ACTA CHEMICA SCANDINAVICA 1 (1947) 581—591

The Effect of Hydrothermal Denaturation of Collagen upon its Reactive Groups

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The denaturation of water-soluble proteins is generally considered to involve the unfolding of the coiled chains of the globular protein. Thereby the reactivity of various protein groups will be altered [1]. Denaturation of the fibrous protein collagen (mammalian) by the shrinkage of the fibres in water of 60—70° C leads to disorganization of the parallelly aligned chains, forming the native protein [2]. The fibrous state is intimately connected with the function of restraining forces between elementary chains and units. By the application of heat, the kinetic energy of the chains will be increased. Hence the cohesive forces will be weakened. The chains will tend to revert to the thermodynamically stable state of a random distribution. The temperature at which shrinkage of collagen takes place is the point at which the disruptive forces exceed the intermolecular cohesion [3].

The mechanism of the shrinkage of collagen fibres has been comprehensively investigated by Wöhlisch [4] from the thermodynamic point of view. Further, the important contributions of Meyer [5], Fauré-Fremiet [6], Küntzel [7] and Grassmann [8] are noted. In earlier papers [7,9] extensive literature references are given.

It would be expected that the reactivity of collagen would be changed by the deep-going alteration of its micellary structure. It is known that the inertness of native collagen fibres toward trypsin is destroyed by denaturation [8]. The elementary composition of the protein, its content of basic groups, more particularly amino ones and its acid binding capacity are not affected [8]. However, it has been indicated in earlier investigations [9] that the reactivity of collagen towards high-molecular compounds, reacting partly by means of co-ordinate valency forces is increased by denaturation.

In a recent critical review of the influence of denaturation of globular proteins upon the properties of the protein groups, Anson [10] points out that it is not possible at the present stage of knowledge of protein structure to define denaturation by means of description of the change in structure of the protein molecule. Anson further remarks that the increase in reactivity of protein groups when a protein is denatured must be explained by some general structural theory. This change is probably connected in some way with the breaking of the bonds between polypeptide chains, although, as Anson points out, our present limited knowledge does not justify further theorization.

The influence of hydrothermal denaturation on the reactivity of mammalian collagen has been investigated, with a view to ascertaining which groups are altered.

These groups functioning as connecting links between the units of the collagen lattice are: 1) *Salt-like links*, due to the attraction of oppositely charged side groups in juxtaposition. This type of cross-link apparently is of secondary importance for the cohesion of the collagen structure compared to: 2) *co-ordination bonds* between H- and O-atoms of the imino- and carbonyl-groups of peptide groups on adjacent protein chains (hydrogen bonds). These links seem to be the principal stabilizing agent of the collagen lattice [11].

MATERIALS AND METHODS

Collagen in the form of calf skin (delimed after regular light liming and made isoelectric (pH 5.5)) was used for the main experiments. Its content of ash was less than 0.05 %. Further, hide powder (Freiberg) was employed for experiments with systems containing reacting substances of high-molecular weight, in order to minimize the topochemical factor. The hide substrate was employed in: 1. Native state. 2. Denatured state. Collagen was denatured by holding the specimens for 5 min. in water of 72° C. All specimens were then dehydrated in two changes of acetone, air-dried and kept so. Further, specimens not acetone-dehydrated were used in some runs. No difference in the behaviour of hydrated and dehydrated specimens was found.

Both native and denatured collagen in the form of calf skin contained identical amounts of protein N; 17.97 %. The figures for hide powder were 17.93 and 17.90 % N respectively.

The reagents used were of analytical grade. The special compounds and extracts employed have been described in earlier papers [12]. The high-molecular fraction of lignosulphonic acid was prepared by the Erdtman »bis»-method [13].

The H-ion concentration was measured at 20° C by means of the hydrogen electrode; checked by the glass electrode. The titration curves plotted in the graphs are the average of at least six separate runs.

EXPERIMENTAL AND RESULTS

Titration curves

Any alteration in the reactivity of the basic protein groups induced by the collapse of the orderly fibre structure will be detected by means of the titration curve. The maximum acid binding capacity of collagen for which equilibrium pH values of ca. 1 are required is not changed by denaturation; this only proves that denaturation does not lead to fission of peptide groups with the formation of new amino and carboxylic groups. From considerations of the disorganization of the collagen chains, taking into account possible steric changes affecting the strength of the polar groups present in internal compensation, the comparison of the acid binding power of native and denatured collagen in a higher pH range, *e. g.*, 3—5, should be expected to be more informative. The technique used was similar to the one described by Harris [14], also found satisfactory in the work of Booth [15] on the influence of denaturation of albumin on its titration curve and employed by Atkin [16] in collagen investigations.

