The rate-determining steps are the formation of the carbamic acid. In the bimolecular mechanism this is from the reaction of a dimer of cyanic acid with an OH^-. The existence of a dimer of cyanic acid has been proposed previously to explain reactions of the acid.[8] Werner and Gray, on the other hand, do not feel that there is evidence for the existence of the dimer.[9] It is felt that the rate equation obtained strongly suggests the existence of the dimer, even though it may be in very low concentration. A dimer of phenyl isocyanate is known to exist.[10] Whether the form of the dimer is NH_2CONCO or NH_2COOCN cannot be distinguished from this work. The dimer is attacked by an OH^- to form an intermediate of the form $\left[\begin{array}{c} O \\ \| \\ NH_2C-OCN \\ | \\ OH \end{array}\right]$ which then dissociates to carbamic acid and OCN^- in the rate-determining step.

Whether cyanic acid is HOCN or HNCO or a mixture of the two has not been completely determined. Infrared spectra of the vapor and liquid appear to show that the acid is HNCO with less than 0.2% HOCN present.[11] In aqueous solution the tautomerism would be more probable than in the vapor or liquid. Werner and Fearon[12] suggest that the mixture is an equilibrium between the two forms and use the formation of the polymers of the acid as proof. The formation of HNCO is favored as the temperature increases.

Evidence supporting the tautomerism is obtained from the temperature dependence of the rate constants (Fig. 6). If it is assumed that only one form of the tautomer can react by a given mechanism, the total concentration of acid used to determine k should be replaced by the concentration of the reacting form. If K_t is the constant for the tautomerism, A_0 = HOCN + HNCO and (HNCO) would be $[K_t/(1 + K_t)]A_0$. This ratio would be included in the rate constant and a linear temperature dependence would not exist. It is believed that the HNCO form is the one that reacts because of steric considerations.

The mechanism proposed is a molecular one and there should be no primary salt effect. Because of the equilibrium which exists between the cyanic acid molecule and the ions, a secondary salt effect might be expected.[13] Since the concentration of OCN^- and HOCN is of the order of 100 times the concentration of H^+ the increased dissociation of HOCN because of the added salt will be very small and will not appreciably change the concentration of un-ionized acid. Under these conditions the salt effect will be very small and less than the experimental error.

Acknowledgment.—The author is indebted to Drs. J. K. Dixon and G. L. Simard for helpful discussions during the early part of this work, and to Dr. Henry Kuivila who first suggested the dimer as an intermediate in the reaction.

(8) (a) T. L. Davis and K. C. Blanchard, THIS JOURNAL, **51**, 1806 (1929); (b) H. W. Blohm and E. I. Becker, *Chem. Revs.*, **51**, 471 (1952); (c) M. Linhard, *Z. anorg. allgem. Chem.*, **236**, 200 (1938).

(9) A. E. A. Werner and J. Gray, *Sci. Proc. Roy. Dublin Soc.*, **24**, 209 (1947).

(10) C. J. Brown, *J. Chem. Soc.*, 2931 (1955).

(11) E. Eyster, R. Gillette and L. Brockway, THIS JOURNAL, **62**, 2326 (1940).

(12) E. A. Werner and W. R. Fearon, *J. Chem. Soc.*, **117**, 1356 (1920).

(13) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, p. 260.

ANNVILLE, PA.

[CONTRIBUTION FROM THE RESEARCH DIVISION, ARMOUR & CO.]

A Non-random Disaggregation of Intact Skin Collagen

BY ARTHUR VEIS AND JEROME COHEN

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Studies of the disaggregation of intact bovine hide collagen show the presence of lateral polypeptide chain cross-linkages of different reactivity and, probably, different distribution along the fibrillar long axis. Under the acid-extraction conditions there appear to be at least two classes of such cross-linkages and the order in which these are broken determines the molecular weight distribution of the solubilized portion. If the extractions are carried out under *p*H and temperature conditions where peptide bond hydrolysis is not significant then discrete soluble fibrillar fragments are obtained which may retain the gross features of the peptide chain configuration present in the native tissue. The onset of peptide bond hydrolysis is readily recognizable.

