

Some Reactions of Esterified Collagen

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By modifying or inactivating specific protein groups, the function of any particular group in various reactions may be studied by comparison with the intact protein as a blank, provided that the method of altering the reactivity of the protein is specific and does not alter other protein groups to any appreciable extent and is attended by a minimum risk of degradation¹. In an earlier investigation of the fixation of cationic chromium complexes by collagen and of the participation of its carboxylic ions², the latter were completely discharged by H-ions in systems with pH values in the range 1.0—1.5, employing acid-resistant olated chromium salts containing added acid of the same pH in the presence of swelling-depressing neutral salts of the same anion. Since the behaviour of such systems is more informative in the pH range 3—4, this method has only a limited usefulness. Hence, the function of the ionic carboxyl groups of collagen in the fixation of various chromium complexes has been investigated by means of intact collagen and collagen with its free carboxyl groups methylated. The esterification of collagen and the reactivity of the resulting products have recently been investigated by Bowes and Kenten³ who employed methyl sulphate and methyl bromide; fourteen or more consecutive methylations with the former agent being required to obtain a fair degree of esterification. However, the details given in the paper of Bowes and Kenten indicate a marked breakdown of the protein during these severe treatments. These reagents as well as diazomethane and 1,2-epoxides, when used as methylating agents for globular proteins, combine not only with the carboxyl groups but also with other groups, *e.g.*, the amino groups and cause hydrolytic breakdown⁴. The same observations have been made in the present investigation on collagen, diazomethane in particular having given only a low degree of methylation of the carboxyl groups. A satisfactory, practically complete esterification of collagen with minimum of side reactions is obtained by the method of Fraenkel-Conrat and Olcott⁴, originally used

by them on globular proteins with very satisfactory results. This method involves treatment of the protein with methanol, 0.05–0.1 *N* in respect to hydrochloric acid, at room temperature and has been found to work satisfactorily on collagen.

MATERIALS AND METHODS

Complete esterification of the carboxyl groups was obtained by shaking 20 g of collagen in the form of acetone-dehydrated pieces of a thin split (the middle layer of corium) of a calf skin, consisting mainly of isoelectric collagen, in 1 000 ml of methanol to which had been added 8.0 ml of 36% hydrochloric acid, intermittently for 6 days at room temperature. After methylation, the collagen was rinsed with 50 per cent aqueous methanol, and placed in one litre of 4 per cent sodium chloride solution, in order to prevent swelling and to facilitate diffusion of the protein-bound hydrochloric acid into the salt solution. The mixture was shaken and kept at pH 5 by the gradual addition of *N* sodium hydroxide. After complete neutralization, the methylated stock was shaken in several changes of water for a few minutes each time, twice dehydrated in acetone for 24 hours and finally dried in vacuum at 60° C. It is important to keep the treatment in water as brief as possible since the methylated collagen swells considerably in water and hydrolysis of the ester may occur gradually. In some experiments the skin was used in the pressed state, containing about 60 per cent of water. The resulting product, otherwise treated as described, showed a slightly lower degree of esterification (0.73 meq. OCH₃ fixed by 1 g collagen). The methylated collagen was in the dry state very similar to acetone-dehydrated intact skin collagen, although somewhat more greyish and stiffer. The most marked difference, compared to intact collagen, was its high swelling power in water.

The analytical characteristics of the intact and methylated preparations of collagen are given in Table 1. The methoxyl content of intact collagen arises from its methionine. The degree of inactivation of the acidic (carboxylic) groups is calculated from the data of the acid binding capacity to be somewhere between 85 and 90%. The acid binding capacity (HCl) of the collagens was determined by treating portions of 0.5 g of the protein in 12.0 ml of 0.1 *N* hydrochloric acid, 0.75 *M* in sodium chloride for 2, 6 and 24 hours, titrating the excess acid (methyl red)⁵. The reaction was complete in 6 hours, since 6 and 24 hours treatments gave identical results. Hence, rather surprisingly no splitting of the ester takes place at pH 1.1.

