The Degradation of Collagen. A Method for the Characterization of Native Collagen

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246–248°. Recrystallization from a mixture of 10 cc. of dimethylformamide and 4 cc. of water gave 0.65 g. (84% recovery) of crystalline product also melting at 246–248°, $[\alpha]^{22}D$ −43.6 ± 0.3° (*c* 1.4, glacial acetic acid).

Anal. Calcd. for $C_{70}H_{98}N_{10}O_{13}$: C, 65.9; H, 6.80; N 11.0. Found: C, 65.5; H, 6.55; N, 11.4.

L-Leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline Trihydrate (VI).—To a solution of 165 mg. (0.0002 mole) of III in 15 cc. of glacial acetic acid was added approximately 100 mg. of palladium black catalyst[11] and the mixture was shaken under 50 lb./in.² of hydrogen pressure at room temperature for 18 hours. The catalyst was then filtered off and washed with 5–10 cc. of distilled water. The clear, colorless filtrate was freeze-dried under high vacuum to give the product as a colorless, fluffy powder; wt. 119 mg. (91%), m.p. 228–233° dec. This was redissolved in 20 cc. of distilled water and the solution filtered to remove a small amount of insoluble material. The filtrate was then again freeze-dried under high vacuum to give 87 mg. (66%) of product as a colorless powder; m.p. 228–230° dec., $[\alpha]^{23}D$ −56.3 ± 0.5° (*c* 0.7, 3 *N* HCl).

Anal. Calcd. for $C_{30}H_{46}N_6O_7 \cdot 3H_2O$: C, 54.9; H, 7.98; N, 12.8. Found: C, 54.8; H, 7.89; N, 12.7.

Paper chromatography using a *n*-butyl alcohol:water:acetic acid (5:3:2) solvent system gave a single, fast running spot (R_f 0.89); however, basic solvent systems, such as 80% aqueous lutidine or *n*-butyl alcohol saturated with 3% ammonium hydroxide, were unsatisfactory in that two fast running spots invariably were obtained (lutidine, R_f 0.58 and 0.83; butanol, R_f 0.61 and 0.92), even after continued hydrogenation of the product to remove contaminating traces of the benzyl ester.

L-Tryptophyl-L-lysyl-L-leucyl-L-alanyl-L-valyl-L-phenylalanylglycyl-L-proline Monoacetate Hexahydrate (VII).—A 50-mg. (0.00004 mole) sample of V was dissolved in 10 cc. of glacial acetic acid and 50 mg. of 10% palladium-on-charcoal catalyst[12] added. The mixture was then hydrogenated and the product was isolated as in the above case. The peptide acetate was obtained as a colorless powder; wt. 33 mg. (77%), m.p. 240–245° dec. (dependent upon rate of heating), $[\alpha]^{24}D$ +15.3 ± 2.5° (*c* 0.8, water).

Anal. Calcd. for $C_{47}H_{68}N_{10}O_9 \cdot CH_3COOH \cdot 6H_2O$: C, 54.2; H, 7.80; N, 12.9. Found: C, 54.2; H, 7.99; N, 12.6.

Chromatography on paper gave a single spot using either *n*-butyl alcohol:water:acetic acid (5:3:2) or 80% lutidine as the solvent system. The spot obtained in the basic system was slower running (R_f 0.69) and more diffuse than that obtained in the acidic system (R_f 0.94). In the case of the *n*-butyl alcohol saturated with 3% ammonium hydroxide system, the spot obtained was too diffuse for characterization.

(11) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 128 (1921).

(12) Baker and Co., Inc., Newark, N. J.

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The Degradation of Collagen. A Method for the Characterization of Native Collagen

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The dye–protein precipitation method for the estimation of polar functional groups on the protein has been applied to an investigation of a highly purified bovine hide collagen and several mildly degraded collagens. There is no difference in the maximum number of basic dye binding sites on each sample per unit weight, but the concentration dependence of the binding is clearly related to the extent of degradation. Less than half of the theoretically available anionic groups are available as dye binding sites in the undegraded material. These become available in stages as the extent of degradation is increased. The thermodynamic functions $\Delta F°$ and $\Delta S°$ have been evaluated for the combination of the undegraded protein with Orange-G and Safranin-O.

The detailed nature of the path by which the connective-tissue proteins, the collagens, are converted into soluble gelatin has been an open question for many years. We have undertaken a re-examination of this problem with particular emphasis on bovine hide collagen. One logical starting point for such an investigation is the characterization of a starting material which represents collagen in its most nearly native state physically and chemically. The present discussion is a description of one method of analysis which gives information on both aspects of such a characterization and which has already led to some interesting information on the initial steps of the degradation.