Various studies of the conversion of collagen to gelatin have had two major objectives: (1) to throw some light on the structure of intact collagen; (2) to explain the variations in the stability of the collagens from different sources.[1-4] Ames' work[1] led him to propose two possible models for collagen, a multi-chain structure in which the peptide chains are held together by non-peptide covalent cross-linkages of some sort and a single chain model in which the peptide chains are too large to be water soluble, but become so as certain particularly weak peptide linkages are broken. In spite of additional data from other quite different points of view[5-7] one could not chose between these alternatives.

Our studies of the mild thermal solubilization of collagen in the acid *p*H range[2] showed, however,

(1) W. M. Ames, *J. Soc. Chem. Ind.*, **66**, 279 (1947); *J. Sci. Food and Agric.*, **3**, 454 (1952).

(2) A. Veis and J. Cohen, THIS JOURNAL, **76**, 2476 (1954); **77**, 2364 (1955).

(3) A. Veis, D. N. Eggenberger and J. Cohen, *ibid.*, **77**, 2368 (1955).

(4) K. H. Gustavson, *J. Amer. Leather Chem. Assoc.*, **50**, 239 (1955).

(5) P. M. Cowan, S. McGavin and A. C. T. North, *Nature*, **176**, 1062 (1955).

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

(7) R. D. Harkness, A. M. Marko, H. M. Muir and A. Neuberger, "Nature and Structure of Collagen," Academic Press, New York, N. Y., 1953, pp. 208–212.

that at any given *p*H in the 5.75 to 2.5 range, prolonged extraction at 60° resulted in the release of only a definite amount of the collagen, different at each *p*H. The experiments reported here were designed to obtain quantitative data on the possible selectivity of the extraction process, and to characterize the range of molecular species obtained. With such data, some clarification of the questions raised above is achieved.[8]

Experimental

Bovine hide collagen was purified from fresh steer hides as described previously.[2] The collagen was shown to be in its native state by electron microscopic examination and dye precipitation studies.

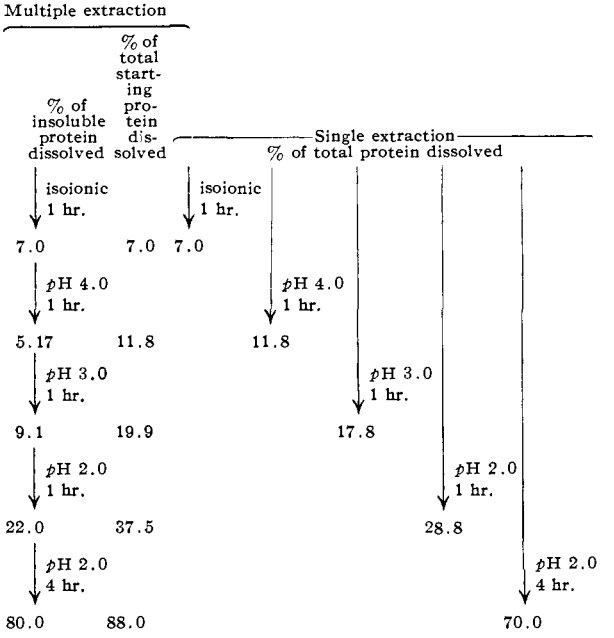
Extraction.—Dried collagen, in the form of porous, fibrous strips, was cut in small, uniform cubes 5 to 6 mm. on an edge. Shredding and grinding were avoided since the mechanical stresses of such processes produce changes in the rate of hydrolysis and the ease of attack with enzymes.[10] The cubes obtained from different parts of the hide but primarily from the thickest section of the back were shaken together until randomly mixed. In the first series of experiments 100 g. portions of the collagen cubes were mixed with one liter of buffer solution in a two-liter round-bottom flask. The resulting slurry was deaerated by putting the flask under vacuum. The vacuum was released suddenly, the pressure forcing liquid into the interfibrillar spaces. This process was repeated until all of the cubes were thrown to the bottom of the container when the vacuum was broken. Each cube of collagen was thus quickly and thoroughly wetted with the buffer solution. After the cubes were equilibrated with their surroundings, extractions were made at 60° for 1 hour at the appropriate *p*H (isoionic, 4.0, 3.0 and 2.0). An extraction was also made for 4 hours at *p*H 2.0. Except in the first instance, where water alone was used, the ionic strength was kept at 0.1. The buffer compositions are given in Table I along with the other extraction data. The resulting slurry was filtered hot through a coarse sintered glass filter. The filtrate was transferred to a sausage casing sac and dialyzed against distilled water until free of diffusible salt, then lyophilized and weighed. The insoluble portion was also dried by lyophilization and weighed. Recovery averaged only about 95% on each extraction due to small fibers trapped within the filter. Nitrogen analyses of the dialysates, except those from the glycine buffers, showed that little diffusible N was lost. The results of the single extractions were in excellent agreement with similar studies reported previously.[2] These extractions were repeated merely to obtain larger quantities of the soluble material for fractionation.

