Citrate-Promoted Helix Formation in Gelatin

2. THE STRUCTURE OF SOME PRECIPITATED GELATINS*

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The modification of the shape of gelatin molecules in dilute solution brought about by citrate and certain other buffers containing aliphatic carboxylic acids is a general reaction for gelatins (Courts, 1962). It was suggested that a slow transformation from a random-coil configuration to a more rigid structure was involved and that, in favourable circumstances, the degree of molecular order was such as to impart properties resembling those of soluble collagen. One such property would be the precipitation of fibres from gelatin-citrate by methods and reagents leading to fibre formation in solutions of collagen-citrate and eucollagen-citrate, e.g. sodium chloride (which interferes with solvation), neutral-salt reagents (which break hydrogen bonds), alcohols and dialysis. It is often observed with reconstituted collagens that what appears macroscopically to be a fibrous deposit may not necessarily correspond to a well-defined microfibrillar structure when viewed in the light-microscope or electron microscope. In many instances macroscopic fibres are seen, on magnification, to be structureless. Thus the gross demonstration of fibre formation or of turbidity is not sufficient to establish a microfibrillar structure, particularly when the protein is heavily hydrated.

Two forms of collagen are distinguishable by the electron microscope (Little, Kelly & Courts, 1962) as either fine or tubular fibrils, thus confirming the detection of two X-ray-diffraction patterns in human-bone collagens by Kelly, Little & Courts (1959). Although the closely analogous wide-angle X-ray-diffraction patterns given by collagen, eucollagen and gelatin indicate a fundamental molecular structure that is unchanged in passing from one modification to another, the electron microscope shows differences. Thus, with reconstituted soluble collagens, a characteristic banded structure with regular spacings about 640 Å apart is invariably detected. These collagens probably resemble the tubular fibrils of bone collagen. The soluble fraction from eucollagen, identified as of

fine fibril form, shows no evidence of this banded structure (Reed & Stainsby, 1961).

In the present work, a gelatin prepared from mature collagen was used in which the large viscosity-time effect (Courts, 1962) suggested that the chance of the formation of tubular fibrils was high.

MATERIALS AND METHODS

Three of the procedures used for attempts to form fibres from gelatin followed the classical methods of protein chemistry: these were dialysis, salt precipitation and alcohol precipitation. In addition, we examined the effects of chondroitin sulphate (Jackson, 1953) and the hydrogen-bond-breaking reagents potassium thiocyanate and sodium salicylate at low molar concentrations (Courts, 1961): all of these substances are known to be effective precipitants of soluble collagens.

A 0.25% solution of acid-processed-pigskin gelatin, purified by a propan-2-ol-fractionation method (Courts, 1959), was prepared. This was kept at 20° in 0.15 M-citrate buffer, pH 3.7, for 12 days. Portions (100 ml.) were then treated as follows:

- (1) The solution was dialysed for 3 days against running tap water. After 1 day the solution developed a turbid appearance indicative of incipient fibre formation, but instead of the formation of the expected fibrous deposits by the third day, small gel aggregates, which settled by gravity, appeared. Then (a) part was freeze-dried, and (b) the remainder was dried under vacuum.
- (2) An equal volume of 30% (w/v) sodium chloride solution was added to the gelatin solution, giving a turbid suspension.
- (3) M-Potassium thiocyanate (50 ml.) was added, giving a fibrous precipitate.
- (4) M-Sodium salicylate (50 ml.) was added, also giving a fibrous precipitate.
- (5) Chondroitin sulphate [50 ml. of a 1% (w/v) solution] was slowly added with stirring, giving a fibrous precipitate. Chemical analysis showed that the collagen and chondroitin sulphate had been precipitated together and X-ray diffraction showed that this precipitate was a mixture of the two components.
- (6) Propan-2-ol was added in 15 amounts of 10 ml. with shaking and cooling of the solution. The precipitate was filtered, washed with propan-2-ol and dried *in vacuo*. X-ray diffraction showed that the salts had not been completely removed, but only the areas in preparations

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free from crystals were photographed under the electron microscope.