The complete inhibition of chromium fixation from solutions containing all the chromium in the form of cations (see Table 2) indicates practically complete inactivation of the carboxyl groups. The content of free carboxyls of moderately limed collagen, such as the present specimen, is generally 0.8–0.9 meq. per g collagen and the number of methoxyl groups, 0.79 meq. per g, evidently closely corresponds with the number of free acidic groups. The shrinkage temperature, *T_s*, was determined on freely suspended strips of substrate, using the shocking method⁶, in order to eliminate as far as possible any complications due to swelling of the methylated collagen during the determination.

The total N content of the modified collagen is close to the theoretical value (17.97) calculated, assuming the methylation of the carboxyl groups. 0.79 meq. of carboxyl groups per g collagen are esterified. The acid binding gives a corresponding figure of 0.77. Of particular interest is the large decrease in the shrinkage temperature of 25° C. By complete inactivation of the acid-binding groups of collagen by means of the non-swelling

Table 1.

Type of collagen	% total N	% ash	% total N on ashfree collagen	meq. OCH ₃ per g collagen	Acidbinding capacity in meq. HCl fixed by 1 g collagen	T _s in °C
Intact	18.13	0.2	18.17	0.03	0.93	62
Methylated	17.87	0.5	17.96	0.82	0.16	37

naphthalene- β -sulphonic acid, specific for ionic protein groups, the electrovalent type of crosslinks in the protein are eliminated, resulting in a lowering of T_s by 16–18°⁷. Accordingly, the very much larger lowering of the hydrothermal stability induced by the esterification of collagen apparently must be due to the rupture or weakening of some other types of cohesive forces besides the salt-links. It is likely that non-ionic crosslinks (coordinate bonds) of the hydrogen bond type are involved.

The unusually large swelling of the isoelectric methylated collagen in water also indicates diminished intermolecular cohesion of the main type of bonds present in collagen, *i.e.*, coordination crosslinks (hydrogen bonds). This view is strengthened by the results from experiments on the combining capacity of the methylated collagen for agents which react with collagen mainly by means of coordinate valency forces (non-ionic).

RESULTS AND DISCUSSION

Before entering into a discussion of the main problem of this investigation, *i.e.*, the function of the carboxyl ions of collagen in the binding of chromium complexes, the results of some experiments on the general reactivity of this modified collagen will clarify the picture.

In comparative experiments with the two types of specimens of Table 1, the fixation of a synthetic tannin of the type of a low sulphonated Novolak resin was determined. Specimens equal to 2.0 g of collagen were shaken two consecutive times in 25 ml of a 10 % solution of the Novolak resin adjusted to pH 4, in order to minimize as far as possible the ionic function of the sulphonic acid group of the Novolak in the reaction. In spite of the fact that part of the binding of the syntan by the intact collagen is due to ionic interaction, which type of acid binding is practically eliminated for the esterified collagen, the latter fixed larger amounts of the sulphonated resin than the intact collagen; the percentages, on the basis collagen, being 72.6 and 60.2 respectively. The shrinkage temperature was elevated by 14° for the intact collagen and by 39° in the case of the modified collagen. The main reactivity of the Novolak sulphonate at pH 4–5 is due to the phenolic groups, probably due to their coordination on non-ionic groups of collagen, as for example, the peptide groups. Hence, it is evident that the coordination reactivity of the esterified

collagen is considerably greater than that of collagen proper, the former possessing a larger number of coordination active sites. These probably result from the rupture of non-ionic crosslinks (hydrogen bonds) in the treatment with methanol.

This is further brought out by the data on the irreversible uptake by the two types of collagen of agents which react with collagen preponderately by means of non-ionic valency forces, such as tannic acid and mimosa tannins. These experiments were carried out with hide powder in its native form and after methylation as described, since the very high affinity of the esterified skin collagen for these natural tannins jeopardized the penetration of the tannins into the interior of the skin because of obstruction by the heavily fixed tannin. Treatments for five days in solutions of these tannins commencing with 5% strength of the solutions and finishing up in 10% solutions, in both instances containing 0.6 eq./l of sodium chloride, gave the following irreversible fixation of tannins, calculated on the weight of collagen: The intact hide powder fixed 71 per cent and the methylated hide powder 107 per cent of tannic acid at a final pH of 4.0. The corresponding figures for the mimosa fixation at a final pH of 5.0, both pH values selected in the range 4–5 in order to minimize hydrolytic demethylation during the extended treatment, were 62 and 109 respectively. These data provide evidence for the occurrence of secondary reactions during the esterification leading to rupture of non-ionic crosslinks of the protein making additional sites available for reaction. However, these changes do not affect the electrovalent reactions, such as those of cationic chromium complexes.