A second series of extractions was carried out on a single portion of collagen. After extraction at 60° at the isoionic *p*H, the insoluble residue was dried, then equilibrated with *p*H 4.0 buffer as before. The *p*H 4.0, 60°, one-hour extraction was made and the residue recovered and successively re-extracted for one hour at *p*H 3.0, and 2.0 and finally at *p*H 2.0 for 4 hours, with the results shown in Table I.

TABLE I

SOLUBILIZATION OF PURIFIED BOVINE HIDE COLLAGEN AT 60°

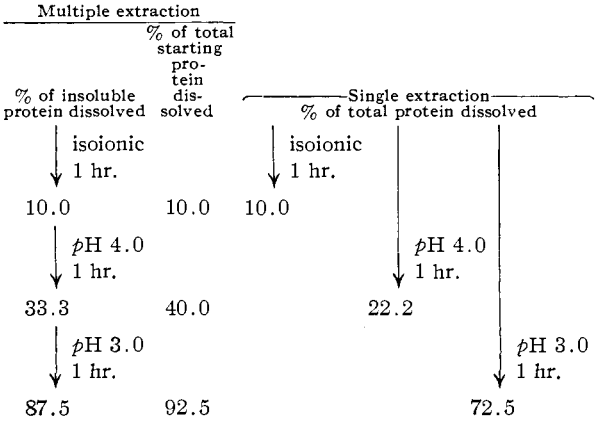
Buffers: ionic strength = 0.1. Composition as indicated by Miller and Golder[11] with adjustment for correct reading at 60°: *p*H 4.0—NaAc, HAc, NaCl; *p*H 2.0 and 3.0—glycine, HCl, NaCl.



The experiment was repeated with the extraction temperature at 80°. These results are shown in Table II.

TABLE II

SOLUBILIZATION OF PURIFIED BOVINE HIDE COLAGEN AT 80°



Molecular Weight Distribution by Fractionation.—For fractionation 200 mg. of the soluble protein was dissolved in 10 ml. of 0.8 *M* NaCl solution at 40°. When the protein was dissolved, anhydrous ethanol (formula 3A) was added from a 40° jacketed buret. The coacervates were collected at the successive alcohol–water ratios indicated in Fig. 1. The precipitates were dissolved in water and reprecipitated from a 2:1 acetone–water mixture (in which the salt is soluble). After several washings the salt-free precipitates were dried and weighed. The results of the fractions are shown in Figs. 1 and 2 for the 60 and 80° extracts, respectively. The fractionation process did not appear to make the protein less soluble. The weight-fraction distribution was not altered when the fractions from one fractionation were recombined and refractionated. A more critical

(8) In the subsequent discussion it will be implied that some of the structural characteristics of mature, intact bovine hide collagen may be retained in at least part of the thermally solubilized protein. As this implication may seem contrary to the experience of those who have studied the low temperature, acid soluble "acid-extract collagens," a few remarks to bring these studies into proper perspective appear to be in order.

Acid-extract collagens, and native tissue collagens, are presumed to be characterized by a very specific set of configurations of polypeptide chains and interchain alignments.[9] This orderliness is retained by the acid extract collagen macromolecules which exist in dilute aqueous solutions at low temperature. Upon heating such solutions above 33°, the ordered polypeptide coils melt out and the peptide chains irreversibly separate, yielding a form of gelatin. The more intractable low temperature acid-insoluble collagens, chemically and structurally quite similar to the acid-extract collagen, must owe their stability to some additional form of organization (cross linkages, twisted supercoils, etc.). It is this feature, absent in the acid-extract collagens, to which our attention has been directed.