Cassel, McKenna and Glime[1] have shown that there are no significant differences in the amino acid analyses of collagen, gelatin or hide powder. In particular, no changes could be found in the composition of the polar amino acid residues other than the loss of some amide nitrogen in the gelatin. Titration curves of insoluble collagen have been used to follow changes in the number of functional groups per unit weight, but drastic treatments are required to demonstrate these changes.[2,3]

Furthermore, as Ames[3] has pointed out, one can barely differentiate between the total nitrogen content of untreated collagen and exhaustively acid treated gelatin and all changes detected can be ascribed to the loss of amide nitrogen. Thus, the methods which have been most commonly used to study the collagen–gelatin transformation lack the sensitivity which is apparently required.

Dye–protein precipitation reactions have been used[4] to determine the number of protein functional groups. Such reactions are sufficiently sensitive to detailed structural features of the globular proteins to allow one to detect minor changes which occur on denaturation.[5,6] These methods have been applied, in modified form, to the present problem.

Experimental Part

A. **Preparation of the Collagen.**—Hide collagen was prepared by a combination of the methods of Bowes and Kenton[2] and of Loofbourow, Gould and Sizer.[7] The hide of a freshly slaughtered steer was immediately chilled in ice-water. The hide was then cut into small strips. These were washed thoroughly with cold water, then extracted with cold 10% sodium chloride. The supernatant was dis-

(1) J. Cassel, E. McKenna and A. Glime, *J. Am. Leather Chem. Assoc.*, **48**, 277 (1953).

(2) J. H. Bowes and R. H. Kenton, *Biochem. J.*, **43**, 358, 365 (1948); **44**, 142 (1949).

(3) W. M. Ames, *J. Sci. Food Agric.*, **3**, 454, 579 (1952).

(4) H. Fraenkel-Conrat and M. Cooper, *J. Biol. Chem.*, **511**, 239 (1944).

(5) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

(6) J. R. Calvin, *Can. J. Chem.*, **30**, 320, 973 (1952).

(7) J. R. Loofbourow, B. S. Gould and I. W. Sizer, *Arch. Biochem.*, **22**, 406 (1949).

carded, the loose dirt was again rinsed out with cold water and fresh 10% sodium chloride was added. After soaking overnight in the salt solution the hide pieces were washed with water and extracted with several portions of ether. The ether was beaten through the hide pieces by vigorous pounding. The hides were again rinsed and were then swollen in cold 2% acetic acid (*p*H 3–4 during the entire process). While the hides were swollen stiff the hair layer was split off below the follicle level. The remaining bits of flesh were also split off the other side of the hide. The pieces were de-plumped with Na_2HPO_4, *p*H 8.5, reswollen with fresh 2% acetic acid, de-plumped with Na_2HPO_4 and washed with dilute ammonium hydroxide solution, *p*H 7.5–8.0. The pieces were finally dialyzed for several days against constantly renewed, cold distilled water. Citric acid–sodium phosphate buffers of *p*H 3.5 could be substituted for the acetic acid extractions. The procollagen extracted in these steps was more readily flocculated from the citrate buffer than from the acetic acid solutions. The collagen pieces were frozen and dried *in vacuo* over P_2O_5 to avoid contraction during the drying process. The resulting solid was light and fibrous and could be shredded easily.

Acid and base titration curves of the dried collagen were similar to those obtained by Bowes and Kenton.[2] Ultraviolet spectra of total acid hydrolysates of the collagen were identical with those obtained by Loofbourow, Gould and Sizer[7] and indicated the absence of aromatic amino acid residues. The total nitrogen content was found to be 18.42%, as compared with the values of 18.49% and 18.6% reported by Ames[3] and Bowes and Kenton[2] for similar preparations.

B. **The Dye-Precipitation Experiments.**—The adsorption experiments were run in essentially the same manner as those of Fraenkel-Conrat and Cooper.[4] Orange-G was used in the acid range with a *p*H 2.2 dibasic phosphate–citrate buffer, $\Gamma/2 = 0.1$, and Safranin-O was used in the basic range with a *p*H 11.5 dibasic phosphate–sodium hydroxide buffer, $\Gamma/2 = 0.1$. The dye–protein equilibration took place in pre-flamed, sealed tubes during a 48-hour period. Diffusion of the dye into the small squares of solid collagen was complete within 36 hours as demonstrated by analysis or visual inspection of the center of the piece. None of the collagen was solubilized under the conditions of these experiments. Temperatures were held at 0.0 ± 0.5° or 25 ± 0.5°.

The amount of dye adsorbed was calculated from the difference in optical density of the supernatant of each blank and sample pair at the absorption maxima of the particular dye used. The Orange-G, equivalent weight 226.2, was 97% active while the Safranin-O, equivalent weight 350.5, was 85% active.