For examination by the electron microscope, specimens were fixed in 10% (w/v) neutral formalin. There is evidence that, in the presence of peptide bonds, formalin produces cross-linking (Little, 1959), but comparison of a large series of specimens, fixed and unfixed, has shown no changes in the X-ray-diffraction pattern that could be attributed to the action of formalin. The fixed specimens were embedded in butyl/isobutyl methacrylate and sectioned, and the embedding medium was removed with pentyl acetate. This is a method that has been used for collagenous tissues because it is necessary for adequate interpretation to remove the embedding medium, and it has been found that with this particular methacrylate and solvent no internal stresses are set up in the specimen (Little, 1961). The use of methyl methacrylate or ethyl methacrylate in the embedding medium, or a more rapid solvent, can cause considerable damage to the specimens. Stereoscopic electron micrographs were taken. Some sections were lightly shadowed before examination because, although such a method of preparation (which consists of coating the surface of the section with a thin film of metal) obscures many specimen details, it was thought that emphasis of the surfaces of cut sections might produce additional information. Plate 1(e) and (i) are taken from nickel-shadowed sections.

RESULTS

Freeze-dried material. Sections of this material showed a very coarse network (Plate 1a). Occasionally more solid areas were observed, but without any apparent internal structure. Very infrequently, in areas that had the appearance of having been pulled out, a tendency to ribbon or fibre formation was observed. Stereoscopic viewing suggested that such 'fibres' were of a tubular type, but there was no appearance of cross-banding.

Material dried under vacuum. This method of dehydration resulted in more solid aggregates. These were usually almost structureless (Plate 1b), but sometimes showed a tendency to form sheets (Plate 1c). Very occasionally such sheets showed a tendency to crack and crinkle, though this crinkling was irregular and had a completely different periodicity from the bands seen in collagen.

Material precipitated with sodium chloride. This gelatin was very hard and tough, and difficult to section. The appearance in section was of a non-fibrillar material similar to the appearance of the specimens dried under vacuum (Plate 1b and 1c).

Material precipitated with potassium thiocyanate. The gelatin produced by this method of precipitation was a fine fibrillar felt-work. Individual pieces of precipitate were bordered by a non-fibrillar modification (Plate 1d). In many details the appearance of the fibres precipitated with potassium thiocyanate was similar to that of fibres precipitated with sodium salicylate and

chondroitin sulphate. Plate 1(e) and 1(f) could be used to illustrate all three.

Material precipitated with sodium salicylate. Plate 1(e) shows a typical non-orientated fibrillar felt, and Plate 1(f) a similar felt with rather more orientation. Like the gelatin precipitated with potassium thiocyanate, that precipitated with sodium salicylate had borders to the pieces, but these were broader and showed the presence of some fibres (Plate 1g). Of the methods used, precipitation with sodium salicylate produced the greatest degree of orientation (Plate 1h). In Plate 1(h) an area has been chosen in which the direction of orientation is almost in the plane of the section. Large areas could be found with this degree of orientation. These fibrils still have a felt-like appearance, but in places there were indications of the collagen cross-banding. This was most clearly seen in some of the less well orientated regions, as shown in Plate 1(i).

Material precipitated with chondroitin sulphate. This precipitated gelatin, though the polysaccharide was still present, showed the same general characteristics of a felt-like fibrillar network. There was none of the definite border regions seen after precipitation with potassium thiocyanate or with sodium salicylate. The appearance of orientated regions was of the type shown in Plate 1(j). Plate 1(k) shows small cracks or discontinuities, fairly frequently observed, that were also seen in the border regions of the specimens treated with sodium salicylate (Plate 1g). They do not seem to be preparation artifacts, and are similar in appearance to small channels that can be observed in sections of anlage cartilage.