Quantities of native and denatured skin in ca. 1 mm cubes, and of hide powder equivalent to 1.00 g collagen were treated in resistance glass flasks with 100 ml of solutions made up as follows: 20 ml 10 % aqueous solution of sodium chloride, x ml 0.1 N HCl-solution and $80 - x$ ml of destilled water. The solutions were shaken at intervals during 24 h and the equilibrium pH values of the filtered solutions were determined. It will be noted that each solution contains two volume percent sodium chloride which is added to equalise the internal pH of the substrate and that of the external solution in order to avoid unequal ion distribution, rapidly set up in salt-free solutions of acid. Corresponding solutions of hydrochloric acid containing salt but *without* added protein were measured as blanks. The difference between the curve showing the effect of added acid on the final pH of the series containing protein and the curve for the blank solution gives directly the acid binding power of collagen at any pH value between 2 and 5. It may be remarked that some difficulty was encountered in the determination of the H-ion concentration of the solutions in the pH range 4—5.5.

Further complementary series were run with a constant amount of hydrochloric acid (10 ml 0.1 N HCl on 1 g collagen) but varying the total volume from 50 ml to 1000 ml. The results of these series showed the same trend as these of the main runs. In the series with denatured hide powder quite large amounts of protein (up to 5 % of the original weight) were brought into solution, accordingly affecting the pH values, especially in the higher pH range. Parallel runs with denatured skin showed average losses of 1 % collagen. Titration curves for the native and denatured calf skin collagen are given in Fig. 1. The curves of Fig. 2 show the effect of denaturation on the acid binding capacity of the specimens.

The fixation of hydrochloric acid by collagen from solutions with final pH values less than 3 is evidently not affected by denaturation. In the systems

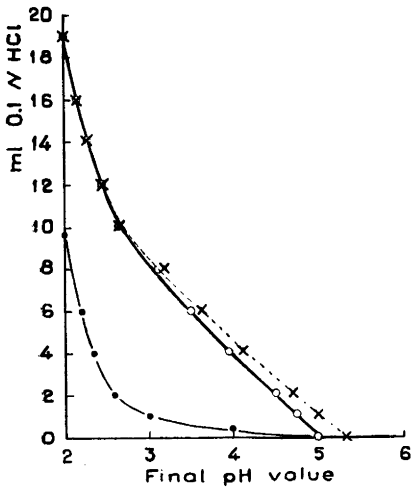

Fig. 1. Titration curves of native and denatured collagen.
—●— *Correction blank for HCl.*
—○— *Native collagen.*
– – × – – *Denatured collagen.*

with equilibrium pH values in the range 3—5, the denatured collagen possesses a slightly greater affinity for H-ions than the native type. However, the increase is not more than 5 % of the value of the maximum acid fixation capacity of collagen at the most. Since the titration of the system: collagen—solutions of sodium hydroxide containing 4 volume percent sodium sulphate, gave irregular values, these curves are not included. However, at pH values > 11, a common curve represents the removal of OH-ions from the solutions by native and denatured collagen, whereas in the pH range < 11 the denatured protein shows somewhat greater affinity for OH-ions;

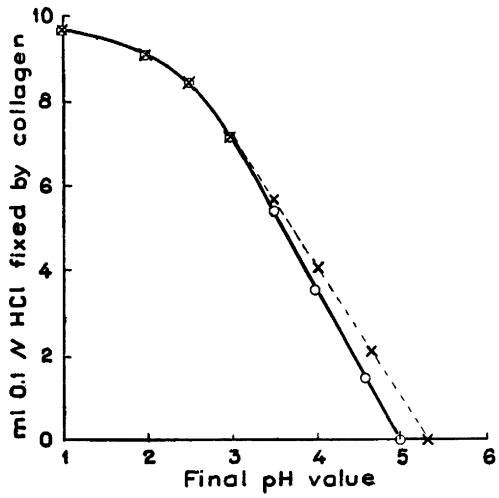

Fig. 2. Fixation of hydrochloric acid by native and denatured collagen.
—○— *Native collagen.*
– – × – – *Denatured collagen.*

the average increase being of the order of 5—10 percent of the total alkali-binding capacity of collagen.