(9) H. Boedtker and P. Doty, Abstracts, 129th Meeting, American Chemical Society, April, 1956.

(10) C. Deasy, *J. Amer. Leather Chem. Assoc.*, **50**, 453 (1955).

(11) G. L. Miller and R. H. Golder, *Arch. Biochem.*, **29**, 421 (1950).

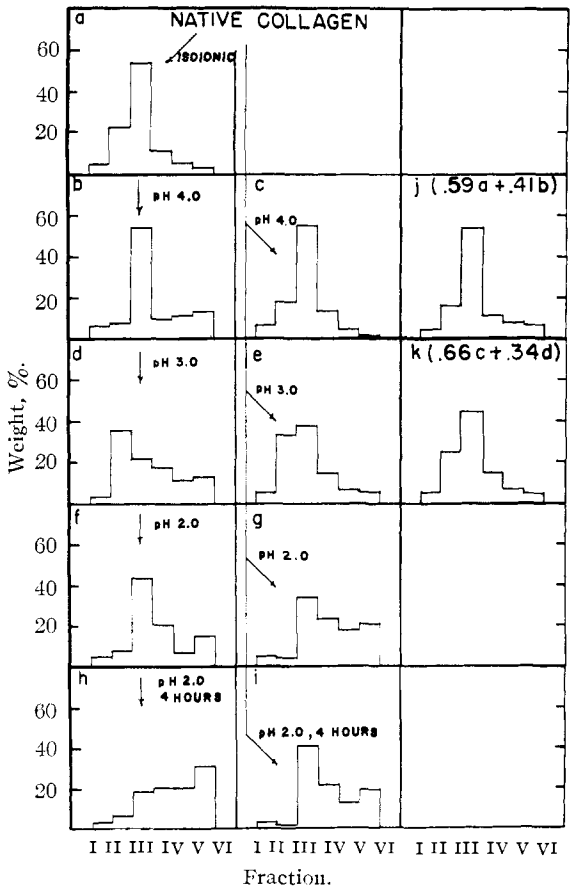


Fig. 1.—Fractionation of 60° extracts as coacervates, at 40°, from the following alcohol–water ratios: I, 2/1; II, 2.5/1; III, 3.0/1; IV, 3.5/1; V, 4.0/1; VI, 20/1.

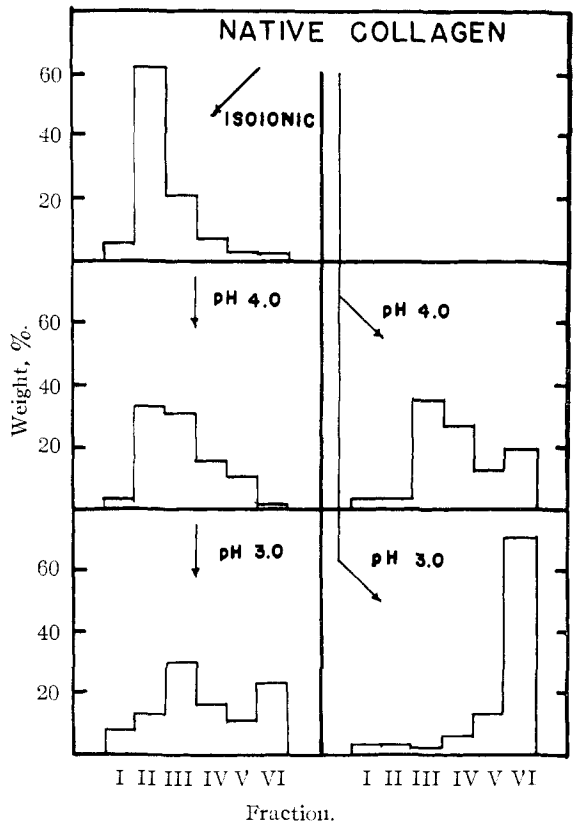


Fig. 2.—Fractionation of 80° extracts as coacervates, at 40°. Alcohol–water ratios as in Fig. 1.