C. **Isoionic Degradation.**—Suspensions, 10% collagen by weight, were subjected to mild degradation by heating to 60° for 1, 2, 3 and 16 hours in distilled water. The *p*H was constant at 6.5 throughout the entire process. At the end of each heating period the suspensions were filtered, the residue solids washed with cold distilled water and lyophilized. The resulting collagen pieces, even after a 16-hour heating period, had the appearance of the original sections.

The filtrate from each extraction was saved and dried by lyophilization over P_2O_5. The fraction of the collagen solubilized in this process was determined for each heating period. The results are given in Fig. 1. It was immediately apparent that the extracted materials were not typical gelatins. A separate report will be made on the interesting properties of these "soluble collagens."

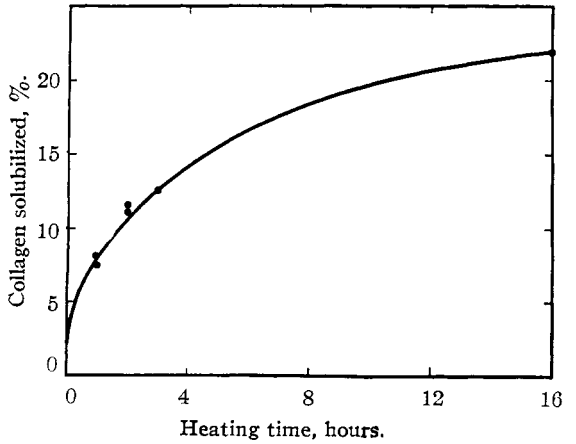
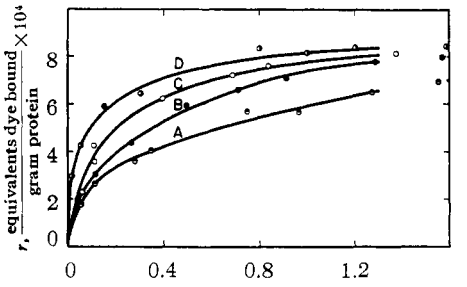


Fig. 1.—Solubilization of isoionic collagen, *p*H 6.5, at 60° as a function of the heating period.

Results and Discussion

The Orange-G binding data are summarized in Fig. 2. At *p*H 2.2 and sufficiently high dye concentrations, Orange-G quantitatively binds with every free cationic group on the protein.[4] Satura-



A, free dye concentration, equivalents/liter × 10^3.

Fig. 2.—Binding of Orange-G by collagen, *p*H 2.2, 25°: A, undegraded collagen; B, collagen heated at *p*H 6.5, 60°: for 1 hour; C, collagen heated 2 hours; D, collagen heated 16 hours.

tion measurements show that there is no significant difference in the maximum number of dye ions bound per gram of solid collagen irrespective of treatment. At low dye concentrations, the extent of binding is clearly dependent upon the dye concentration, the affinity for the dye increasing regularly with the extent of disintegration of the collagen structure. These data are most clearly demonstrated in a plot of r/A *vs.* r as in Fig. 3; r is the equivalents of dye bound per gram of collagen and A the concentration of the dye in equivalents per liter. The curvature of each plot is an indication of the interaction between bound anions or suggests that two types of cationic groups are involved in the binding process, one readily available to the dye, the other available only with difficulty. Fig-

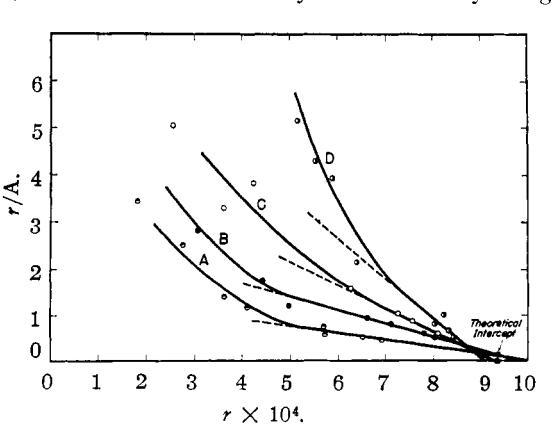


Fig. 3.—Alternative plot of Orange-G binding data for extrapolation to n and kn: A, undegraded collagen; B, collagen heated 1 hour; C, collagen heated 2 hours; D, collagen heated 16 hours.

ure 3 emphasizes even more clearly than the direct representation of the data that, within experimental limits, the maximum number of equivalents of dye bound per gram is not altered by the mild degradation. The average r intercept is equivalent to 93×10^{-5} equivalent bound per gram of collagen, in excellent agreement with the analytical data[2] for the sum of the arginine, histidine and lysine residues, 93.5×10^{-5} equivalent/gram.