Reactivity towards certain complex compounds

Since the titration curves prove that denaturation only slightly modifies the reactivity of the acid binding protein groups, it was deemed of interest to ascertain the behavior of the two types of mammalian collagen towards reactants predominantly reacting with hide protein by means of electro-valent forces. For that purpose a formaldehyde condensed naphthalene sulfonic acid consisting of a mixture of about equal parts of the di- and tri-nuclear disulfonic compounds was employed [17]. On account of the absence of phenolic and other co-ordination active groups in this compound, complications due to secondary reactions of nonionic nature should be eliminated as far as possible. The second type of sulpho-acid used was the high-molecular fraction of lignosulphonic acid.

Portions of 2 g of collagen in the form of calf skin cubes were shaken in 100 ml portions of 5 % solutions of these acids for 48 h, this being sufficient to attain equilibrium. The final pH values were 1.4 and 1.2 for the solutions of naphthalene- and ligno-sulphonic acids respectively.

A great number of basic chromic salts have been investigated. Two typical runs are given. A solution of 67 % acid chromic sulphate* with composition corresponding to the empirical formula $Cr_2(OH)_2(SO_4)_2 \cdot Na_2SO_4$, and a solution of 67 % acid chromic chloride corresponding to the formula $Cr_2(OH)_2Cl_4 \cdot 2NaCl$ were employed at a concentration of 1 eq. Cr per l.

The ratio of solution to substrate was 100 to 2 and the time of interaction 144 h. All series were run at room temperature (ca. 20° C). The treated stock was washed free from water-soluble matter, dried and analyzed by the usual methods for determination of the agents irreversibly fixed by collagen. The sulfur content of the sulfo-acid treated specimens was further determined, by the method of Grote-Krekeler. This determination is important for the following series in which the fixation of vegetable tannins by sulpho-acid saturated collagen is investigated. For the differentiation of irreversibly fixed vegetable tannins and sulpho acids in the resulting product, accurate S-figures are required [17]. The findings are shown in Tables 1 and 2.

Table 1. Fixation of chromic salts by collagen.

Type of compound	meq. Cr fixed by 1 g collagen in the form of:	
	Native collagen	Denatured collagen
67 % acid chromic chloride	3.3	3.3
67 % acid chromic sulphate	4.2	4.15

* % acidity = valencies of Cr in combination with acid groups (SO_4; Cl), expressed in per cent.

Table 2. Fixation of sulpho acids by collagen.

Type of sulpho-acid	% Sulpho-acid irreversibly fixed by: Native collagen	Denatured collagen
Condensed naphthalene sulphonic acid (Final pH 1.4)	29.8	29.3
High-molecular fraction of lignosulphonic acid (Final pH 1.2)	50.6	50.3

According to these data, denaturation of collagen does not alter the ionic protein groups involved in the fixation of low-molecular chromic salts and sulpho-acids.

Some types of chromium compounds are for their fixation by collagen dependent upon its co-ordinate function beside the availability of electrovalent protein groups. The fixation is a function of the state of the co-ordinate valency centres of hide protein which are probably located on the peptide groups. The afore-mentioned type of chromic salt brought to a high degree of basicity, leading to increased molecular size of the chromium complexes, was applied as previously described. The 40 % acid chromic sulphate and the 33 % acid chromic chloride, with a composition corresponding to the empirical formula: $Cr_2(OH)_4Cl_2 \cdot 2NaCl$ were employed in solutions containing 1 eq. Cr per l.

Further, the interaction between collagen and a highly aggregated sulphato-sulphito chromiate, mainly consisting of uncharged and negatively charged chromium complexes, corresponding to the formula: $Na_2(Cr_2(OH)_2(SO_4)_2(SO_3))$ was investigated. In reactions of solid proteins with solutions containing highly aggregated solutes, the topochemical influence of the protein substrate is a complicating factor particularly marked in denatured skin. The shrinkage and twisting of the micelles should lead to a reduction of interfibrillar and intermicellar space. The diffusion of the large molecule into the interior of the substrate will be retarded and impede the further progress of the reaction. By the use of finely divided hide powder, this topochemical complication may be largely eliminated. Table 3 contains the data.

Table 3. Fixation of aggregated chromic salts by collagen.

