every third amino acid residue of collagen is generally considered to be a pyrrolidine residue. This proportion is an integral part in recent models of collagen commencing with Astbury's classical model [9], and up to the helical structure principle of Pauling and Corey [10], although they have recently suggested occasional units of one residue of prolines of four residues [11].

The effect of the hypro content of collagen on its stability and reactivity was first examined in the light of Klotz's concept [12], according to which the hydroxy groups of the residues of aliphatic amino acids form hydrogen bonds of the type: $-OH\cdots\overline{O}CO-$ in some globular proteins. This linking should affect the reactivity of the carboxyl ion. The reactions of bovine and cod skin collagen with the cupric ion and with a number of cationic, anionic and non-ionic complex compounds of chromium, dyestuffs and non-electrolytes have been studied in the isoelectric zone. The results could not readily be reconciled with an extension of the Klotz concept to the collagens. Moreover, according to Bear [13], the electron-optical researches suggest the presence of the prolines in the interbands of the protofibrils, whereas the bands should contain the residues of the dicarboxylic amino acids. Hence, formation of the hypro-hydroxy—carboxyl bond appears improbable for steric reasons. Bear's concept has recently received strong experimental support chemically by Zahn's [14] isolation of dinitro-diphenyl-sulphone-*bis*-lysine derivatives and data of Schroeder *et al.* (15) provide some evidence that the peptides in partial hydrolysates of gelatin and collagen may contain the sequence: glycine-pro-hypro-glycine frequently. The nature of the effect of the hypro residues on the organization of collagen and the type of bond which the hypro forms have been studied on modified bovine collagens. A preliminary account of this work is given in the following note

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The Presence of Interchain Links Between Hydroxy and Keto-Imide Groups in Collagen

K. H. GUSTAVSON

Garverinäringens Forskningsinstitut, Stockholm, Sweden

The function of the hydroxyproline (=hypro) residue, the imino acid unique for collagen, has come to the forefront by the observation that the hypro content is directly related to the organization and reactivity of collagens [1]. Its possible participation in interchain links is obvious. An attempt of characterization of such cross-links has been made, primarily by the study of the effect of inactivation of the hydroxy groups of collagen on its properties and behaviour to certain reactants. The results of the investigation of collagen with its amino groups completely and its hydroxy groups to the maximum extent acetylated indicate that a part of the hydroxy groups forms a strong bond with the oxygen of the keto-imide groups (hydrogen bond). A preliminary account of the main findings forming the basis for this conclusion will be given in this note.

Both bovine hide powder and calfskin were used. The N-acetylation and the exhaustive acetylation (N- and O-acetylation) of the collagen were carried out according to Green *et al.*[2]. Their analytical procedures were also used. The N-acetylated collagen contained the ε-amino groups completely inactivated (0.39 mmole acetyl per g collagen). The exhaustively acetylated collagen had in addition 80 % of its hydroxy groups blocked, containing 1.78 mmole acetyl per g collagen. The O-acetylation was 1.39 mmole/g collagen. The aliphatic amino acids and tyrosine account for 0.60 mmole OH/g collagen. Assuming that these residues are preferentially acetylated, at least 0.79 mmole/g collagen of hypro should have been acetylated. Hence, out of the 1.07 mmole hypro present in the original collagen, three hydroxy groups out of four have at least reacted. The total N content of the acetylated collagen was 16.5 % and the acid binding capacity 0.68 mmole HCl per g protein, while the corresponding values for the intact collagen were 18.0 and 0.90.

The stability of collagen, measured by its shrinkage temperature, T_s, was not changed by N-acetylation. The N- and O-acetylated collagen commenced to shrink at 40—44° C, while the intact collagen showed T_s of 64—66° C. Other properties, such as the degree of swelling, were profoundly changed by the exhaustive acetylation also. These findings indicate that by acetylation of hydroxy groups *interchain* crosslinks are broken. It is also of interest to note that the T_s obtained for the O-acetylated collagen coincides with that of the skins of most cold-water fishes (cod[1]), suggesting that the hydroxy group resisting acetylation forms a stronger bond than the rest of the hydroxy groups, and that this particular group is mainly responsible for the stabilization of the teleost collagen.

Indications of the nature of the other partner in the interchain bond were obtained by investigating the linking of polyphenols such as condensed tannins (Mimosa) by collagen and O-acetylated bovine collagen. It has been proved conclusively that the main reaction in the irreversible fixation of these tannins by collagen involves hydrogen bonding of the polyphenols on the keto-imide group (the carbonyl oxygen), since collagen and hydrated modified polyamides with the —CO—NH— link as the predominant reactive group show the same trend of reaction towards the condensed type of vegetable tannins [3]. By the N-acetylation, the fixation of mimosa tannin is slightly decreased, due to the elimination of the small fraction of tannin fixed by the amino groups, by their acetylation. The exhaustively acetylated collagen possesses markedly *greater* binding capacity for the mimosa tannins than intact collagen, the increase being of the order of 50—75 %. Thus, the intact collagen fixed 60 % mimosa tannins, on the basis of collagen, while the O-acetylated collagen fixed 95—105 %. A number of other vegetable tannins showed all increased binding, percentages increase of 15—40 having been recorded according to the degree of affinity of the individual tannins for the —CO—NH-link. In view of these data, amplified by our present knowledge of tanning reactions, the rupture of links between the hydroxy groups and the keto-imide group is indicated to occur in the O-acetylation.