The experiments with the chromium compounds were carried out with the two types of skin collagen, employing portions of hydrated skin equal to 2 g of collagen, which were shaken for 6 hours in 50 ml of the solution of chromium salt containing 0.6 eq./l Cr, if not otherwise noted. Making the solutions 0.5 *N* in regard to neutral salts by the addition of sodium chloride to the solutions of chromium chlorides and sodium sulphate to those of the chromium sulphates, swelling of the substrate was prevented. The amounts of chromium fixed by the intact and the methylated collagens, and the increase in shrinkage temperature of the tanned specimens are given in Table 2 which also lists particulars of the nature of the compounds employed and the reaction.

The composition of the various compounds in regard to the electrochemical state of the chromium complexes was determined by the ion exchange technique using the sodium salt of Dowex 50 and the hydrochloride of Amberlite, IRA-400⁸. The increase in the shrinkage temperature, ΔT_s , determined by the shocking method is a measure of the degree of stability of the chromed protein structures, the + sign denoting improved and the – sign impaired degrees of hydrothermal stability. It is to be noted that solutions no. 1, 2, and 3 contained all the chromium as positively charged complexes, whereas the chromium in the remaining solutions existed predominantly as non-cationic complexes.

The data show that for the fixation of the cationic chromium complexes of the chloride, perchlorate and sulphate the presence of the ionic carboxyl groups of collagen is essential. By prolonging the time of reaction, a slight fixation of cationic chromium gradually takes place, probably mainly due to the hydrolytic splitting of the ester link which frees carboxyl groups available for the binding of cationic chromium. This is demonstrated by Fig. 1 which

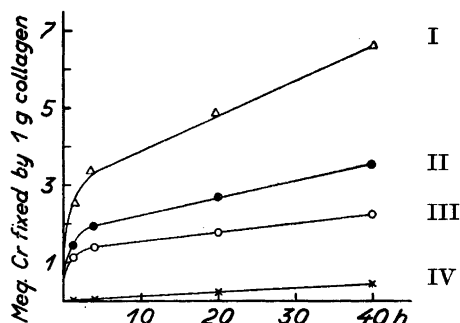


Fig. 1. The fixation of chromium by intact collagen and methylated collagen at various time of interaction.

Sulphito-chromium sulphate (no. 6 of Table 2) with 72% of its chromium in the form of non-cationic complexes:

I — Δ — Δ — intact collagen

II — \bullet — \bullet — methylated collagen.

Cationic complexes of 66% acid Cr-chloride (no. 1 of Table 2):

III — \circ — \circ — intact collagen

IV — \times — \times — methylated collagen.

shows the fixation of chromium by the two types of collagen, as a function of time, for a typical cationic agent and a typical coordination-active agent, largely composed of non-cationic chromium. The gradual, but marked, augmentation of the fixation of the sulphito-chromium sulphate is characteristic for reactions of the coordination type which require more time for the attainment of equilibrium than the ionic type of reactant, represented by the basic chromium chloride. The linear function of the slight fixation of the latter compound with time is in line with a gradual freeing of carboxylic groups by hydrolysis of the ester.

Whereas equilibrated dilute solutions of basic chromium sulphates of the type given in Table 2 (no. 3), which are composed mainly of cationic sulphato-hydroxo-chromium complexes, do not react with the esterified collagen, highly concentrated solutions of these compounds (Table 2; no. 4) possess some affinity for collagen devoid of carboxylic groups. By increasing the chrome concentration of these basic sulphates, non-ionic and anionic chromium complexes are formed; in the present instance (no. 4) the main constituent is of non-cationic nature. This latter type of complexes is thus responsible for the uptake of chrome by the methylated collagen, probably by means of coordination of these complexes on groups in the collagen other than the carboxyl groups. Such a dual nature of the chromium fixation by intact collagen from concentrated

Table 2.

