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THE DISPERSION OF THE COLLAGEN SYSTEM OF PROTEINS BY CONCENTRATED SALT SOLUTIONS

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SUMMARY

Solubility, optical rotation and viscosity measurements have been performed on collagen after dispersion with KI and $CaCl_2$. This dispersion has been shown to involve a partially reversible denaturation. A threshold salt concentration has been demonstrated below which no denaturation takes place and no collagen is eluted from dextran gel (Sephadex G-50). Above this threshold concentration collagen has been fractionated on the gel and gives an elution pattern which is repeated at several concentrations of salt. It is suggested that a solution of native collagen is a continuous (or discontinuous) spectrum of protein aggregates with varying degrees of inter- and intramolecular cohesion.

INTRODUCTION

It has been known for a considerable time that neutral salts can cause profound changes in the structure and solubility of collagen. Early investigation[1] showed that hide powder was solubilised by a variety of halides and GUSTAVSON[2] showed that this involved the breakdown of intermolecular structure when he demonstrated that the shrinkage temperature of collagen fibres was lowered after equilibration in 2 M NaCNS and $NaClO_4$. The collapse of the native structure was confirmed by the finding that the laevorotation and viscosity of ichthyocol were lowered by 2 M KCNS (see ref. 3), by KBr and KI (see ref. 4) and by $CaCl_2$ (see ref. 5).

The effect of salts on the physical properties of collagen corresponds in many respects to the changes which occur when solutions of soluble collagen are heated to temperatures in excess of 45°. DOTY AND NISHIHARA[6] followed the change in viscosity and optical rotation with time at 35° and found that these parameters decreased at equivalent rates. They concluded this implied that the protein molecule was dissociating into its components by an all or none process. More recent work on calf skin tropocollagen[7,8] suggests the process to be more complicated. ENGEL[7] has shown that whilst viscosity and the initial slope of angular dependence of light scattering (tan α), both of which are dependent upon axial ratio, decrease at about the same rate, Mw (weight average molecular weight) decreases more slowly. STEVEN AND TRISTRAM[8], in studies on the urea denaturation of soluble calf skin collagen, have shown that the fall in viscosity and laevorotation proceeds at different rates and they suggest that the

changes observed are due to a two stage process, first depolymerization without the loss of helical structure and second the completion of denaturation by the loss of helical structure. The work reported in the present paper suggests that at 35° only the first stage proceeds with a measurable velocity.

The molecular weight of native soluble calf-skin collagen was found by light scattering measurement to be 360000 (DOTY AND NISHIHARA[6]). These workers produced evidence that the native molecule was made up of two components, α and β, present in equal molecular proportions. They determined the molecular weights as 120000 (α) and 230000 (β), values which are in good agreement with those reported (α, 115000; β, 215000) by ENGEL[7], who has suggested that the difference between the molecular weights of the native collagen and the sum of the isolated components might be evidence that a small proportion of larger aggregates of collagen exists in solutions of native soluble collagen.

This paper describes some of the changes induced in the collagen system of proteins by salt denaturation and the partial separation of components of the system.

EXPERIMENTAL

Preparation of collagen

The acetic acid soluble fraction of calf-skin was prepared by the method of STEVEN AND TRISTRAM[9]. The thrice reprecipitated material was desalted by dialysing against running tap water for seven days and distilled water for two days. A freeze dried sample yielded 0.46 % ash and 17.98 % nitrogen, corrected for ash.

Reagents

$CaCl_2$ and KI (BDH AR reagent grade) were used for viscometry and polarimetry. $CaCl_2$ went into solution at pH 5.5 and was standardised by permanganate titration. KI solutions were stabilised by very small amounts of thiosulphate. For gel fractionation reagent grade KI was used. All other materials, including buffer materials, were reagent grade.

Buffers

Borate buffers, pH 5.5–7.5, were prepared by adding 0.2 N NaOH to 0.2 M H_3BO_3 to give the required pH. For acetate buffer (pH 5.5), 0.2 M sodium acetate was adjusted to that pH with 0.2 N acetic acid.