The binding of cationic Safranin-O by collagen is less clearcut (Figs. 4 and 5). According to titration curves and amino acid analyses[2] there are 126×10^{-5} equivalent/gram of free carboxyl groups of which about 49×10^{-5} equivalent/gram are tied up as amide residues leaving 79×10^{-5} equivalent/gram available for binding cationic dyes. The arrangement of the free carboxyl groups in the untreated material seems to be such that only about half of these are available as binding sites. The heating process renders the remaining groups available and the Safranin-O binding increases to nearly the quantitative value. Prolonged extraction (16 hours) brings about a decided increase in the total number of groups available, probably due to the loss of amide nitrogen.

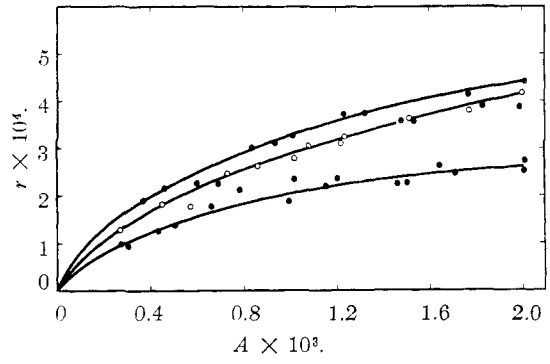


Fig. 4.—Binding of Safranin-O by collagen, pH 11.5, 25°: ●, undegraded collagen; O, collagen heated at pH 6.5, 60° for 1 hour; ⊙, collagen heated at 2 hours.

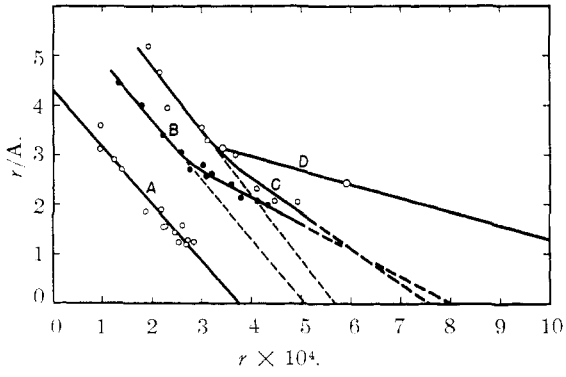


Fig. 5.—Alternative plot of Safranin-O binding data for extrapolation to n and kn: A, undegraded collagen; B, collagen heated 1 hour; C, collagen heated 2 hours; D, collagen heated 16 hours.

The degradation process appears to be initiated in a reorientation of the collagen fiber bundles. No hydrolytic peptide bond disruption was detected in the insoluble residue of the isoionic degradation.

A change in temperature of 25° did not significantly alter the extent of binding of Orange-G so that $\Delta H^0 = 0$ and the negative free energy of binding may be attributed to a positive entropy term which apparently is related to the degree of disorganization of the collagen. The positive entropy of anion binding by serum albumin has been attributed to the release of water bound to the protein.[8] This is almost visibly evident in the case of collagen–Orange-G binding at pH 2.2. In buffer alone, the collagen imbibes water and becomes swollen stiff. When the anionic dye is added, making a negligible change in the ionic strength of the solution, the dyed fibers contract to the compact form which they exhibit at neutral pH. This contraction is also apparent during the usual vegetable tanning process for leather.

An extrapolation of the Orange-G binding data in the low dye concentration range, as ln r/A *vs.* and A, yields a limiting value of $[r/A]_{A=0,\ r=0} = 5.74$ for the untreated collagen. According to Klotz and Urquhart[9] $[r/A]_{A=0} = kn$ where k is the intrinsic binding constant for the first dye ion bound and n the maximum number of sites for binding. If we assume $n = 93.5 \times 10^{-5}$ equivalent/gram of protein then $\Delta F^0_{298} = -5160$ cal./mole and $\Delta S^0_{298} = +17.35$ e.u./equivalent of dye bound, with $\Delta H^0 = 0$. These values are in good agreement with similar values tabulated for the binding of other anions by serum albumin.[8] All of the heat treatments make ΔF^0 a progressively larger negative number. Similarly, for Safranin-O binding by undegraded collagen we find $\Delta F^0_{298} = -5590$ cal./mole and $\Delta S^0_{298} = +18.75$ e.u., choosing $n = 3.75 \times 10^{-4}$ equivalent/gram from the extrapolated data of curve A, Fig. 5.

It should now be possible to compare different preparations of "undegraded" collagen by the dye binding method described to point out the preparation which yields the lowest intrinsic binding free energy as the least degraded. This also indicates a fresh approach for the comparison of collagens from different sources.

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