No.	Type of chromium compound	Type of collagen	Complex composition			Final pH	meq. Cr fixed by 1 g collagen	ΔT_s in °C
			cat-ionic	non-ionic	an-ionic			
1	66% acid Cr-chloride	Intact	100	0	0	2.9	1.70	+27
1	corresp. to: $\text{Cr}_2(\text{OH})_2\text{Cl}_4 \cdot 2\text{NaCl}$	Methylated	100	0	0	2.8	0.00	-6
2	66% acid Cr-perchlorate	Intact	100	0	0	2.8	1.42	+23
2	corresp. to: $\text{Cr}_2(\text{OH})_2(\text{ClO}_4)_4 \cdot 2 \text{NaClO}_4$	Methylated	100	0	0	2.7	0.00	-4
3	66% acid Cr-sulphate	Intact	96	4	0	3.0	1.96	+38
3	corresp. to: $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot \text{Na}_2\text{SO}_4$	Methylated	96	4	0	2.8	0.04	-6
4	66% acid Cr sulphate as no 3 except	Intact	42	48	10	2.7	2.84	+38
4	5.0 eq./l Cr	Methylated	42	48	10	2.7	0.69	-4
5	33% acid Cr-chloride	Intact	32	68	0	3.5	4.24	+26
5	corresp. to: $\text{Cr}_2(\text{OH})_4\text{Cl}_2 \cdot 4\text{NaCl}$	Methylated	32	68	0	3.5	0.38	-4
6	Sulphito-Cr-sulphate	Intact	28	50	22	4.5	3.72	+37
6	corresp. to: $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot \text{Na}_2\text{SO}_4 \cdot 2\text{Na}_2\text{SO}_3$	Methylated	28	50	22	4.5	1.65	+12
7	Phthalato-Cr-sulphate	Intact	32	68	0	4.6	3.01	+34
7	corresp. to: $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot \text{Na}_2\text{SO}_4 \cdot (\text{NaOCO})_2\text{C}_6\text{H}_4$	Methylated	32	68	0	4.6	0.92	+2

solutions of basic chromium sulphate containing at least one mole of neutral sulphate per mole of chromium oxide, was indicated in earlier investigations of the reactivity of collagen with discharged carboxyl ions (at pH = 1.5). The present findings corroborate this view. The main reaction is the ionic attraction of the chromium cations to the carboxyl ions of collagen, followed by the penetration of the discharged carboxyls into the chromium complex with the formation of a covalent-coordinate link with chromium. In highly concentrated solutions an additional reaction of the non-cationic chromium complexes which does not require the ionic carboxylic groups of collagen, is shown to take place. The second type of chromium fixation is probably of coordinated nature, and does not lead to any marked stabilization of the protein lattice. Chlorides of low basicity which do not show this non-ionic type of reaction, conform with the explanation given, since they do not form any appreciable amounts of non-cationic chromium complexes.

From the solutions of basic chromium sulphates complexed by means of phthalate and sulphite which form mixed chromium complexes of non-ionic and anionic nature, the esterified collagen fixes such complexes, evidently by groups other than the carboxyl groups. Accordingly, a large part of the binding of sulphito-sulphato-chromium compounds by intact collagen must then occur through non-ionic protein groups (coordination)⁹.

As a further illustration of this type of fixation of chromium complexes by collagen, the reactivity of such a sulphito compound consisting to three fourths

of non-ionic chromium and to one fourth of anionic chromium is of interest. Solutions of this compound, which contained three moles of sodium sulphite per one mole of chromium oxide, did not react with the carboxylic ions of a cation-exchanging resin, Amberlite IRC-50, equilibrated with sodium ions at pH 7. This proves that the charged carboxyl group cannot penetrate into the nucleus of these non-cationic complexes. The fixation of this compound by collagen devoid of carboxyl groups was 3.6 meq. Cr per g collagen compared with 6.3 meq. per g intact collagen. In addition to peptide bonds and cationic protein groups as potential sites for the fixation of non-cationic chromium complexes, the possible participation of hydroxy groups*), such as those of serine and hydroxyproline, in the binding of these complexes by collagen is indicated by data from interaction of modified polyamides with these chromium compounds (10).

