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Evidence for the Presence of the —OH···OC-Link in Collagen from its Fixation of Non-ionic Chromium Complexes

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Data on the fixation of various chromium complexes, particularly nonionic sulphito-chromium sulphates by intact collagen, on the one hand, and by denatured and otherwise modified collagens, particularly hide protein of various degrees of acetylation (O- and N-acetylated), on the other, are in harmony with the postulate that a considerable part of the hydroxy groups of mammalian collagen are internally compensated by stable hydrogen bonds with the oxygen atoms of the keto-imide groups of adjacent chains.

The presence of an interchain crosslink of the hydrogen bond type, between the hydroxy and keto-imide groups in collagen has been suggested in earlier papers [1,2], in which experimental findings supporting this assertion were briefly discussed. The excellent correlation between the content of hydroxyproline and the degree of hydrothermal stability of collagens of a variety of teleostean and mammalian skins [3,4] was one of the principal arguments, further emphasized by the finding that by exhaustive acetylation of bovine collagen (N- and O-acetylation), its shrinkage temperature was lowered by 25° C [1]. Moreover, the changed reactivity of the bovine collagen caused by the inactivation of the hydroxy groups of collagen was found to conform with the postulated crosslinkage [2]. In the present paper, some additional data considered by the author to be indirect proof for the existence of such a stabilizing bond are presented.

EXPERIMENTAL

Materials. a) Substrates. Mammalian collagen in the form of hide powder (Lyon) and modified specimens of this hide powder were employed as substrates. Hide powder collagen of various degrees of acetylation formed the most important substrate of the modified collagens. In the N-acetylation and the exhaustive acetylation (N- and O-acetylation) of the collagen, the technique of Green *et al.*[5] was used. A sample of an intermediate degree of acetylation (complete N-acetylation and about 40 % of the

hydroxy groups acetylated) was included in the series. The treatment was identical with that of the exhaustive acetylation, except that the time of acetylation was reduced from 12 days to 6 days. Further, collagen (hide powder) with its carboxyl groups esterified exhaustively, prepared by the methylation technique of Fraenkel-Conrat and Olcott [6], described earlier [7], was included in the series. Finally, hide powder which had been heat-denatured in water of 70° C for one minute [8], and as a representative of the lyotropic salt effect [9], hide powder pretreated for 4 days in 2 M solution of sodium perchlorate of pH 5.5 at 20° C, subsequently washed and freed from the salt, were employed. The analytical characteristics of these specimens of collagen are given in Table 1.

Table 1. Some analytical characteristics of the specimens of collagens (on 1 g ash- and fat-free dry substance).

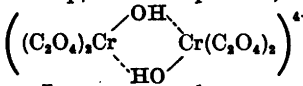
Substrate	% Total N	meq. N-acetyl	meq. O-acetyl	Acid binding capacity in meq. HCl	*T*s in °C
Hide powder (Blank)	18.05	0.00	0.00	0.92	65
N-acetylated hide powder	17.9	0.39	0.00	0.76	66
Partially O-acetylated hide powder	17.2	0.39	1.01	0.72	44
Exhaustively O-acetylated hide powder	16.6	0.39	1.25	0.60	40
Esterified hide powder *	17.3	0.00	0.00	0.11	35
Heat-denatured hide powder (shrunk)	18.0	0.00	0.00	0.92	—

* 2.83 % methoxyl on the protein basis (corrected value).

It should be noted that sodium perchlorate in 2—4 M solutions has proved to be the most excellent lyotropic agent among the numerous neutral salts of this type tested. Its main action on collagen is directed on the non-ionic crosslinks (hydrogen bonds) which are ruptured. In the present instance, the loss of collagen incurred in the brief treatment amounted to 22 % of the original amount. Finally, a modified polyamide [10-12], based on the copolymerisate of adipic acid-hexamethylene salt (60 %) and caprolactam (40 %), containing 12.1 % N, was selected since it supplies a substrate with its reactivity restricted to the coordination faculty of the keto-imide group, the most frequently occurring protein linkage. This special polyamide was made into woolly fibres by stirring the hot saturated methanol solution of the polyamide into water. Its base binding capacity (at equilibrium pH of 12) was only 0.02 meq. per g polyamide [10-12].