Collagen solutions for viscometry and polarimetry

A suspension of salt free collagen in distilled water was added to the calculated volume of standard salt solution in a volumetric flask. The volume was made up with distilled water and solution accomplished by stirring or shaking mechanically for 48 h. After filtration, the protein content was estimated; a concentration of approx. 0.1% was used throughout. At this concentration, any increase of the total volume caused by solution of the collagen was considered negligible.

Polarimetry

A Hilger standard polarimeter was used. Measurements in salt solution were carried out at room temperature (20°). For heat denaturation studies, a jacketed 1-dm cell was held by an external water bath at the required temperature, ± 0.1°. 30 min

were allowed for equilibration at each temperature. For heat denaturation the collagen was dissolved in 0.2 M acetate buffer (pH 5.5).

Viscometry

All measurements were made at 25° ± 0.1° using an Ostwald viscometer with a flow time of 52 sec for water.

Fibre formation

Collagen was dissolved in 0.2 M acetate buffer (pH 5.5). Aliquots were pipetted into test tubes and standard NaCl or KI added to give the required concentration. Fibres were allowed to form by warming the tubes to 37°, the time at which a precipitate first appeared being measured.

Protein estimation

Viscometry and polarimetry solutions were analysed by the microkjeldahl method, the conversion factor of 17.98% nitrogen being used. Effluent from gel fractionation was analysed by a modification of the Folin method (LOWRY *et al.*[10]), absorption being measured at 750 mμ.

Solubility measurements

10-ml aliquots of a 0.1 N acetic acid solution of collagen of known concentration (approx. 0.25%) were run from a burette into Visking tubing and precipitated by dialysis against 1 l of the appropriate salt solution at 7° for 10 days, with regular agitation. The material was then washed from the sacs with a small volume of water and centrifuged at 3000 rev./min for 20 min; the nitrogen content of the supernatant collagen was measured by the microkjeldahl method.

Gel fractionation

Sephadex G-50 (Pharmacia, Uppsala (Sweden)) was used. Equilibration of the gel, solution of the collagen, and elution were normally performed with the same strength of KI solution. Buffering did not appear to be necessary at any stage. Columns 2 cm × 21 cm were prepared using gravity only for packing. 8 mg protein was normally applied to the column, and 2-ml cuts were collected by automatic fraction collector.

RESULTS

Specific rotations in $CaCl_2$ and KI are given in Fig. 1. There is a threshold concentration for each salt below which there is no change in rotation, this concentration being 0.5 M for each salt. Above the threshold concentration KI has a more marked effect than $CaCl_2$ on optical rotation and hence upon helical structure although the limiting value for each salt was identical (−96°). These changes are closely paralleled by those occurring during thermal denaturation (Fig. 2), when no effect was observed below 27° with a limiting value of −125° at 55°. It was clear however on attempting renaturation either by cooling or removal of salt by diffusion that there was a major difference between thermal and salt denaturation. On removal of salt, even from the limiting (4 M) solutions, the optical rotation was restored to the original value, whereas after cooling for 18–24 h the optical rotation was −183°. It would thus appear that in

salt denaturation certain bonds remain which make possible the restoration of helical structure whereas these bonds do not survive thermal denaturation.

Viscosity measurements (Fig. 1) showed that at salt concentrations above 0.5 M a very rapid fall in viscosity occurred, the limiting value being reached at about 1.5 M salt. Unlike rotation, however, viscosity only reached about one third of its original value on removal of salt.