The behaviour of collagen with its cationic as well as its anionic groups completely inactivated by means of irreversibly fixed polymetaphosphoric acid towards basic chromium salts is of interest in this connection. This polymetaphosphated collagen fixes chromium compounds irrespective of their electrochemical type. The reaction occurs probably through reactive centres of the polymetaphosphate chain held by the protein. This indirect binding of cationic chromium complexes does not lead to an improved degree of stability of the original collagen-compound or only a minor one. However, the sulphito compound mentioned elevates the shrinkage temperature by 23° C upon 4 hours' interaction. Solutions of the basic sulphate (no. 3), equilibrated at a concentration of 0.6 eq./l Cr, only slightly increases this property (4–5° C), whereas the solution of this sulphate applied *immediately* after diluting the stock solution of 6.0 eq./l Cr to the aforementioned concentration of 0.6 eq./l Cr raises the shrinkage temperature by 25° C. The directly diluted solution contained as the dominating constituent, non-ionic chromium complexes. Apparently this additional fixation involves the non-ionic protein groups, resulting in additional stabilization of the collagen lattice. These observations add further strength to the concept advanced.

Concluding Remarks. The main finding of this investigation affords an additional proof of the governing importance of the carboxyl ions of collagen for the fixation of cationic chromium complexes. This important reaction appears at the present state of our knowledge to be initially an ionic reaction between the positively charged chromium complexes and the negatively charged carboxyl groups of collagen. The discharge is followed up by the most important reaction for the stability of the resulting protein-compound formed, the penetration of the carboxyl group into the chromium complex, entering into

*) Professor John T. Edsall should be credited with the suggestion of residues of hydroxyamino acids as *loci* for the binding of tanning agents by collagen (personal communication, 1948).

covalent-coordinate linking with chromium. By attachment of two or more carboxylic groups of adjacent protein chains to two or more chromium atoms of one polynuclear chromium complex, an exceedingly stable crosslinking of the protein chains should be effected.

The high degree of stability of the chrome-carboxyl link exhibited by chrome leather tanned by means of cationic chromium compounds is also evident in the chromium compound formed between cationic chromium complexes and cation exchanging resins carrying the carboxyl ion as functional group. These are absolutely resistant towards neutral salts and only a very small part of the chromium fixed by the resin is removed upon prolonged and repeated digestions with 5 *M* solutions of hydrochloric acid. A great excess of strong complexing agent containing hydroxy and carboxylic groups leads to a gradual removal of the fixed chromium in both instances, through the mass action effect of the added complexing agent.

SUMMARY

The carboxyl group of collagen has been completely esterified by means of methanol made 0.1 *N* in respect to hydrochloric acid, following the method of Fraenkel-Conrat and Olcott. The esterified collagen contained 0.79 meq. methoxyl per g collagen and its HCl-binding capacity was reduced by 0.77 meq. H-ion. The methylated collagen commenced to shrink at 37° C, compared with 62° for the intact collagen. It swelled considerably in water.

Investigations of the reaction of various chromium compounds with intact and esterified collagens show:

1. Dilute solutions of basic chlorides, perchlorates and sulphates of chromium, containing cationic chromium complexes, have practically no affinity for collagen with its carboxyl groups completely inactivated by esterification.

2. From concentrated solutions of basic chromium sulphates of the type: $\text{Cr}_2(\text{OH})_m(\text{SO}_4)_n \cdot \text{Na}_2\text{SO}_4$, which contain a large percentage of non-ionic and anionic chromium complexes, the esterified collagen fixes small amounts of chromium. This fixation does not appreciably improve the hydrothermal stability of the protein. The reaction is indicated to involve non-ionic protein groups by coordination. This implies a dual nature of the chrome fixation by collagen from concentrated solutions of basic chromium sulphates.

3. From solutions of sulphito-sulphato-chromium compounds and of basic chromium sulphates complexed by means of sodium phthalate, with non-cationic chromium as the main constituent, esterified collagen fixes irreversibly large amounts of these complexes, in some instances amounting to more than

half of the value of chromium fixed by intact collagen. This fixation markedly improves the hydrothermal stability of the modified collagen.

4. The results prove the dominating function of the ionic carboxyl groups of collagen in the fixation of the most important chromium compounds, *i.e.*, positively charged chromium complexes. The final result is probably a multi-point crosslinking of adjacent protein chains through the incorporation of two or more carboxylic groups of different chains into one polynuclear chromium complex with direct attachment of the carboxyl group on the chromium.

5. The similarity of chrome-collagen compounds and chrome-carboxylic resin compounds is stressed.

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