b) Interacting agents (Tanning agents). In order not to obscure the main points of the issue involved, only a few examples selected from the numerous tanning agents and complex salts investigated will be given. The reaction of some aromatic compounds with a selective affinity for the hydroxy group will be separately treated in another connection. The main reagents employed were chromium salts, representing various types of reactivity. The typical cationic chromium complexes, the sulphato-oxo-chromium cations, with a composition corresponding to the empirical formula $(Cr_3(OH)_2SO_4)^{3+}$ were supplied by an equilibrated solution of the 67 % acid chromium sulphate $(Cr_3(OH)_2(SO_4)_3) \cdot Na_2SO_4$, at a concentration of 0.4 eq./l. It consisted of 98 % of cationic chromium complexes [13] and is a compound specifically restricted to the carboxyl ions of collagen for its irreversible fixation. A solution of 67 % acid chromium chloride, corresponding to the empirical formula $Cr_3(OH)_2Cl_4 \cdot 2NaCl$ (Ref.[13]) equilibrated at 1.0 eq./l Cr, which contained mainly cationic chromium complexes, represents an agent predominantly fixed by the carboxyl ions of collagen but this reaction, however, is supplemented at the concentration used, by secondary reactions involving the non-ionic protein groups. An extremely basic chromium chloride, corresponding to the formula $Cr_3(OH)_4Cl_2 \cdot 2NaCl$ (Ref.[13]) employed in a solution containing 0.6 eq./l Cr, and composed of 70 % non-ionic chromium complexes and 30 % cationic ones, was included, and also the corresponding chromium perchlorate, at the same chromium concentration, containing 68 % non-ionic chromium complexes and 32 % cationic ones. In the reactions of these solutions with collagen, the non-ionic complexes take a prominent part. Another type of non-ionic chromium complexes is present in the solutions of the sulphite-complexed basic chromium sulphates,

which were prepared by adding 2 moles, and 2.5 moles of sodium sulphite per mole Cr_2O_3, to a salt-free 67 % acid chromium sulphate solution, applied at a concentration of 0.8 eq./l Cr. The complex composition of the solution corresponding to the stoichiometric ratio: $Cr_2(OH)_2(SO_4)_2 \cdot 2Na_2SO_3$, was 80 % non-ionic chromium, 12 % cationic and 8 % anionic complexes [7]. The corresponding figures for the more highly sulphite-masked solution was 86, 0, and 14. The pH values were 4.2 and 4.8, respectively. A similar solution of complexed basic chromium sulphate, in which sodium phthalate was the effective masking agent, employed 1 mole phthalate per mole Cr_2O_3 at 0.8 eq./l Cr and contained highly aggregated chromium complexes, 65 % in the non-ionic form and 35 % in the form of cationic complexes [14]. Finally, a solution of sodium tetraoxalatodiol-chromiate [15,16], 0.8 eq./l Cr and pH 5.6, containing the anionic chromium complex:



was selected as a typical anionic agent.

In some complementary experiments the tanning with a solution of mimosa extract (the condensed type of vegetable tannin), 5 w/v % tannins, pH 4.8, was studied.

Methods. Portions equal to 1.0 g protein, completely hydrated, were shaken with 40 ml of the solutions of the tanning agents for 48 h after which time the equilibrium of the system had been attained in the majority of the series. For some series, notably the oxalato-chromiate, two weeks' reaction time was required, and one week for the mimosa tannage. After complete reaction, the substrates were freed from soluble matter, the point being indicated by the conventional analytical tests on the exhaust water. The analytical determinations were carried out by conventional methods.

RESULTS

It has been established previously [12], that the vegetable tannins of the condensed type, such as the mimosa tannins are predominantly fixed by the keto-imide link of collagen; their binding by polyamides, with the —CONH-group as the sole reacting group, being the crucial evidence [12]. It has also been shown that by heat denaturation of collagen (thermal shrinkage [8]), or by pretreatment of collagen with lyotropic agents in concentrated solution, subsequently restoring the protein to its isoelectric state, the irreversible binding of vegetable tannins is greatly increased [9]. By these modifications of collagen, additional coordination sites (hydrogen bonding) on the non-ionic protein groups, internally compensated in the intact collagen, are made available to the tannins by the rupture of crosslinks, in which such groups are involved.[17]

Table 2 contains a resumé of data on the fixation of mimosa tannins by the various substrates.

Table 2. The fixation of Mimosa tannins by various substrates (in % tannin fixed by the protein).