EXPLANATION OF PLATE 1

Plate 1. Electron micrographs of fibre networks deposited from a reverted gelatin. Magnification: $\times 2100$. a, Material dialysed and freeze-dried; b, material dialysed and dried under vacuum, showing complete absence of structure; c, material dialysed and dried under vacuum, and demonstrating sheet formation; d, material precipitated with KSCN, showing a fibrillar network with a non-fibrillar border; e, material precipitated with sodium salicylate, showing a typical non-orientated area (nickel-shadowed section); f, material precipitated with sodium salicylate, showing improved orientation; g, material precipitated with sodium salicylate, showing a pattern of fibril formation with a non-orientated border; h, material precipitated with sodium salicylate, showing a region of extensive orientated network; i, material precipitated with sodium salicylate, showing cross-banding in a poorly orientated region (nickel-shadowed section); j, material coprecipitated with chondroitin sulphate showing an orientated, fibrillar network; k, material coprecipitated with chondroitin sulphate, showing appearance of small channels; l, material precipitated with propan-2-ol, showing a definite fibrillar network without orientation.

Material precipitated with propan-2-ol. This precipitated gelatin was of a different type from the preceding ones. The fibres were more definite, though with little preferred orientation. Plate 1(l) is typical of the results observed. These fibres showed various diameters, as is observed in naturally-occurring collagenous tissues, and the cross-banding was definite.

Material obtained in the absence of citrate (control conditions). No fibres have been obtained with gelatin under the above series of conditions in the absence of citrate.

DISCUSSION

Investigations of the viscosity of dilute solutions of buffered gelatins (Courts, 1962) suggested that in citrate buffer the gelatin reverted to a more ordered configuration. Of the many ways of confirming such a change, the precipitation of fibres together with a study of their structures by the electron microscope appeared likely to provide the most convincing evidence for molecular order. The seven precipitation conditions used in the present work gave three distinct fibre forms. In considering these fibre forms it is important to recognize that the precipitates, like the reconstituted collagens, are heavily hydrated so that the protein component is never more than 1% by weight. The role of such water of hydration may therefore be critical to the final structure of the precipitated fibre.

Water can be stabilized in biological materials (so-called 'bound water') through hydrogen-bonding. In this form the water molecules are orientated (Berendsen, 1962). With collagen-water systems it seems most likely that the protein molecules are hydrogen-bonded through orientated water shells. The strength of such a protein...H...water...H...protein bridge would be weaker than a protein...H...protein bridge, the energy required to disrupt it being inversely proportional to the size of the water shell. Our observations with hydrated-collagen and hydrated-gelatin systems suggest that the behaviour of various hydrogen-bond-breaking reagents is dependent on the quantity of bound orientated water between protein molecules. Disorientation of the water component should, in fact, allow the subsequent formation of stronger hydrogen-bonded regions.

The first three methods used, dialysis followed by freeze-drying or by drying under vacuum, and precipitation with sodium chloride, failed to give a fibrillar structure. These processes have in common the displacement of citrate with the consequent rendering of groups available for the formation of hydrogen bonds with water, no further ordering of molecules having occurred. Some loss of orientation is also possible and

certainly the precipitates formed do not correspond to true fibre formation.

Potassium thiocyanate and sodium salicylate are both reagents recognized as being capable of breaking hydrogen bonds. They would prevent immediate hydrogen-bonding of protein to water and, at low concentrations, would so allow more ordering to take place. The stronger hydrogen bonds between molecules would then have the opportunity to form. Sodium salicylate was more effective in causing orientation. The reason for this may be either that sodium salicylate forms an ionic complex with the gelatin [cf. Brown, Conden & Glynn (1958) for a similar observation on collagen salicylate] or that it was used at a concentration nearer to that necessary for maximum precipitation. The effectiveness of these hydrogen-bond-breaking reagents is a function of concentration, as was shown for the action of phenol on nylon by Little (1959), and a threshold concentration may be expected below which they lose their influence. Chondroitin sulphate, even though it was precipitated with the collagen, had the same general effect, and so it may possibly, under certain conditions, behave as a hydrogen-bond-breaking reagent. Likewise propan-2-ol effectively removes adsorbed and hydrogen-bonded water, and the present results suggest that this is a condition for the production of smooth fibres with regular bonding. Once the strong regions of hydrogen-bonding between molecules are formed, they would not readily be broken by subsequent wetting.