In gelatin, the main part of these interchain hydrogen bonds on the —CO—NH— links apparently have been severed since its binding capacity for mimosa tannins reaches values of an order of 140 % tannin on the protein, figures [5] comparable with those by the polyamide [4] and considerably higher than those found for the O-acetylated collagen. These data support the present thesis that by acetylation of the hydroxy group of collagen the hydrogen bond which it forms with the carbonyl oxygen of the —CO—NH-link is split, consequently increasing the number of potential sites for hydrogen bonding of the polyphenolic tannin on the collagen.

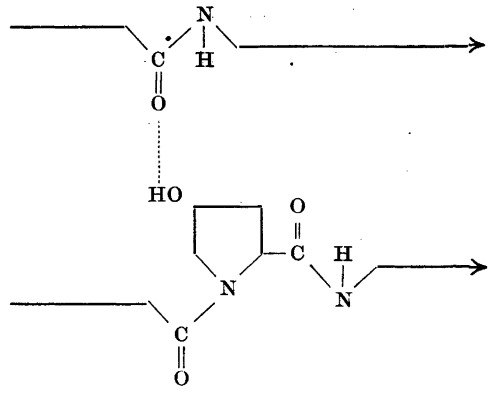
Additional support of this view is given by data forming the first experimental indication of the participation of the hydroxy group of collagen in tanning processes, more particularly for the binding of non-ionic chromium complexes. The fixation of tanning agents by the hydroxy groups of polyvinyl-alcohols has been demonstrated by Elöd and Schachowskoy [6].

The sulphito-chromium sulphates, obtained by adding 2—2.5 moles of Na_2SO_3 per each mole Cr_2O_3 to a solution of the 67 % acid chromium sulphate, described elsewhere[7], contain preponderantly non-ionic complexes and small amounts of negatively charged chromium complexes, as proved by electrophoresis and ion exchange. They are prone to aggregate. These chromium complexes are practically inert to the modified polyamide [7]. However, they have been found extremely useful as specific

agents for the evaluation of the coordination faculty of collagen [4], since their fixation by collagen is mainly governed by the number of free sites available on the protein for hydrogen bonding. By the heat-denaturation of collagen, for instance, the non-ionic chrome fixation is increased by 50—75 %, indicating the rupture of cross-links in collagen and the formation of additional sites for the binding of these complexes [4]. Since the polyamide does not bind the sulphite-complexes[7], and steric hindrance due to the molecular size is improbable from comparison with other agents of greater molecular weight which are fixed by the polyamide [3], it appears that some other groups than the keto-imide link must be instrumental for the fixation of the nonionic sulphito-sulphato-chromium complexes.

Collagen (hide powder) treated for 24 h with the sulphito complexes at a concentration of 20 g Cr_2O_3 per liter fixed 21.8 % Cr_2O_3 of its own weight. The O-acetylated collagen showed very much lower fixation, or 15.2 % Cr_2O_3. It should be noted that the cationic, and gradually even the anionic protein groups, are involved in the reaction. However, the main fixation has been indicated to be located on non-ionic protein groups. The hydroxy group is indicated to form the principal site of binding, in view of the marked decrease of the chrome fixation resulting from its acetylation and inactivation. This finding, with due consideration of numerous other facts which cannot be enumerated in this note, supports the main thesis of this paper that the hydroxy group of collagen, probably mainly that of hypro, is able to form a strong interchain link with the —CO—NH-group of the type.

The rupture of such a bond in the various pretreatments mentioned and by heat denaturation would satisfactorily explain both the increased fixation of vegetable tannins (by the freed —CO—NH—bond) and of the non-ionic complex chromium compounds (by the freed OH—bond) as well as the effects of the blocking of the hydroxy group, *i. e.*, the impaired hydroxy-coordination of the chromium complexes, but increased binding of the keto-imide-attached vegetable tannins and the large T_s decrease ($\triangle T_s$: —22° C) found. In view of the marked reactivity of native collagen for the agents mentioned, it is probable that only a few of the total number of the hydroxy and keto-imide groups are able to form the type of crosslink suggested.



That the hypro residue mainly supplies the hydroxy group is made probable by the following facts. The content of aliphatic hydroxyamino acids is somewhat higher in teleost collagen than in the bovine type [8]. However, the T_s values follow the trend of the hypro content, being lowered with decreasing content of this imino acid [1]. If the aliphatic hydroxy group should form the main interchain link, the T_s values should show the reverse trend. The evidence for the crosslinking being *intermolecular* has already been given.

Professor R. S. Bear, Department of Biology, M. I. T., Cambridge, Mass., has kindly informed the author that the type of bond suggested is compatible with the steric conditions in the present model of the collagen helix [9].

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