requirement has been pointed out by Sayre.[12] He showed that for a heterogeneous polymer the distribution obtained in a given fraction depended upon the distribution and relative amounts of the species present in the original polymer mixture. Accordingly, a more representative fractionation–distribution pattern is obtained by taking relatively large cuts and refractionating these. The 1 hour, 60° isoinic extract was fractionated as illustrated in Fig. 1a, then each fraction was put through the same fractionation scheme and the new weight–fraction distribution curve calculated. Each fraction so obtained came within 5% of the amount indicated in Fig. 1a and the weight-average molecular weight was within 10%, the experimental error, of that of the material obtained in the single fractionation series. Because of the broad range covered by each fraction (as described below) no other refractionations were made. A ±10% error in distribution is sufficiently precise for the ensuing discussion.

Molecular Characterization.—Angular light scattering measurements were made in a direct reading Ludox calibrated photometer[3] in a 10-ml. capacity semi-cylindrical cell. The cell was supported in a jacket held at 40°. Readings were taken at convenient intervals in the range θ = 30–135°. The soluble protein was dissolved in 1.0 M KCl and centrifuged at 40° and 26,000 × g for 5 hours. The refractive index increment in 1.0 M KCl was found to be $(dn/dc)^{40°}_{\lambda=4360}$ = 0.1758 upon direct measurement in a Rayleigh Interferometer.

Viscosity measurements were made in Cannon–Fenske U-tube capillary viscometers at 40.00° ± 0.02°. The viscometers had flow times of about 200 sec. for water at 40° and shear gradients of about 1000 sec.$^{-1}$.

The average molecular parameters, computed from these data, are given in Table III for several fractions of the soluble protein. These data must be interpreted with caution because of the heterogeneity indicated by the broad range in M_w within a given fraction (Fig. 3). In general M_w has the following ranges: fr. II, 2.0 × 10^6; fr. III, 2.0 × 10^6 – 2.5 × 10^5; fr. IV, 2.5 × 10^5 – 5.0 × 10^4; and fr. VI, <5 × 10^4. The light scattering data were extrapolated in Zimm plots to $[Kc/R(\theta)]_{C=0}$ and plotted *vs.* $\sin^2(\theta/2)$. The fraction II plots (*e.g.*, Fig. 3a) were all curved and were treated as stiffly extended coils according to the method of Peterlin.[13] The stiffness parameter, x (ranging from 0 for a rod to ∞ for a random coil), was found to be between 40 and 50 for each fraction II. The values of root-mean-square end-to-end extension, $(\overline{R_z^2})^{1/2}$, and of extended chain length, $(\overline{L_z^2})^{1/2}$, show the fraction II material to be dense asymmetric structures.

The $[Kc/R(\theta)]_{C=0}$ *vs.* $\sin^2(\theta/2)$ plots for fraction III gave straight lines (Fig. 3b), except for a minor drop at 30–45°, undoubtedly due to polydispersity. The slopes of the lines are given in Table III in terms of the z-average radii of gyration. These values are considerably higher than those reported by Gouinlock, Flory and Scheraga[14] for alkali precursor-gelatins of nearly equivalent molecular weights, indicating the presence of more asymmetric molecules. The viscosity data indicate that all of fraction III is not of equivalent structure. A plot of ln $[\eta]$ *vs.* ln M_w (Fig. 4) shows that the data fall on either of two straight lines. The protein extracted under the most drastic conditions, *i.e.*, *p*H 2.0, 4 hr., has a value for α, defined by the empirical relationship $[\eta] = KM^\alpha$, of 0.89, in the range obtained for most random coil polymers. This is to be compared with the value 0.885 obtained by Pouradier and Venet for alkali-precursor gelatin.[15] The extracts obtained under milder conditions show a much lower value, α = 0.264. As shown previously the viscosities of these extracts depend upon the viscometer shear gradient.[3] Consequently, these

(12) E. V. Sayre, *J. Polymer Sci.*, **10**, 175 (1953).

(13) A. Peterlin, *ibid.*, **10**, 425 (1953).

(14) E. V. Gouinlock, P. J. Flory and H. A. Scheraga, *ibid.*, **16**, 383 (1955).

(15) J. Pouradier and A. M. Venet, *J. chim. phys.*, **49**, 238 (1952).