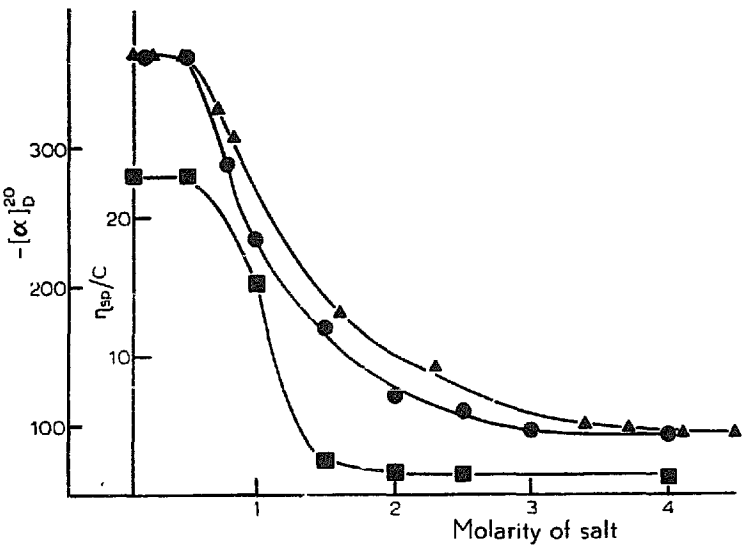

Fig. 1. Specific rotation and viscosity of collagen in concentrated salt. Δ—Δ, Laevorotation in calcium chloride solution pH 5.5; ○—○, laevorotation in potassium iodide solution pH 7.0; ■—■, reduced specific viscosity (concentration in g/100 ml) in calcium chloride. Abscissa, molarity of salt; ordinate, $-[\alpha]_D^{20}$ and η_{sp}/c.

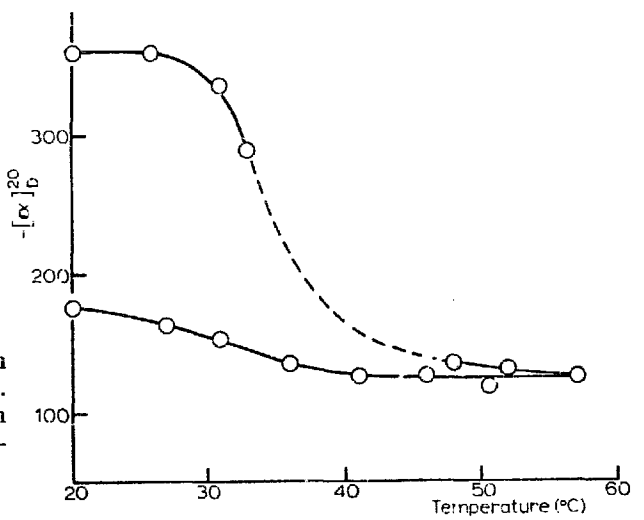

Fig. 2. Change of specific rotation of collagen in acetate buffer (pH 5.5) with temperature. Upper line, heating; lower line, cooling; broken line, period of precipitation. Abscissa, temperature; ordinate, $-[\alpha]_D^{20}$.

These findings are in major agreement with those of ENGEL[7] who found that $-[\alpha]_{405}$ which was 700° in native collagen fell to 375° at 37.8° and recovered to 820° after 20 h at 4°, whereas viscosity was restored to only a fraction of the native value.

Fibre formation

The contrasting effects on fibre formation of the salts such as KI and NaCl can be seen in Fig. 3. In the absence of salt, fibre formation commences after 18 ± 2 min. In 0.1 M salt solutions the times are 7 min and 18 min for KI and NaCl respectively. In 0.35 M NaCl fibre formation commenced after 14 min at 37° whereas in 0.35 M KI fibre formation was completely inhibited and did not occur after removal of the KI

by diffusion and resolution of the collagen in buffer. GROSS AND KIRK[11] showed that certain anions accelerated the heat precipitation of collagen and placed them in order of decreasing effectiveness (*viz.*: SCN^-, HCO_3^-, I^-, F^-, Cl^-). This is borne out in the present work which shows that low concentrations of iodide accelerate the formation of collagen fibres.

In the cold calcium chloride on the other hand enhanced the solubility of collagen (Fig. 4) and in 3–5 M $CaCl_2$ up to 3 g per 100 ml of collagen could be brought into solution.

