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The Denaturation of Acetic Acid-Soluble Calf-Skin Collagen

CHANGES IN OPTICAL ROTATION, VISCOSITY AND SUSCEPTIBILITY TOWARDS ENZYMES DURING SERIAL DENATURATION IN SOLUTIONS OF UREA

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The thermal conversion of collagen into gelatin is accompanied by dramatic changes in the physical and biological properties of the parent protein, e.g. a marked decrease in the values of the optical rotation and viscosity and an increase in the ease with which proteolytic enzymes can hydrolyse the derived gelatin as compared with the original collagen (Boedtker & Doty, 1956). These authors suggested that the highly organized structure of the collagen triple-helix was converted into the random-coil configuration of gelatin by a process of thermal depolymerization. This process carried out at neutral pH takes place with the minimal rupture of peptide bonds (Steven & Tristram, 1962b).

Gustavson (1956) indicated the mechanism by which concentrated solutions of urea at neutral pH caused the depolymerization of collagen. Courts (1958) has also drawn attention to the importance of various hydrogen-bonding agents, including urea, in the conversion of collagen into gelatin. Work on the soluble collagens by Orekhovich & Shpikiter (1957, 1958) has shown that treatment with urea induced physical depolymerization and formation of gelatin, the latter protein having the physical properties associated with the corresponding gelatin produced by thermal shrinkage of the original collagen. Piez (1961) reported the isolation of two types of both the α - and β -components from collagen depolymerized by either heat- or urea-pretreatment.

It seemed worth while to investigate the physical changes brought about by serial urea-denaturation

of acetic acid-soluble collagen. The normal effect of concentrated solutions of urea on soluble globular proteins is to increase the laevorotation and viscosity (Neurath, Rupley & Dreyer, 1956; Schellman, 1958; Hamaguchi, 1958; Rovery, Gabeloteau, Vernejoul, Guidoni & Desnuelle, 1959). Schellman (1958) suggested that these changes reflected an alteration from a helical structure to a random-coil configuration. The physical changes which accompany urea-denaturation of collagen are in the opposite sense to those observed in globular proteins. The high values of laevorotation and viscosity which are typical of collagen are converted to the lower values found in gelatins (Boedtker & Doty, 1956). The present work describes these changes during the serial urea-denaturation of acetic acid-soluble collagen.

MATERIALS

Acetic acid-soluble calf-skin collagen was prepared as described by Steven & Tristram (1962a). The protein contained 18% of total nitrogen on an ash- and moisture-free basis.

Crystalline salt-free trypsin (Armour, batch no. 22191) and crystalline pepsin (Armour, batch no. 22306) were used in this study.

Urea obtained from British Drug Houses Ltd. was recrystallized from ethanol.

Physical measurements were made on a Hilger standard polarimeter by using the sodium D line, and an Ostwald viscometer with a flow-time of 69 sec. for water was used to determine the viscosity of the collagen solutions, relative to water. All physical measurements were made at 18°.

RESULTS

Purified soluble collagen was dissolved in 0.1M-acetic acid (final pH approx. 3.5), the nitrogen concentration determined by micro-Kjeldahl analysis and the protein content calculated from the value of 18% of total nitrogen. The collagen solution (20 ml.) was then added to a series of flasks in which an appropriate weight of urea had previously been placed. The reagents were mixed with gentle agitation over 24 hr. before measurements were taken. The dilution effect of urea was taken into account before the values of laevorotation and viscosity were calculated, as described by Steven & Tristram (1959). The observed changes in laevorotation and viscosity are presented in Fig. 1 as a percentage of the initial values of native collagen and are plotted against the initial and final urea molar concentrations.

Fig. 1. Changes in optical rotation (O) and in viscosity (●) of collagen during serial urea-denaturation. The initial protein concentration was 2.4 mg./ml.