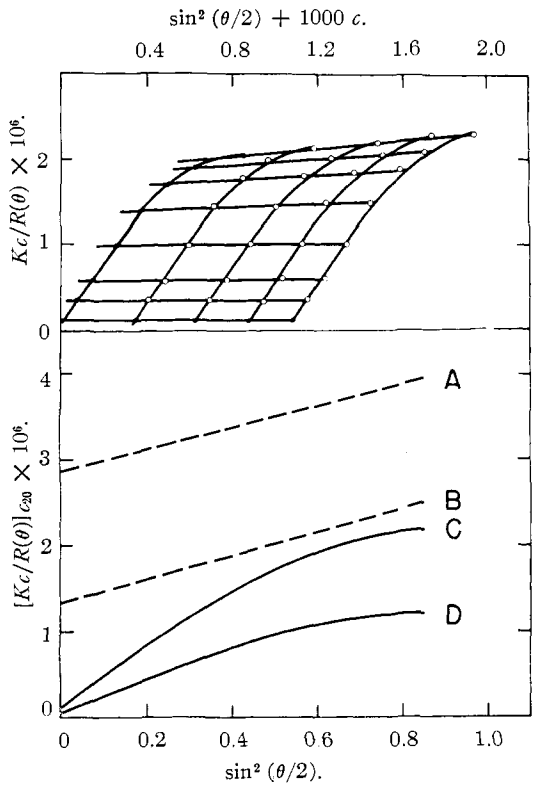


Fig. 3.—Typical light scattering data of the characterization study: (a) (top) Zimm plot for a 60° extract, fraction 2; (b) (bottom) extrapolated $[Kc/R(\theta)]_{c=0}$ *vs.* $\sin^2(\theta/2)$ plots for fraction 3 (A and B) and fraction 2 (C and D), showing the range in variation of the data.

extracts were treated in terms of rods or stiffly extended molecules while the *p*H 2.0 extracts were considered to be coils. In every case the mass per unit length estimates are minimal values since the length used is a greater than z-average dimension while the molecular weight is a weight-average dimension. Without some means of determining the polydispersity and the M_z/M_w ratio (possibly as large as 3), no better estimate of the mass per unit length can be made. There is, of course, the further possibility that the thickness is not uniform over the entire length of the molecule.

Discussion

From the standpoint of the fractionation and extraction data, and without regard to any previous conception of the macromolecular organization of collagen, two facts are evident. First, extraction of intact collagen at *p*H's $\geq$ 3.0, at 60°, resulted in the solubilization of nearly equivalent total amounts of the collagen at each final *p*H whether the extraction was done in a stepwise fashion or not. Second, the molecular weight distribution of the solubilized matter was such that the distribution obtained in a single extraction to a given *p*H was nearly the same as that obtained by adding together the distributions in the serial extracts to that final *p*H (Fig. 1, compare C, J and E, K). However, the molecular weight distribution of the total was different at each *p*H. The 0.1 ionic strength buffer had sufficient capacity to maintain the *p*H constant throughout each extraction at *p*H $\geq$ 3.0. At *p*H's < 3.0 a more extensive breakdown occurred involving the uptake of acid and the probable rupture of polypeptide chain bonds.

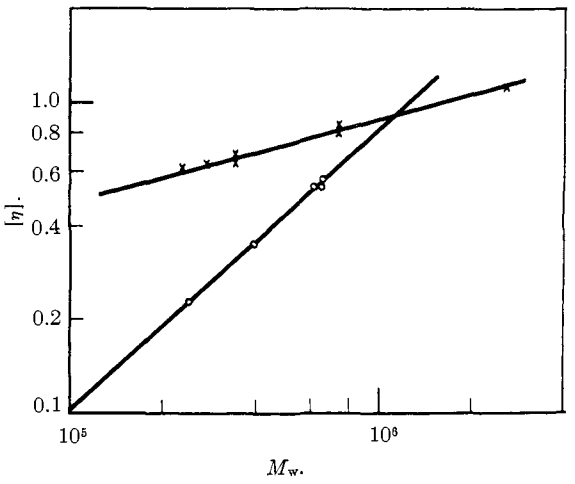


Fig. 4.—Intrinsic viscosity *vs.* weight average molecular weight. M_w determined by light scattering: O, *p*H 2.0 extracts; X, all others.