Type of compound	meq. Cr fixed by 1 g collagen in the form of: Native collagen	Denatured collagen
33 % acid chromic chloride	5.6	7.8
40 % acid chromic sulphate	6.4	8.0
Sulphito-sulphato-chromiate	7.1	10.9

Evidently the denatured collagen fixes greater amounts of aggregated chromium compounds than the native protein. An exceptionally large effect

is evident in the fixation of sulphito compound which according to experimental information available mainly reacts by means of co-ordinate valency. The increased fixation of these types of compounds is evidently caused by structural alterations induced by the shrinkage of collagen. Since agents predominantly attached to collagen by means of its acidic groups (carboxyl), for example, simple chromic salts, or by means of its basic groups, for example, simple sulpho-acids, are unaffected by denaturation it follows that the increased fixation of highly aggregated substances by denatured collagen is indicative of activation of non-ionic valency forces in the denaturing process.

The fixation of vegetable tannins

These complicated polyphenols are generally conceived to react both by means of the electro-valent basic groups and the co-ordinate *loci* of collagen. Methods for the differentiation of the main types of fixation have been proposed [17, 18]. The increased reactivity of co-ordinate peptide bonds resulting from the disorganization of the protein chains in hydro-thermal denaturation is strikingly demonstrated by data from the fixation of vegetable tannins by collagen in the native and the denatured state.

Portions of hide powder equal to 2 g collagen were treated for 3 days in 50 ml portions of solutions of vegetable tanning extracts containing 1 % tannin. The tanned hide powder was sucked free from solution and further treated for 3 days in 50 ml of solutions containing 2 % tannin. Further treatments of 4 days each were carried out in solutions of 3, 5 and 10 % strength in tannin. The tanned hide powder was washed free from water-soluble matter (negative gelatin-salt test). The content of irreversibly fixed tannin was obtained by analysis of the dried hide powder for ash and collagen (N × 5.62). The difference between 100 and the percentages of these constituents being taken to be irreversibly combined tannins. Table 4 contains some typical data.

Table 4. Fixation of vegetable tannins.

Type of vegetable extract	Final pH	% irreversibly fixed tannin on collagen basis by:	
		Native collagen	Denatured collagen
Wattle bark	4.6	42.1	67.3
Quebracho wood	5.5	46.2	70.5
Sumach	4.0	34.4	46.8
Tannic acid	3.4	65.2	88.0
Myrobalan	3.0	66.7	100.4

These data demonstrate the far-going influence of the denaturating pretreatment on the fixation of vegetable tannins by hide protein. A differentiation between the effect of the ionic protein groups (basic) and the co-ordi-

nate ones (peptide) was attempted in series with collagen containing its basic groups completely blocked by irreversibly fixed condensed sulpho-acids.

Native and denatured hide powder were treated in solutions of vegetable tanning extracts as described in the foregoing series. Further, both types of hide powder were in two consecutive treatments saturated with the high molecular fraction of lignosulfonic acid at final pH values of 1.4 in order to block the electro-valent groups. The pretreated hide powder contained 51.2 and 50.4 % sulpho-acid fixed by collagen in native and denatured state respectively. The sulpho-acid hide powder did not change the pH value of 0.1 *N* HCl-solution, proving the complete absence of free basic groups. The specimens were then treated with vegetable tannins in the same manner as described for the blank hide powder. After removal of reversibly attached matter from the stock by washing, it was dried and analyzed. From complete analysis of the stock including the content of S, the percentage of lignosulpho-acid fixed by protein was obtained (S × 14.7 = fixed lignosulpho-acid). The rest of the irreversibly fixed matter represented the vegetable tannins fixed by the non-ionic protein groups; the »peptide fraction». Table 5 contains the data.

Table 5. Fixation of tannins by hide powder and hide powder with inactivated basic groups, in native and denatured state.

Type of tannin	Substrate	In % on collagen basis: Total matter irreversibly fixed by collagen	fixed sulpho-acid	fixed vegetable tannin
Sulphited quebracho	Native collagen	54.8	0	54.8
	Sulpho-acid »	88.7	51.2	37.5
	Denatured »	84.1	0	84.1
	Sulpho-acid saturated denatured collagen	108.3	49.1	59.2
Myrobalan	Native collagen	76.7	0	76.7
	Sulpho-acid »	101.6	51.2	50.4
	Denatured »	113.1	0	113.1
	Sulpho-acid saturated denatured collagen	126.7	49.6	77.1

Table 6. Allotment of vegetable tannins to the electro-valent and co-ordinate groups of collagen.