A sample of the original collagen solution was brought to pH 7 with 0.1N-sodium hydroxide and then converted into gelatin by placing it in a water bath at 100° for 1 hr. The gelatin was cooled and the rotation and viscosity were determined at pH 3.5 in 0.1M-acetic acid solution. These values were identical with the corresponding values obtained from collagen denatured with urea at initial concentrations greater than 6M. The optical rotation (-140°) of collagen treated with 6M-urea was unaltered after dialysis, and in good agreement with the value quoted by Doty & Nishihara (1958) for the gelatin derived from thermal treatment of soluble calf-skin collagen. This may be taken as evidence for the irreversible nature of collagen denaturation brought about by treatment with 6M-urea at acid pH.

The action of trypsin and pepsin on native soluble collagen and on collagen after thermal denaturation or treatment with urea (3M and 6M) was determined as follows. Solutions of these enzymes were added to the protein solutions so as to produce an enzyme: substrate ratio of approximately 1:20. The change in viscosity induced by the enzyme was recorded at 18° for a period of

Fig. 2. Trypsin digestion of collagenous proteins. The enzyme:substrate ratio was approximately 1:20, and the digestion was carried out at pH 3.5 in 0.1M-acetic acid. A, Acetic acid-soluble collagen or 3M-urea-treated soluble collagen after dialysis (concn. of protein, 2 mg./ml.); B, 6M-urea-treated soluble collagen after dialysis (concn. of protein, 10 mg./ml.); C, soluble collagen which had been converted into gelatin by thermal degradation at neutral pH by heating for 1 hr. at 100° (concn. of protein, 10 mg./ml.). All protein solutions had an initial viscosity of 12–15 mp.

Fig. 3. Pepsin digestion of collagenous proteins. The conditions were the same as in Fig. 2, and A, B and C have the same meanings as in Fig. 2.

1 hr. at pH 3.5 in 0.1M-acetic acid. At acid pH trypsin has a greatly decreased activity, but these conditions have the advantage that soluble native collagen may be studied in the absence of a lyotropic agent, such as CaCl_2 , which was used by Hodge, Highberger, Deffner & Schmitt (1960). The results of these enzyme studies are presented in Figs. 2 and 3; native acetic acid-soluble collagen and the protein obtained after dialysis of collagen treated with 3M-urea were equally resistant to enzyme attack as shown by a very small change in viscosity similar to the change observed by Hodge *et al.* (1960). The thermally denatured collagen and dialysed 6M-urea-treated collagen were attacked by both trypsin and pepsin; this behaviour is in keeping with the proposed conversion of collagen into a gelatin by urea at concentrations greater than 6M.

In the present viscosity study a control experiment was carried out in which the trypsin was inactivated with di-isopropylfluorophosphonate before the addition of the enzyme to the gelatin dissolved in 0.1M-acetic acid, pH 3.5. No change in viscosity was observed in this control. This demonstrated that the change in viscosity of the thermally degraded and 6M-urea-treated collagen was in fact due to the enzymic hydrolysis of peptide bonds caused by trypsin at this low pH rather than to the presence of a contaminant enzyme in the trypsin preparation.

DISCUSSION

High concentrations of urea caused the conversion of soluble collagen into a protein with physical

and biological properties which were almost identical with the corresponding heat-denatured collagen, or gelatin as it is more usually termed. It is suggested that both heat- and urea-depolymerization produce similar if not identical gelatins. This supports the claim by Steven & Tristram (1962b) that the thermal transition of collagen to gelatin at neutral pH is a physical depolymerization involving the rupture of only a minimal number of peptide bonds. Urea is well known to rupture hydrogen bonds but not peptide bonds.