If the bonds responsible for the insolubility of collagen were equal in strength and distributed randomly or uniformly throughout the structure, one would expect to have found that the fraction of the protein solubilized was the same for the serial and single extraction at any given *p*H in the non-

TABLE III

A. CHARACTERIZATION OF ISOIONIC EXTRACTS, 60°

Fraction	$M_w \times 10^{-5}$	$(\overline{S^2}_z)^{1/2}$, Å. radius of gyration	Assumed shape	X, stiffness parameter	$(\overline{L^2}_{z+1})^{1/2}$, extended chain length	$\frac{M_w}{L_{z+1}}$
2	83.3	2410	Stiff coil	48	30800	270
3	3.45	314	Rod		1088	315
4	2.05	345	Rod		1200	170

B. VARIATION IN MOLECULAR WEIGHT WITHIN A FRACTION, 60°, FRACTION 3

Extraction conditions	Serial extractions: $M_w \times 10^{-5}$	$(\overline{S^2}_z)^{1/2}$, Å.	$[\eta]$, (100 cc.)/g.	$(\overline{L^2}_z)^{1/2}$ or $(\overline{R^2}_z)^{1/2}$*	$\frac{M_w}{L_{z+1}}$
Isoionic	3.45	314	0.65	1088	315
*p*H 4.0	26.3	1050	1.10	5100[a]	515
*p*H 3.0	7.45	444	0.85	1530	482
*p*H 2.0	2.71	320	.64	1110	244
*p*H 2.0, 4 hr.	6.35	449	.54	1100*	..
*p*H 4.0	7.45	444	.81	1530	482
*p*H 3.0	2.32	371	.61	1285	180
*p*H 2.0	6.30	416	.56	1020*	..
*p*H 2.0, 4 hr.	3.98	347	.35	850*	..

[a] Stiff coil, Peterlin's-parameter $m = 5$, L_z = extended length.

hydrolytic range. This is obviously not the case, for at *p*H 3.0 17.8% of the native collagen was dissolved while only 9.1% of the previously extracted collagen was solubilized. Aside from the unlikely prospect that the heat treatment and solubilization of a part of the collagen renders the remainder more resistant to further solubilization, one is forced to conclude that intact skin collagen is, in some sense, a heterogeneous structure. The two alternative models proposed by Ames[1] remain: either the collagen is composed of a series of dis-

crete molecules of closely related chemical structure, parts of which become successively soluble as some particularly labile bonds break; or the intact fibrous structure is composed of segments which may vary in their degree of integration into a larger macrostructure. By "integration" one broadly infers the presence of inter-peptide chain (or intermolecular) cross linkages which may vary in density and/or in type.

The fractionation–distribution experiments summarized in Fig. 1 (compare B, D, F and C, E, G) and Fig. 2 provide the key data by which a choice may be made between these two alternatives. These data show that even when extensive peptide bond hydrolysis accompanies the primary solubilization reactions, the route by which the solubilization is carried out to reach the same set of equally drastic conditions determines the molecular weight distribution of the solubilized protein. Further, where hydrolysis occurs at *p*H 4 and 3, at 80° fragments of larger molecular weights are isolated from the soluble products of the serial extraction than from those of the single extractions.

As a first approximation the solubilization of a set of open (non-cross-linked) peptide chains may be considered as follows. Assume that there is a system of peptide chains with $n + 1$ units and n bonds per chain. It is not necessary that the "unit" be thought of as an amino acid residue, but rather a larger chain segment between the more labile bonds. Let s_0 = initial number of insoluble peptide chains; s = number of intact chains at any later time, t; p_i = number of bonds i intact at time t ($p_i = s_0$ at $t = 0$); χ_i = rate constant for dissociation of bond i; and finally let the hydrolysis conditions be such that $-ds/dt = (\Sigma\chi_i)s$ and $-dp_i/dt = \chi_i p_i$ so that $dp_i/ds = \chi_i p_i/s\Sigma\chi_i \equiv \alpha_i(p_i/s)$. Integrating this last equation, one finds $p_i/s_0 = (s/s_0)^{\alpha_i}$ or $p_i = s_0 R^{\alpha_i}$ where R is the fraction unhydrolyzed. If we assume that each bond broken results in the solubilization of both fragments then $(1 - R)$ is the weight fraction of protein solubilized. Linderstrom-Lang[16] has shown that for the case of peptide bond hydrolysis in a linear polypeptide where all χ_i's are equal, the number of molecules S_q in which bond q has broken while all other bonds are intact is