Collagen, as recognized histologically in mammalian tissues, has three main components: the protein collagen, at least one mucopolysaccharide (Partridge, 1948), and lipoprotein (Leach, 1958). The present results suggest that, in addition to their other recognized functions, the mucopolysaccharides may have the necessary role of breaking hydrogen bonds to allow fibre formation. Citrate has been found in many collagenous tissues. Bone, which is frequently mineralized shortly after fibre formation is completed, may contain up to 1% by weight of citrate, which suggests that it has been trapped there after the fibre formation was completed. It has often been suggested that the citrate in bone is related to calcification (Neuman & Neuman, 1958), but convincing evidence has not been produced. The present work suggests that citrate may have an important role in the production of collagen fibrils.

SUMMARY

1. Electron micrographs have demonstrated fibre formation in an acid-processed gelatin which had reverted to a collagen-type of structure in citrate.

2. The type of fibre produced depends on the method or reagent of precipitation.

3. The possible function of citrate in bone formation is discussed.

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Microbial Growth on C₁ Compounds

5. ENZYME ACTIVITIES IN EXTRACTS OF *PSEUDOMONAS* AM1*

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Studies on the labelling of intermediates during the short-term incubation of methanol- and formate-grown *Pseudomonas* AM1 with [¹⁴C]methanol and [¹⁴C]formate (Large, Peel & Quayle, 1961) showed that the first intermediate to acquire radioactivity from the respective radioactive growth substrates was serine. As a result of studies on the labelling patterns of radioactive compounds isolated from short-term incubations, a scheme was proposed (Large, Peel & Quayle, 1962*a*) for the formation of C₃ and C₄ compounds by a pathway involving (a) the hydroxymethylation of glycine to give serine, and (b) the carboxylation of a C₃ derivative derived from serine to give a C₄ dicarboxylic acid such as oxaloacetate or malate. Alternative hypothetical pathways were proposed by which the glycine required for this sequence of reactions could be formed. Later work (Large, Peel & Quayle, 1962*b*) showed that the mechanism by which C₄ units could be formed from C₃ units involved an irreversible carboxylation of phosphoenolpyruvate to oxaloacetate catalysed by a phosphoenolpyruvate carboxylase (EC 4.1.1.31). This paper reports an examination of extracts of *Pseudomonas* AM1 to determine whether other enzymes implicated in the proposed pathway are present, and to compare their activities in the same organism grown on different substrates.

* Part 4: Large, Peel & Quayle (1962*b*).

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MATERIALS AND METHODS

Maintenance and growth of the organism. The maintenance and growth of cultures of *Pseudomonas* AM1 on methanol were described by Peel & Quayle (1961). For formate-grown cells, 0.1 M-sodium formate was substituted for 0.5% (v/v) methanol as the carbon source.

Preparation of cell-free extracts. Methanol- and succinate-grown cells were disrupted by ultrasonic disintegration and formate-grown cells by crushing the frozen bacteria in a Hughes (1951) press; both procedures have been described by Large *et al.* (1961). The bacterial extracts were then centrifuged at 25 000g for 10 min. in the high-speed head of an International refrigerated centrifuge (model PR-2), and the supernatant was used for assay.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. Sodium [¹⁴C]formate was purified as described by Large *et al.* (1961). L-[¹⁴C]Serine was used without further purification. 3-Phospho[¹⁴C]glycerate was obtained by incubating extracts of formate-grown *Pseudomonas oxalaticus* with sodium hydrogen [¹⁴C]carbonate and ribulose 1,5-diphosphate (Quayle & Keech, 1959*b*). D-[¹⁴C]Glycerate was obtained from 3-phospho[¹⁴C]glycerate by treatment with Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951). The resulting [¹⁴C]glycerate was purified by paper chromatography in butan-1-ol-propionic acid-water (47:22:31, by vol.) (Benson *et al.* 1950).

Chromatographic analysis and radioautography. The solvent systems of Kornberg (1958), phenol-90% (w/v) formic acid-water (500:13:167, w/v/v), and of Benson *et al.* (1950), butan-1-ol-propionic acid-water (47:22:31,