Fig. 1 indicates that the rotation remains constant in urea up to a concentration of 3M. This would suggest that the collagen fold of the individual molecules remains intact since the triple-helix is thought to confer the high value of laevorotation associated with native collagen (Ramachandran & Kartha, 1955; Todd, 1960). This is further supported by the fact that dialysed 3M-urea-treated collagen was indistinguishable from native soluble collagen when subjected to enzymic digestion and the reaction followed by the change in viscosity. The rotation of collagen in 3M-urea was found to be -440° (typical of soluble calf-skin collagen); the rotation decreased rapidly to -140° (typical of a gelatin) when the urea concentration was increased to 6M.

The viscosity changes observed in the denaturation of collagen by increasing concentrations of urea may be divided into three stages as follows: (1) Up to 3M-urea, a rapid fall in viscosity was observed which may be associated with the rupture of intermolecular hydrogen bonds. This is suggested because the optical rotation shows no change until after the 3M-urea concentration has been reached, which may be taken as evidence that the triple-helix remains intact at this stage. (2) From 3M- to 6M-urea the viscosity fell rapidly to that observed for the corresponding thermally denatured collagen. This part of the viscosity curve may be related to the rupture of the intramolecular hydrogen bonds and consequent collapse of the triple-helix since the corresponding values of laevorotation also show a sudden fall to that of a gelatin. The two graphs are closely similar over the range of 3M- to 10M-urea concentration. (3) From 6M- to 10M-urea caused no change, which indicated the completion of the depolymerization at about 6M-urea concentration. The final values of these physical constants agreed well with those of thermally denatured collagen at this pH.

It is further suggested that two parameters (inter- and intra-molecular hydrogen bonding) contribute to the high viscosity of acetic acid-soluble collagen. Doty (1957) published a diagrammatic representation of the intramolecular hydrogen bonds which were considered to be essential for the maintenance of the collagen triple-helix. Inter-

molecular hydrogen bonds are considered by the present authors to cause association between neighbouring triple-helices of dissolved collagen. Under the conditions examined in this work the two parameters have almost equal contributions to the resultant viscosity of a dilute solution of collagen.

Native soluble collagen is not extensively attacked by proteolytic enzymes other than collagenase (Mandl, 1961) whereas gelatin is readily digested. Hodge *et al.* (1960) and Hodge & Schmitt (1961) have described the localized attack of trypsin on the so-called 'end-regions' of native tropocollagen with the result that end-to-end polymerization can no longer take place. At concentrations of urea lower than 3M, the depolymerizing action of the hydrogen-bonding agent was insufficient to cause the collapse of the triple-helix in collagen, and as a result trypsin and pepsin had no more action on the urea-treated protein after dialysis than on native collagen. At concentrations of urea greater than 6M the denatured protein was easily attacked by these enzymes in a similar manner to the thermally denatured collagen or gelatin. This would indicate the formation of a gelatin-like protein from collagen in the presence of concentrated urea, which is in agreement with the interpretation placed on the changes in laevorotation and viscosity.

SUMMARY

1. Serial urea-denaturation of soluble calf-skin collagen dissolved in 0.1M-acetic acid has been described. The reaction course has been followed by measuring the changes in laevorotation, viscosity and enzyme digestibility (after dialysis) of the protein dissolved in 0.1M-acetic acid in the presence of increasing concentrations of urea.

2. Soluble collagen has been denatured to produced gelatins of similar physical properties by either heat- or urea-depolymerization. This observation supports the previous claim by the authors that the thermal conversion of collagen into gelatin at neutral pH is a physical depolymerization accompanied by the rupture of only a minimal number of peptide bonds.

3. Urea-depolymerization of soluble collagen to form a gelatin proceeds by the rupture of hydrogen bonds in two distinct steps. The first step involves the rupture of intermolecular hydrogen bonds. The second step is irreversible at pH 3.5 and involves the rupture of intramolecular hydrogen bonds with the consequent destruction of the collagen triple-helix structure and the formation of gelatin.

4. The influence of inter- and intra-molecular hydrogen bonds on the resultant viscosity of dilute solutions of collagen were approximately equal under the conditions studied in this work.

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