—	—

Table 4. Properties of peroxide precursor gelatins and controls

1st extractions were made in 1.7M-H₂O₂, 60° for the indicated times; 2nd extractions were made in 4M-sodium salicylate at 20° for 1 day.

	2½ hr.		3 hr.		3½ hr.		3 hr., 60° Water	3½ hr., 60° 0.1N-HCl
	1st	2nd	1st	2nd	1st	2nd		
Gelatin yield (%)	22	18	36	17	49	20	2	30
Viscosity (cP)	3.8	6.8	3.2	7.3	2.8	6.3	—	3.7
Gel strength (g. Bloom)	179	203	188	206	152	185	—	230
H ₂ O ₂ content (% w/v)	0.7	Nil	0.8	Nil	0.8	Nil	—	—

Table 5. *N-Terminal amino acid residues of heated collagen*

Measurements were made on washed, reagent-free samples. Values are expressed as mole/100 000 g. of collagen.

	Treatment				
	1.7M-H ₂ O ₂ 2 hr., 60°	H ₂ O ₂ then water 2½ hr., 60°	Water 3 hr., 60°	0.14M-H ₂ SO ₄ - Na ₂ SO ₄ , 80° 40 min.*	Soluble eucollagen, citrate extract
Glycine	0.21	0.23	0.18	0.35	0.86
Serine	0.08	0.10	0.04	0.05	0.16
Threonine	0.08	0.13	0.02	0.08	0.12
Alanine	0.07	0.05	0.00	0.10	0.10
Aspartic acid	0.06	0.06	0.03	0.05	0.14
Glutamic acid	0.11	0.10	0.01	0.08	0.13
Others	0.10	0.05	0.00	0.05	0.10
Total	0.71	0.72	0.28	0.76	1.62
10 ⁻³ C _n	139	138	350	132	62

* From Courts (1960).

Table 6. *Effect of peroxide on lyotropic action in gels*

Reagent	Time for complete gel liquefaction at 18° (min.)	
	In water	In 1.7M-H ₂ O ₂
None	>2000	>2000
2M-Urea	>2000	12
M-NaBr	>2000	30
2M-NaBr	20	4
0.5M-Sodium salicylate	>2000	35
0.7M-Sodium salicylate	80	15
0.9M-Sodium salicylate	18	8

reactions. The yield of gelatin was less than 1% compared with 54 and 61% respectively when Na₂SO₄ was absent.

Analyses of the *N*-terminal amino acid residues were made on the insoluble fraction of collagen after heating in 1.7M-H₂O₂ at 60° and the insoluble fraction after further heating in water at 60°. The fractions gave results (see Table 5) equivalent to C_n 139 000 and C_n 138 000 respectively, values which closely resemble the C_n 132 000 for a similar collagen heated in 0.4N-H₂SO₄ in the presence of Na₂SO₄ (Courts, 1960).

In a gelatin prepared by the action of 1.7M-H₂O₂ on collagen at 60° (Table 1), certain amino acids appear to have been affected by H₂O₂ treatment. Thus the methionine content has been considerably decreased and the proportion of histidine is also lower. On a gram-residue basis both of these decreases are equivalent to 4½ and 3½ g.residues/10⁵ g. of gelatin respectively. The hydroxy amino acids have higher contents, serine increases by 7 and threonine by 4½ g.residues/10⁵ g. of gelatin.

Of those amino acids that were subjected to 1.7M-H₂O₂ at 60°, no alteration could be detected by paper chromatography with glycine, serine, proline, hydroxyproline or aspartic acid. Glycylglycine was also unaffected. On the other hand,

methionine completely disappeared and was replaced by a spot corresponding to threonine. The histidine spot was reduced in intensity when compared with the control and a second spot corresponding to serine accompanied the histidine on the chromatogram.

Effect of peroxide on gelation

Table 6 shows that where a 'weakly active' hydrogen-bond-breaking reagent is used at too low a concentration, the liquefying effect on a gelatin gel is negligible. The addition of H₂O₂ greatly increased the efficiency of the lyotropic reagents. This was also seen with 2M-NaBr which liquefied the standard gel five times more quickly in the presence of 1.7M-H₂O₂. No lyotropic action could be detected with H₂O₂ alone up to 4M.

DISCUSSION

Two soluble fractions that are fundamentally different in character may be derived from collagen. These are gelatin and acid-soluble collagen. The conversion of ox-bone collagen (C_n 350 000) into eucollagen (C_n 62 000) enhanced the gelatin yield from 2 to 100% (as defined by Courts, 1960) and the dispersion into 0.1M-citric acid from about 0.2 to about 30%. When eucollagen is solubilized no significant change in C_n occurs whether the resultant form is gelatin or soluble eucollagen and hence no primary bonds are broken in either process. This may be illustrated by:

The enhanced solubility of eucollagen into a fibre-forming material with a reduced specific viscosity equivalent to soluble collagens (Courts &

Stainsby, 1959) suggests the reversion of developed collagen into a type of fundamental unit.