Separation of components of soluble collagen on cross-linked dextrans (Sephadex G-50)

When soluble collagen in solution in 0.5 M KI or acetate buffer (pH 5.5) was applied to a column of Sephadex G-50 no protein passed through the gel. On eluting

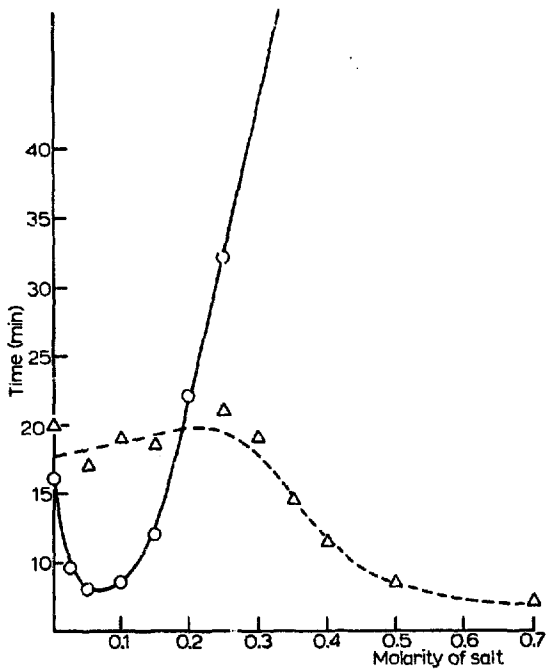

Fig. 3. Precipitation time of collagen dissolved in acetate buffer (pH 5.5) warmed to 37° in the presence of KI (O—O) and NaCl (Δ—Δ). Abscissa, salt concentration (M); ordinate, time for precipitation (min).

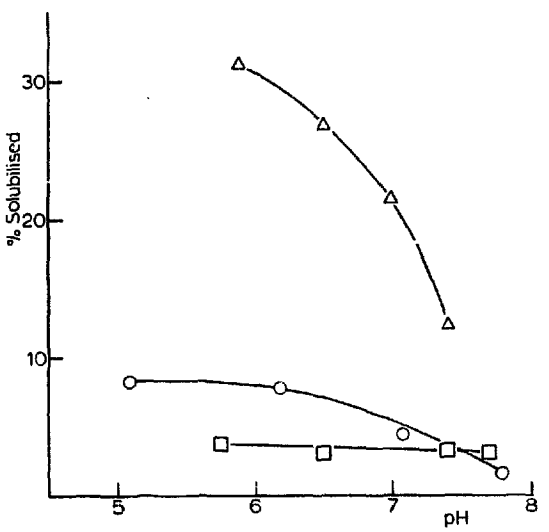

Fig. 4. Solubility of collagen in calcium salts. □—□, 0.2 M borate buffer alone; O—O, with 0.0023 M calcium chloride; Δ—Δ, with 0.25 M calcium acetate. Abscissa, pH; ordinate, solubility as percent added collagen.

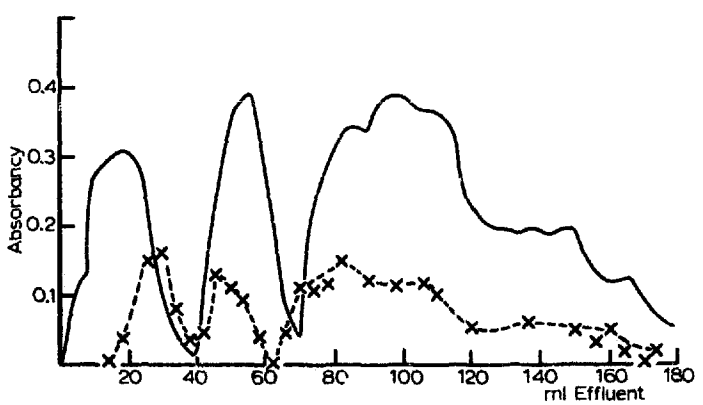

Fig. 5. Elution pattern of collagen in KI from Sephadex G-50. Continuous line, 3 M KI; broken line, 1 M KI. Abscissa, millilitres of effluent; ordinate, absorbancy at 760 mμ.