No.	1	2	3	4	5	6	7	8
Type	*Esterified hide powder*	*Polyamide*	*Hide powder (=h.p.)*	*Shrunk h.p.*	*H.p. pretreted in 2 M $NaClO_4$*	*N-acetyl. h.p.*	*Partially O-acetyl. h.p.*	*Exhaustively acetylated h.p.*
% fixation	124	97	60	96	118	56	78	93

In Table 3 the data on the fixation of the chromium compounds by the various substrates are summarized.

Table 3. The fixation of chromium complexes by the various substrates (in % Cr_2O_3 fixed by the protein).

No.	1	2	3	4	5	6	7	8
Solution	*Polyamide*	*Esterified collagen*	*Hide powder (=h.p.)*	*Shrunk h.p.*	*H.p. pretreted in 2 M $NaClO_4$ solution*	*N-acetyl h.p.*	*Partially O-acetylated h.p.*	*Exhaustively acetylated h.p.*
67 % acid Cr-sulphate	0.2	0.1	10.2	11.0	11.9	8.2	7.8	7.0
67 % acid Cr-chloride	0.3	0.4	6.6	8.8	6.2	6.5	7.6	7.8
33 % acid Cr-chloride	2.5	3.1	12.4	18.1	21.3	11.2	15.5	18.7
33 % acid Cr-perchlorate	7.2	2.6	8.4	14.1	15.9	7.7	10.0	12.4
Sulphito-Cr-sulphate ($2SO_4$:$1Cr_2O_3$)	0.2	21.0	23.9	37.0	40.7	19.2	—	20.8
Sulphito-Cr-sulphate ($2.5SO_3$:$1Cr_2O_3$)	0.9	40.6	29.4	53.8	60.3	25.8	24.1	22.7
Phthalato-Cr-sulphate	4.5	7.0	17.2	27.5	30.4	15.2	—	15.8
Oxalato-chromiate	0.1	13.6	6.7	12.4	13.9	5.9	—	5.1

DISCUSSION

The data on the fixation of the vegetable tannins by the various collagen preparations indicate that by the pretreatments in which rupture of crosslinks of the hydrogen bond type has been proved to occur, such as in the series Nos. 1, 4, 5, and 8, the combining power of the collagen for these coordination agents is increased by about 50 % of the values obtained from the intact collagen. It should be noted that exhaustive acetylation, *i. e.*, acetylation involving the hydroxy group, is as effective in augmenting the fixation of the polyphenolic agent as is the straight breaking of crosslinks in the collagen lattice by supplying energy to the system. Also the methylation of the carboxyl groups of collagen (No. 1) which is carried out at a pH value as low as 1 in methanol, results in the breaking of bonds in which groups forming binding sites for the vegetable tannins are freed, and additional binding sites made available to the tannins. As already mentioned, the very marked tannin uptake by the polyamide, *i. e.*, by means of the —CO—NH-link, supports the concept of the increased tannin fixation resulting from the pretreatments mentioned being mainly due to the free sites of reaction on the keto-imide groups resulting from the dislocation of hydrogen bonds in which the peptide link partakes. Obviously, these systems supply no information whether the additional coordination sites on the —CO—NH-groups are due to the breaking of hydrogen bonds between two adjacent keto-imide linkages, or to the rupture of the hydroxy-keto-imide link, or to both reactions. These findings of the changed reactivity of the modified collagens towards an agent which is attached chiefly to the keto-imide group are important for the elucidation of the reaction of non-ionic chromium complexes with collagen.

For a discussion of the data in Table 3, and particularly to enable conclusions to be drawn from them, it is advisable didactically to discuss the data obtained with solution No. 1 first, *i. e.*, the cationic chromium complexes which do not react with substrates lacking in carboxyl ions, such as the polyamide and the esterified collagen [11]. By heat denaturation, the ionic groups of collagen are not made more reactive or increased [8]. Hence, the cationic chrome fixation should not be affected by the hydrothermal shrinkage of collagen, as is the case [8]. The severe lyotropic pretreatment (No. 5), inducing very great swelling of the collagen, evidently results in secondary breaking of cross-links in which the carboxyl group is involved, the result being slightly increased uptake of the cationic chromium complexes.

By inactivation of the amino group by acetylation (No. 6), the chrome fixation is lowered. This decrease is of the order generally found for collagen in which the amino group has been removed or inactivated [18]. This impairment has been shown to be a secondary effect of the decreased uptake of the protolyzed acid of the system, which change retracts on the chrome fixation that is intimately bound up with the removal of protolyzed acid by collagen [18]. The same trend, but still more pronounced is shown by the O-acetylated specimens which indicates that the hydroxy group is apparently involved in the creaction.