The amino acid composition of soluble eucollagen is in general similar to the analyses obtained by Eastoe (1955) and Eastoe & Leach (1958) for bovine collagens and gelatins. Two differences are of possible significance. The hydroxyproline value at 14.9% is appreciably above that of 14.4% obtained for a purified gelatin (Eastoe, 1955) and higher than the values of 12.6–14.3% generally reported. A hydroxyproline value of 14.9% has recently been reported by Eastoe (1960) for a gelatin that had received an unusual pretreatment. The implications that a high hydroxyproline content indicates a high degree of purity in the collagen group of proteins has been discussed by Courts & Kragh (1960). Most of the tyrosine found in native collagen (about 1%) may be regarded as contributed by the non-collagen impurities and this is indicated by the appreciably lower tyrosine values reported for gelatin (0.2–0.6%) (Bowes, Elliott & Moss, 1953; Eastoe & Leach, 1958). However, Gross (1957) finds that dissolved collagens always have at least 0.3% of tyrosine, so that the low tyrosine value of 0.15% for soluble eucollagen would suggest it to be particularly free from impurity. Although the methods used in the preparation of soluble eucollagen are likely to be very effective in removing extraneous materials, the method might have given rise to a collagen fraction with an atypical amino acid composition.

The hydrothermal conversion of soluble eucollagen fibres into gelatin involves two stages. The first is a sharp-melting phenomenon. At this stage, the inability of the 43° melt to gel and the high viscosity of its citric acid solution (Gallop, 1955; Doty & Nishihara, 1958) indicates that the molecular organization associated with soluble collagens has not been disrupted. The interchain forces disrupted are likely to be of the van der Waals type and this change is mainly reversible.

The second stage at 44° is molecular disorientation and transition to gelatin. This change is for the most part irreversible. Although these observations were made on reconstituted eucollagen fibres, in which water represents about 95% of the total mass, it is possible that similar, but less well-defined, stages occur with native collagen. Flory & Garrett (1958) and Witnauer & Fee (1959) regard the shrinkage of collagen to be purely a melting phenomenon. Witnauer & Fee (1959) have predicted that, if melting is the uncoiling of helices, then the temperature for the shrinkage of intact collagen in water should be very nearly that for soluble collagen (i.e. isolated helices) at the same pH. This view is exactly confirmed with eucollagen in which the shrinkage temperature of 44° in water (Courts, 1960) is the same as that of the reconsti-

tuted soluble eucollagen at the same pH. However, the initial melting of the reconstituted fibres at 43° cannot be regarded, by itself, as the uncoiling of the collagen helix. Any subsequent molecular disorientation is likely to be strongly dependent upon environment and, as such, this stage would be the rate process in native collagen, which Weir (1949) suggested.

The development of turbidity in citrate extracts of eucollagen between pH 4 and 5 is best explained as isoelectric behaviour. The production of maximum turbidity at pH 4.33 (± 0.03) would therefore correspond with the isoelectric point (pE). The shift, $pI - pE = -0.5$, which has been induced by citrate ions (or molecules) is small compared with the value of -4 determined by Jackson & Neuberger (1957) for acid-processed gelatins. The small shift is, however, predictable from the known strong buffering capacity of alkali-processed gelatin (pI about 4.8) in the isoelectric region (Kenchington & Ward, 1954) where addition or removal of hydrogen ions incurs a relatively small pH change.

The precipitation of fibres of soluble eucollagen by dilute forms of hydrogen-bond-breaking reagents is of some interest, since similar quantities incorporated into a gelatin gel, a largely hydrogen-bonded system, would interfere with gelation. Thus, even in a concentrated (5.65%, w/v) gel, 0.1 M-sodium salicylate reduced the gel strength by 17% and 0.1 M-potassium thiocyanate reduced the strength of the same gel by 10% (A. Courts, unpublished work). The turbidity induced by potassium thiocyanate in soluble eucollagen is somewhat different from isoelectric behaviour and the charge distribution along the protein molecule is unaltered. About half the molar concentration of salicylate is required to produce the same maximum turbidity effect as potassium thiocyanate. A similar relationship occurs in the results of Brown *et al.* (1958) in T_g depression studies. The reconstitution of fibres from solubilized collagens by salicylates or thiocyanates has not previously been reported. The ability of soluble eucollagen to reconstitute as fibres by dialysis, by salting out and by forming complexes with chondroitin sulphate indicates a structural similarity with soluble collagens, in support of the reduced specific viscosity data of Courts & Stainsby (1959). The electron micrographs of reconstituted soluble eucollagen from human bone (Little *et al.* 1961) did not reveal any banded structure.