with 3 M KI (the salt concentration required to promote complete breakdown of the triple helix), the elution pattern suggested the presence of three components (Fig. 5 solid line). When the experiment was repeated in which the column was eluted consecutively, with (a) 0.5 M (b) 1.0 M and (c) 2 M KI it was found that no protein was eluted with 0.5 M KI and that three components appeared on eluting with 1.0 M KI (Fig. 5 dotted line). Even after 24 h continuous elution with 1.0 M salt no further protein was obtained. When the salt concentration was raised to 3 M the triple elution pattern was again observed, and this was again observed when the column was subsequently eluted with 3 M KI. The height of each peak was equivalent to the difference between the heights of the two series in Fig. 5.

After exhaustive washing with 3 M KI it was estimated that 19% of the nitrogen originally applied to the column had not been recovered. The absorptive power of Sephadex is considered to be small in concentrated salt solutions[12] and if it is to be assumed that the Sephadex gel permits the elution of dissociated molecules only it seems possible that the protein system found in native soluble collagen is one which contains several orders of complexity which are dissociated when salt concentration reaches certain distinct threshold values. The highest orders which result in fibre formation when the system is relaxed at 37° do not survive in KI beyond a threshold concentration of 0.35 M.

DISCUSSION

The specific rotation of protein solutions must be interpreted with care as salt or intermolecular attractions may cause changes in rotation due to strain in the protein molecule without altering its overall configuration[13]. In the present experiments the possible effect of molecular interaction was minimised by the use of low concentrations of protein although it was impossible to maintain constant ionic environment. It is clear that the fall in laevorotation and viscosity of solutions of collagen is due to randomisation of the helical structure in a manner which resembles heat denaturation (*e.g.* melting out of gelatin).

If salt denatured collagen was dialysed to remove salt it precipitated from solution as the concentration of salt approached zero and the protein, on redissolving at pH 3–3.5, had the high laevorotation of native soluble collagen. In sharp contrast heat denatured protein did not completely recover helical conformation and the laevorotation rose to only −150° to −200° after long periods at room temperature.

Salt denaturation however, is not completely reversible since the viscosity of the regenerated (dialysed) protein was only about one third of the original value, and solutions would no longer form fibrils or gel at 37° (see ref. 11).

DOTY AND NISHIHARA[6] postulated that the randomization of soluble calf skin collagen occurred at temperatures above 35° as an all or nothing process. The present work, in agreement with that of STEVEN AND TRISTRAM[8] suggests that denaturation is at least a two stage process involving (a) the breakdown of intermolecular forces (*e.g.* H-bonds) and (b) intra-molecular forces (H-bonds) (*cf.* STEVEN AND TRISTRAM[8]). The obvious polydispersity of the protein system suggests too that the process of denaturation may be far more involved than the formation of α- and β-components[3] or even α-, β-, and γ-components[14]. Indeed the results of the chromatographic experiments on Sephadex are best explained on the basis of the concept that soluble collagen is a continuous (or semi-continuous) spectrum of protein aggregates[15]. The use of salts

(*e.g.* KI) of increasing molarities induces the depolymerisation of those aggregates, the cohesive forces of which have been overcome by the H-bonds breaking activity of the solvent system.

The physico-chemical nature of this partially reversible salt denaturation is at present unknown. GUSTAVSON[2] pointed out that salts such as KI and $CaCl_2$ were largely unionised in concentrated solutions (0.005 M $CaCl_2$ is 8 % unionised and has a solubilising effect even at this concentration (*cf.* Fig. 5)), and he postulated that such salts by competing for the co-ordinating sites in proteins acted as hydrogen bond breakers and thereby caused denaturation. PFEIFFER[16] had earlier described a series of co-ordination compounds between molecular $CaCl_2$ and amino acids whereby the solubility of the amino acids was enhanced.

