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Cross-linking of collagen and gelatin during acetylation

GREEN, ANG AND LAM[1] found that treatment of bovine hide powder with a mixture of acetic acid and acetic anhydride caused acetylation of the amino groups and, more slowly, the hydroxyl groups. A related method using ethyl acetate, acetic anhydride and formic acid has been reported[2]. The acetylation of keratin (human hair) with hot 16% (v/v) acetic anhydride in acetic acid was reported to result in a product that showed less supercontraction in aq. LiBr than did not-acetylated keratin[3]. The method of GREEN *et al.*[1] has been used to modify collagen for tanning studies[4–6]. CASSEL AND MCKENNA[7] used it to study the roles of the amino and hydroxyl groups in the swelling of collagen (bovine corium), and found that acetylated corium showed reduced swelling at pH 2–12. The reduced swelling at low pH was attributed to a reduced net positive charge due to blocking of amino groups, and at high pH to blocking of the hydroxyl groups which would otherwise ionize. The latter cannot be correct because aliphatic hydroxyl groups of proteins are not titratable at pH 12, and because the 22-h soaking at that pH undoubtedly hydrolyzed all the ester groups. Complete hydrolysis of *O*-acetyl groups of acetylated collagen was accomplished by GREEN *et al.*[1] and by BLACKBURN AND PHILLIPS[8] under similar conditions.

KENCHINGTON[9] reported that treatment of gelatin by the method of GREEN *et al.*[1] resulted in an insoluble product, and proposed that cross-linking occurs through formation of ester bonds. We had simultaneously made the same observation, and obtained evidence for amide cross-links. KENCHINGTON, in a private communication, has informed us that he also has found amide cross-linking in acetylated gelatin. We present our findings separately, as our evidence relating to the question of ester cross-links involves data on ester-like links in the original collagen at variance with data of others.

After acetylation, commercial flake gelatin swells but does not dissolve readily in hot water. Heating for several days at 50° gradually brings the gelatin into solution. The original gelatin dissolves in water at 50° in a few minutes. The acetylated gelatin has the properties typical of a polymer lightly cross-linked. The reduced swelling of acetylated collagen found by CASSEL AND MCKENNA[7] can be explained by cross-linking. The reduced shrinkage of acetylated keratin may also be explained by cross-linking, although we have not sought proof in this case. A reasonable mechanism for cross-linking consists of 2 steps: (1) reaction of acetic anhydride with protein carboxyl groups to form mixed anhydrides: (2) reaction of the mixed anhydrides with amino or hydroxyl groups to form intermolecular (and perhaps intramolecular) amide or ester links. Amide cross-links were indicated by the observation that the reagent of GREEN *et al.* does not insolubilize gelatin in which the amino groups previously had been acetylated in water at pH 9. In the same way we had found earlier that thermal insolubilization of gelatin is due to the formation of amide cross-links[10].

The possibility of ester cross-links accompanying the amide links was investigated by use of hydroxylamine. Flake gelatin, bovine corium or bovine achilles tendon was acetylated with acetic acid – acetic anhydride (40:60, v/v) for 5 days at room temperature, then soaked in acetic acid for 2 h, in 4 changes of acetone and 6 daily changes of cold water. The pieces of gelatin or collagen were then soaked for 24 h in 4 M $NaClO_2$ and then in 3 M NH_2OH at pH 8.6 for 16 h at room temperature. This treatment resulted in dissolution of all the gelatin and about 50% of the corium

or tendon. Similar treatment of non-acetylated gelatin, corium or tendon dissolved all of the gelatin and 80–90 % of the corium. The solutions and insoluble materials were dialyzed at 4° against a large volume of 1 % acetic acid for 1 or 2 days and then against water, with mixed ion-exchange resins in the outer compartment, to remove hydroxylamine. The same results were obtained whether a mixture of H^+ and OH^- or of Na^+ and Cl^- resins were used, or if acetic acid was not used before the resin. The solutions were freeze-dried and the insoluble material was air-dried. The soluble portions were analyzed for hydroxamic acid by the method of BERGMANN AND SEGAL[11]; the insoluble portions were not analyzed. There was no increase in hydroxamic acid content in the acetylated materials as compared with the controls (Table I). Apparently there were no ester cross-links in the soluble parts; the insoluble parts cannot contain ester cross-links after the hydroxylamine treatment, but may have had such links before. It is probable that the large portion of acetylated collagen that is insoluble in hydroxylamine contains amide cross-links.

TABLE I

HYDROXAMATE CONTENT OF ACETYLATED TENDON, CORIUM AND GELATIN

Sample	*Hydroxamate (moles/10⁵ g)*
Tendon, acetic cross-linked, 2 runs	0.4, 0.5
Tendon, control	0.5
Corium, acetic cross-linked, 3 samples, 4 runs	0.5, 0.4, 0.7, 0.5
Corium, control	0.3
Gelatin, acetic cross-linked, 2 runs	0.5, 0.7
Gelatin, control	0.4

As the hydroxamate values for the soluble controls are as little as one-tenth of those reported by others for different collagens[12,13] we cannot exclude the possibility of some ester cross-links until the disagreements have been explained. The present hydroxamate value for the controls are somewhat smaller than we had reported previously[14]. The molecular weight of the solubilized control corium collagen (completely gelatinized, of course) was found to be about $34 \cdot 10^3$ by sedimentation-diffusion and $30 \cdot 10^3$ by osmotic pressure. The molecular weights were determined using acetate buffer containing 0.5 M $NaClO_4$, at 30°, to prevent aggregation. These values correspond to about ¼ of an individual collagen molecule, neglecting the possibility of branching. If the breaks indicated by the molecular weight represent the presence of ester-like links in the original corium, their non-appearance as hydroxamates may be due to loss by dialysis or to decomposition. The breaks may also result from the action of the acetylating mixture on the protein. The similarity between the osmotic and sedimentation molecular weights suggests that the non-dialyzable material is fairly homogeneous.

That only about 50 % of the acetylated corium, compared to 80–90 % of the control, dissolved in hydroxylamine is indicative of non-uniformity in this material. At what levels of organization these differences arise is not yet known. It was noted that squares of corium after cross-linking, hydroxylamine treatment, washing, and drying in air were of nearly the same area as before treatment, but were thinner, more

brittle, translucent, and had grainy surfaces. Cross-linking of corium or tendon with a water-soluble carbodiimide, followed by treatment with perchlorate resulted in products of which only 30 % could be dissolved in hydroxylamine. Amino acid analyses showed little difference between the soluble and insoluble portions of corium or tendon cross-linked with either anhydride or carbodiimide.

The function of the acetic acid in the acetylating mixture was proposed by GREEN *et al.*[1] to be of a catalytic nature. Our observations suggest that its function is to swell the collagen or gelatin to permit penetration of the anhydride. Flake gelatin in acetic anhydride did not swell and was not acetylated, while freeze-dried gelatin, which is porous, was 50 % acetylated, under the same conditions.

We are grateful to Professors O'FLAHERTY and DEASY of the University of Cincinnati for the gift of corium, and to Mr. F. WISSLER and Mr. J. FRENCH and Mrs. C. OPALINSKI for the molecular-weight data.

Part of this work was carried out during 1953–1956 under Contract No. Da-007-MD-298 between the California Institute of Technology and the Department of the Army.

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Received August 16th, 1962