Reactions between proteins of the collagen group and 'oxidizing agents' has been studied in most detail with sodium periodate (Desnuelle & Antonin, 1946; Grassmann & Kuhn, 1955; Gustavson, 1958; Hörmann & Fries, 1958; Hörmann, 1960). Investigations with hydrogen

peroxide appear to be more limited. Orekhovich & Shpikiter (1957) state that 6M-hydrogen peroxide irreversibly modifies procollagen into simpler components which may be related to gelatin. There appears to be little evidence to indicate that changes that these 'oxidizing agents' bring about in collagen are due to oxidation of the molecule or that periodate and peroxide behave in the same way.

Bovine collagen is not solubilized in 1.7M-hydrogen peroxide at 20°, but undergoes extensive solubilization when a hydrogen-bond-breaking mechanism is used in conjunction with the peroxide. This mechanism can involve either heat or certain lyotropic reagents. In both instances the collagen is converted into gelatin. It appears likely that the course of this conversion is, in the initial stages, similar to the collagen into eucollagen reaction (Courts, 1960) as instanced by the substantial gel-forming and viscometric properties. It is interesting that, whereas in the presence of 0.7M-hydrogen peroxide at 60°, gelatin has undergone some secondary degradation, the collagenous residue has not and this residue undergoes a further conversion into high-grade gelatin in the absence of hydrogen peroxide, either in water at 60° or in 2M-sodium salicylate at 18°. This reaction presents further evidence that eucollagen can retain molecular integrity through hydrogen bonding against a reagent that will degrade the molecularly disorientated form, gelatin. This was implied by Courts (1960) in connexion with acids and alkalis. The collagen-1.7M-hydrogen peroxide system equilibrates at pH 5.5. When 8M-urea was introduced to lead to extensive solubilization of the collagen the system was at pH 8. When 4M-sodium salicylate was used to solubilize the collagen, the system was again pH 8. It is considered unlikely that hydrogen ions play a major part in these reactions since the pH of the systems is generally near neutrality. Hörmann (1960) has reported the dissolution of collagen by hydroxylamine in conjunction with lithium bromide or potassium thiocyanate, but not alone.

Kalyankar, Vaidyanathan & Giri (1955) have studied the action of 0.1M-hydrogen peroxide on certain amino acids in the presence of iron salts at 25°. They explain their results in terms of the dissociation of peroxide as free radicals (Haber & Weiss, 1934). The disorientation of collagen by a hydrogen-bond-breaking mechanism induced by free hydroxyl radicals would be a useful model to collate the apparently parallel reactions of collagen with hydrogen peroxide and hydroxylamine in the presence of lyotropic salts and of sodium periodate and hydrogen peroxide at elevated temperatures.

Little *et al.* (1961) have investigated by electron microscope the irradiated collagen described by

Bowes *et al.* (1960). With hydrated material, the fibre bundles coalesce and become structureless. The production of free hydroxyl radicals in aqueous systems by ionizing radiation points to an analogy with the mechanism proposed for the collagen-peroxide system.

SUMMARY

1. Controlled alterations to the structure of bone collagen by alkali has led to increased solubility in acid buffer solutions, resembling a reversion of the developing processes.

2. In this solubilized form, the collagen-type structure has been maintained, as shown, for example, by the ability to re-form fibres. After its derivation from eucollagen, this form is termed 'soluble eucollagen'.

3. Soluble eucollagen was isoionic at pH 4.8 and gave an ultimate isoelectric point at pH 4.3 in the presence of acetate, citrate, lactate and mandelate solutions.

4. The amino acid analysis indicated a typical collagen of high purity.

5. Hydrothermal melting and disorientation of reconstituted soluble eucollagen occurred in two distinct stages.

6. Hydrogen peroxide dissolved intact collagen at 60° to give, initially, high-molecular-weight fragments. Only limited oxidation occurred.

7. Hydrogen peroxide catalysed the conversion of collagen into gelatin at 21° by hydrogen-bond-breaking reagents, e.g. sodium salicylate.

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The Action of Anions on Catalase Peroxide Compounds

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Catalase, like other haemoproteins, shows two kinds of chemical behaviour in respect of its haem iron, namely complex-formation and oxidation-reduction. The role of these two types of reaction in the catalytic activity (both catalatic and peroxidatic) of this enzyme has been the subject of some controversy. According to Chance (cf. Chance & Fergusson, 1954), the intermediates involved in the catalytic reactions are essentially complexes (similar to the fluoride or cyanide derivatives);

according to George (1952, 1953*a*), they should rather be considered as higher oxidation states.

The chemical nature of these intermediates (catalase peroxide compounds I and II) can be most usefully investigated by examining the reactions they undergo. The best-known group of such reactions comprises those with hydrogen donors, the behaviour of which has been analysed in terms of six reaction types (Keilin & Nicholls, 1958; Nicholls, 1959). These six types of donor