The more concentrated solution of the 67 % acid chromium chloride which contained preponderantly [13] cationic chromium complexes, was intentionally selected, since it presents a more complicated type of reaction. Apart from the primary ionic fixation, the main reaction, the occurrence of another mode of reaction is indicated at the chromium concentration employed. It should be noted that from dilute solutions of this chromium compound the chrome fixation is entirely ionic, located solely on the carboxyl ions of the collagen. Thus, the increased reactivity of the non-ionic protein groups resulting from heat denaturation or the lyotropic salt effect augments the chromium uptake from the more concentrated solution by about 25 % of the amount fixed by the intact collagen. The N-acetylation has no effect on the reaction, but after O-acetylation of collagen an increased chromium fixation is noted. Apparently, come groups are freed by the exhaustive acetylation which fix the non-ionic somplexes present in the solution of the basic chloride.

A more favourable case for the interpretation of the reaction mechanism is the extremely basic chromium chloride (33 % acidity) solution, in which non-ionic chromium complexes predominate [13]. It possesses only a slight affinity for the keto-imide groups (the polyamide). Hence, the drastically increased chrome fixation, effected by the thermal shrinkage and particularly by the lyotropic pretreatment, must be due to the freeing of some other group than the keto-imide linkage in these denaturations. In view of the inert behaviour of these chromium complexes to the polyamide, it is difficult to explain the marked effect of the O-acetylation *i. e.*, the inactivation of the hydroxy groups, on the uptake of chromium. It is possible that the presence of some other protein group is required for its fixation by means of the —CO—NH-link which is absent in the polyamide. At the present state of our knowledge, the reaction cannot be explained.

The corresponding perchlorate (33 % acid) shows a great affinity for the polyamide, the amount of chromium fixed in this instance being about 85 % of the amount fixed by collagen. This marked increase of the binding of chromium (from 8.4 to 12.4 % Cr_2O_3), resulting from the inactivation of the hydroxy groups, is satisfactorily accounted for by the additional reaction sites on the —CO—NH-links formed by the breaking of the —OH----OC-NH- bonds in the O-acetylation.

The data from the series with solutions of the non-ionic sulphitochromium sulphates supply the *crucial* experimental support for the view postulating the presence of a stable link: the hydroxy group being internally compensated by a strong hydrogen bond to the oxygen atom of the keto-imide group. The polyamide has *no* affinity at all for the sulphito complexes [11], while the esterified collagen binds greater amounts of these complexes than the intact collagen [7], accounted for by the extensive rupture of coordinate crosslinks (hydrogen bonds) in the heavy swelling taking place in the methylation process, as mentioned already (T_s lowering of 30° C). By the thermal shrinkage and the lyotropic pretreatment, the fixation of the non-ionic sulphito complexes by collagen is tremendously increased [8,9]. The most convincing proof of the participation of the hydroxy group in this reaction concerns the effect of the O-acetylation which results in a very marked decrease of the irreversible fixation of chromium by collagen. The only explanation which conforms with all the facts cited and with other findings which cannot be enumerated in this connection is that the —OH----OC-bond in collagen is broken by the acetylation of the hydroxy group and thus the compensation on the —CO—NH-group is removed and it is made coordinatively reactive. This will result in the augmentation of reactions on the keto-imide group (by hydrogen bonding), as for instance the uptake of polyphenolic vegetable tannins and the non-ionic chromium perchlorates, while the fixation of the non-ionic sulphito-chromium complexes, which rely on the hydroxy groups for their attachment to collagen, is impaired.

It is to be noted that the pretreatments of collagen which would be expected to rupture the postulated crosslink, setting free both the OH group and the —CO—NH-group have a very favourable effect on the binding of the sulphito complexes, and indicate that they are bonded to the freed OH-groups.