The denaturing action of calcium chloride and potassium iodide may not be by

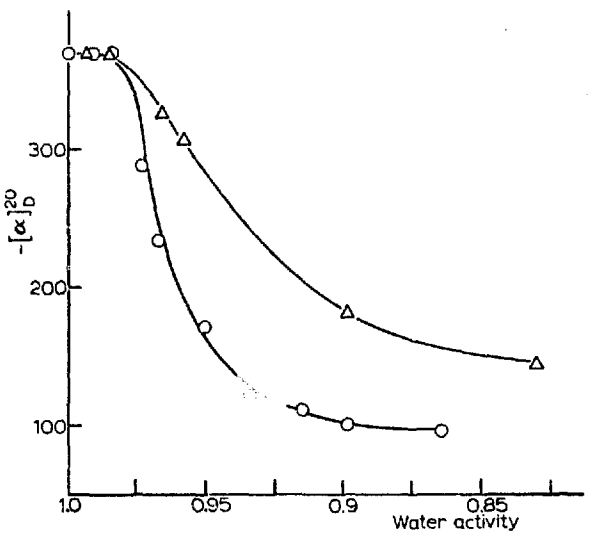

Fig. 6. Laevorotation of collagen in $CaCl_2$ (△—△) and KI (○—○). Abscissa, water activity of solvent; ordinate, $-[\alpha]_D^{20}$.

direct effect upon the sites of hydrogen bonding but an influence on the environment by lowering the water activity of the solvent. HARRINGTON AND SCHELLMAN[17] proposed that the lowered water activity of the solutions of lithium bromide increased the hydrogen bonding in ribonuclease whereas BIGELOW AND GESCHWIND[18] stated that lithium bromide caused a denaturation which they could not relate directly to a decrease in water activity. In the present work the plot of laevorotation against water activity (Fig. 6) suggests that any relationship must be far from simple.

If soluble collagen does exist as a single molecular species made up of α- and β-components denaturation might normally be envisaged as a progressive depolymerization of each of the associated chains. The results here presented suggest that there is a wide variation in the rate of denaturation and in the resistance to denaturation of components of the collagen system of proteins. When soluble collagen is separated on Sephadex (G-50) and 1 M KI, three component fractions were obtained while a considerable amount of protein remained absorbed on the gel. Successive elutions with 2 M and 3 M KI yielded further fractions which separated as three components in each case. This can best be explained by assuming that there exists in solutions of collagen a spectrum of collagen aggregates with a wide range of stability towards denaturants such as salts and heat. This is in harmony with the views on collagen

metabolism advanced by JACKSON AND BENTLEY[15]. These workers maintain that there exists, in collagenous systems, "a continuous spectrum" of aggregates with varying degrees of molecular cohesion (or cross linking); the degree of which depends upon the biological age of the constituent molecules. The recent work of PIEZ *et al.*[19] also implies that the same subunits are found in extracts of collagen of different maturities and that the stability of combination varies with respect to the biochemical age of the protein molecules.

The viscosity of a solution of collagen is said to be a function of the axial ratio of the molecules present. The finding that viscosity does not return to its initial value after the removal of salt or on cooling after thermal denaturation[7] suggests that the axial ratio, hence the degree of polymerization is not restored. This may be explained if it is assumed that the higher orders of the collagen system which contribute to the initial high viscosity of native soluble collagen and are responsible for the initiation of fibre formation at 37° are destroyed by salt.

The nature, composition and homogeneity of the various fractions obtained from Sephadex columns are now under investigation.

No phenomenon analogous to salt denaturation is known *in vivo* but COURTS[20] has suggested that, since no enzyme system is known to be associated with the massive rate at which uterine collagen is dispersed after parturition, some form of catalysed hydrogen bond breaking mechanism may be involved. WOESSNER[21] has suggested similarly that in the involuting uterus there is a process of denaturation prior to proteolysis occurring.

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