Type of tannin	State of collagen	% tannin on collagen basis fixed by: Electrovalent groups	Co-ordinate groups
Sulphited quebracho	Denatured	24.9	59.2
	Native	17.3	37.5
	Additional fixation due to denaturation	7.6	21.7
Myrobalan	Denatured	36.0	77.1
	Native	26.3	50.4
	Additional fixation due to denaturation	9.7	26.7

In Table 6 the allotment of the fixed vegetable tannins to the two main types of protein groups is given. The figures show that the parts of the tannins which are fixed by non-ionic protein groups mainly account for the additional fixation due to the denaturative treatment. However, with some vegetable tannins both types of tannin fixation are proportionally increased by denaturation. Evidently the reactivity of the tannins is too complex to be formulated in a simple manner [19].

DISCUSSION

From the plausible assumption that the main cohesive forces in the collagen super-structure consist of: 1. salt-like links between oppositely charged acidic and basic groups on adjacent protein chains, and: 2. cross-links due to co-ordinate valency forces between $\overset{|}{\underset{|}{CO}}\cdots\cdots\overset{|}{\underset{|}{HN}}$ groups in juxtaposition, the following explanation of the reported findings may be advanced. The hydrothermal denaturation of collagen fibres involves a folding of the collagen chains. By the distortion of the structure, the distance between the chains may in certain points be so greatly enlarged that the force does not suffice for bridging the chains. Interlocking links located on the protein backbone (hydrogen bonds) are primarily affected and ruptured. The free valency forces on the liberated groups (hydrated), no longer internally compensated, will then be available for reaction and the heat denatured collagen will hence possess greater potency of co-ordinate reactivity of the peptide bonds than the original type of collagen.

The electrostatic intermolecular forces are not affected or only slightly so, by the shrinkage of the chains. The slightly increased H-ion fixation in the pH range in the vicinity of the iso-electric zone of collagen is probably due to a weakening of the salt-like cross-links by the widening of the gap between chains in the coiling. Diminished internal compensation of the electro-static force should connote increased reactivity of the polar protein groups towards external ions, as, *e. g.*, hydrogen ions. In systems of high ionic concentration, as, for example, acid solutions of low pH values, the greater reactivity of these easier available protein groups is not apparent for obvious reasons.

Collagen of fish skin (teleost) does not show increased affinity for the high-molecular compounds investigated in denatured state [19]. This finding as well as other differences between mammalian and teleost collagen, *e. g.*, the latter's low degrees of hydrothermal and tryptic resistance, are considered to indicate the non-participation of co-ordinate links on peptide bonds in this type of collagen [19].

It was found in earlier investigations [9] that by heat denaturation of globular proteins, as, *e. g.*, albumins, their affinity for substances, reacting electrovalently as well as co-ordinately, is greatly impaired. This finding may indicate that by the »opening up» of the globular structure and the setting of the denatured protein into structures of higher degree of orientation of elementary units, certain reactive protein groups are inactivated. Their function as interlockers of individual chains should then explain the decreased reactivity of globular proteins resulting from denaturation. It is noteworthy that the reactivity of casein which is not denaturable is not affected by hydrothermal treatment.

The data are in accord with the views of the mechanism of denaturing advanced by Astbury and Lomax [20], assuming interrelationship between the native and denatured forms of the globular and fibrous proteins.

SUMMARY

The hydrothermal denaturation (shrinkage) of collagenous fibres does not alter the maximum acid binding capacity of collagen. However, a comparison of the titration curves of native and denatured fibres shows that the H^+-fixation of collagen in the pH range 3—5 is slightly increased by denaturation, as is also the uptake of OH-ions from solutions of final pH values < 11.

The irreversible fixation of simple condensed sulpho acids is not affected by the treatment, neither is the uptake of basic chromic salts of low degree of aggregation. Highly aggregated chromium compounds show preferred affinity for denatured collagen. The vegetable tannins show the same trend. The last two mentioned types of compounds are for their reaction with proteins partly dependent upon co-ordinate valency forces, probably located on peptide groups. The increased fixation of these compounds by denatured collagen is probably a direct result of the rupture of intermolecularly co-ordinated cross-links on the peptide groups (hydrogen bonds), caused by the distortion of the fibre structure in the denaturation process. Evidence is presented in support of the view that in hydrothermal denaturation of collagen links of the hydrogen bond type are ruptured and the peptide bond made accessible for co-ordination of external reactants.

The data are in accord with the views of Astbury and Lomax on the mechanism of denaturation of globular and fibrous proteins.

A research grant from *Statens Tekniska Forskningsråd* for the year 1943 for the investigation of the reactivity of fibrous proteins is gratefully acknowledged.

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Received August 1, 1947.