It was deemed of interest to include in these series compounds with a tanning potency expected to be similar to that of the sulphito-chromium sulphate. The solution of the phthalato-chromium sulphate, obtained by the addition of one mole of sodium phthalate to each mole Cr_2O_3 of the 67 % acid salt-free chromium sulphate solution was used. The phthalate is a powerful complexing agent for chromium [19], and also markedly increases the size of the masked chromium complexes by bridging the chromium atoms [19]. By the extent of masking employed, about two thirds of the cationic sulphato-oxo-chromium cations were converted into non-ionic phthalato-chromium complexes [14]. As to the reactivity of these complexes, it should be noted that they have a marked affinity for the polyamide, about two thirds of the amount of chromium taken up by the esterified collagen being fixed. This fact is of importance from the point of view of the non-reactivity of the non-ionic sulphito complexes with the polyamide, since their inertness cannot be due to

their large molecular size, hindering diffusion into the polyamide, in view of the very large fixation of the still more highly aggregated phthalato-chromium complexes, and also of vegetable tannins with molecular weight of one to two thousand. The uptake of the phthalato complexes by collagen is slightly impaired by its N-acetylation, which is probably due to the indirect effect of the blocking of the amino groups on the cationic chromium fixation as already mentioned. By exhaustive acetylation (including the OH group), no further lowering of the chromium uptake than that noted for the N-acetylated specimen is found. Since by the O-acetylation, the hydroxy group is inactivated, while the keto-imide group is activated coordinatively, the independence of the chromium fixation noted in this instance might be interpreted as implying that both the protein groups which form this hydrogen bond, function as binding sites for the phthalato-chromium compounds. The increased reactivity to be expected from the freeing of the —CO—NH-link from its compensation by the hydroxy group, is then balanced by the blocking of the potential coordination sites on the OH groups. For the non-ionic chromium compounds specifically directed on to the keto-imide group, the O-acetylation results in a heavy increase of the chrome fixation, as earlier noted. The final result of the moderate lowering of the amount of chromium fixed, compared to intact collagen, effected by the inactivation of the hydroxy groups and the activation of the keto-imide group, should thus exclude a reaction exclusively governed by the keto-imide-coordination but favour the occurence of two reactions of the opposite trend, almost counteracting each other.

Finally, the combination of the tetraoxalato-diol-chromiate with the various substrates represents the reactions of an anionic complex. This has no affinity for the polyamide, excluding the participation of the polypeptide link in its attachment to collagen. By the esterification of collagen the chrome fixation is more than doubled. Besides making a greater number of cationic protein groups available for the chromiate by the discharge and inactivation of the carboxyl ions in the esterification, the freeing of non-ionic protein groups by dislocation of various hydrogen bonds in the collagen structure (swelling) is probably responsible for the increased reactivity noted, further supported by the data on the effect of the denaturation processes. By exhaustive acetylation the chrome fixation is impaired to a greater extent than by N-acetylation, indicating that the hydroxy groups are taking some part in the binding of this type of chromium complexes. The marked influence of the heat denaturation of collagen and the lyotropic effects on the binding of the oxalato complexes, practically doubling the fixation, should be viewed in the light of the inability of the —CO—NH-groups of the polyamide to react with these complexes. Moreover, considering that the reactivity of the cationic protein groups is not altered by these pretreatments of collagen [9], the conclusion seems justified that the hydroxy groups form sites for the binding of a part of the oxalato-chromiate, particularly in the later stage of reaction. For the participation of the cationic protein groups, adequate experimental proofs have been adduced earlier [15,16]. It is of interest to note that the reaction of the oxalato compounds with collagen occurs at a very slow rate [16], several weeks of tanning being required for attainment of equilibrium, while the corresponding point in the tanning with cationic complexes, *i. e.*, reactions restricted to

the free carboxyl ions of collagen, is already reached after only a few days [20]. The participation of hydroxy groups gradually freed from their compensation and made available to the oxalato complexes would satisfactorily explain the time factor. A similar trend of the rate of fixation is shown by the sulphito-chromium compounds. Further, some type of coordination of the hydrogen bond type is probably involved in the reaction since a considerable part (about a third in the present instance) of the oxalato complexes fixed by collagen are removed on treatment with 6—8 *M* solutions of urea, which specifically removes hydrogen bonded compounds from their attachment on collagen [21].

Summing up, the different trends of the data on chrome fixation by collagen after its acetylation and denaturation, with regard also to the behaviour of the chromium compounds studied with the polyamide (coordination on the —CO—NH-group), appear to be adequately explained by the assumption of the presence of a cross-link of the type —OH----OC<(NH—)(—) in collagen, which is severed by certain pretreatments of collagen. It is obvious that the postulated link, which is supported by numerous other findings [1,2], does not detract from the importance of the hydrogen bridges between adjacent keto-imide groups, generally conceived to be the principal stabilizing crosslinks in proteins.

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