$$S_q = R^{(q-1)/n} S_0(1 - R^{1/n})[2 + (1 - R^{1/n})(n - q)] \quad (1)$$

The total number of bonds broken, b, is given by

$$b = s_0(n - \sum_n R^{\alpha_i}) = ns_0(1 - R^{1/n}) \quad (2)$$

Returning to the question of solubilization, if each bond broken in an intact molecule solubilizes the fragments of that molecule then the number-average molecular weight of the soluble species is

$$M_N' = \frac{n(s_0 - s)}{(s_0 - s) + b} \quad (3)$$

or

$$M_N' = \frac{n(1 - R)M_0}{(n + 1) - nR^{1/n} - R} \quad (4)$$

(16) K. U. Linderstrom-Lang, "Les Proteins," 9th Conseil Chim. Solvay, 1953, pp. 247–294.

where M_0 is the molecular weight of the peptide between bonds. Similarly the weight-average molecular weight, M_w' of the soluble protein is given (for all χ_i equal) by eq. 5.

$$M_w' = \frac{\sum_1^n q^2 R^{(q-1)/n}(1 - R^{1/n})[2 + (1 - R^{1/n})(n - q)]}{\sum_1^n qR^{(q-1)/n}(1 - R^{1/n})[2 + (1 - R^{1/n})(n - q)]} M_0 \quad (5)$$

If one chooses n large enough the summations in (5) may be replaced by integrals and approximate values of M_w' obtained. M_N' and M_w' are plotted as a function of R in Fig. 4 for $n = 1{,}000$. It is of interest to note that the ratio M_w'/M_N' is not equal to two for this random depolymerization except at the very beginning of the solubilization process. In any event, Fig. 5 shows clearly that one could not expect to find increases in the fraction of higher weight material as $(1 - R)$ increased for a system consisting of discrete molecules with similar distributions of heat-labile bonds. Nor should one expect any different distributions in serial and single extracts. Further, if there is a distribution of molecular weights in the insoluble protein then the highest weight species will still have the greatest probability of disappearing in the early stages of the solubilization process.

A different result is obtained from the second alternative model. Assume a system of parallel rods, held together by transverse linkages at random intervals along each chain. Let there be a distribution of rod lengths such that $M_w/M_N = P > 1$ and let

N_i = no. of rods which are i-mers
N = total no. of rods = $\Sigma_n N_i$
α = fraction of momomers which may participate in cross-linkages, the same for each rod or $i\alpha$ cross-linkages per i-mer

The total number of cross linkage sites is thus $\alpha \sum_n iN_i$ while there are $(\alpha/2\Sigma_n)\ iN_i$ cross-linkages possible. If we set $p_0 = \alpha \sum_n iN_i$ = the number of sites bonded in the native protein and p = number of sites bonded at any time t then there are $(p_0 - p)$ cross-linkage sites free at time t. To free a rod from the macro-structure every cross link to the structure must be broken. Hence, since $(1 - p/p_0)$ is the probability that a site will be free, the fraction of i-mers in solution is given by eq. 6.

$$\frac{N_i'}{N_i^0} = (1 - p/p_0)^{\alpha i} \quad (6)$$

while the total weight dissolved is

$$\sum_n iN_i' M_0 = \sum_n iN_i^0 (1 - p/p_0)^{\alpha i} M_0 \quad (7)$$

and the weight fraction solubilized, $(1 - R)$, at any extent of reaction p/p_0 is

$$(1 - R) = \frac{M_0 \sum_n iN_i^0(1 - p/p_0)^{\alpha i}}{M_0 \sum_n iN_i} = \frac{M_0}{W} \sum_n iN_i(1 - p/p_0)^{\alpha i} \quad (8)$$

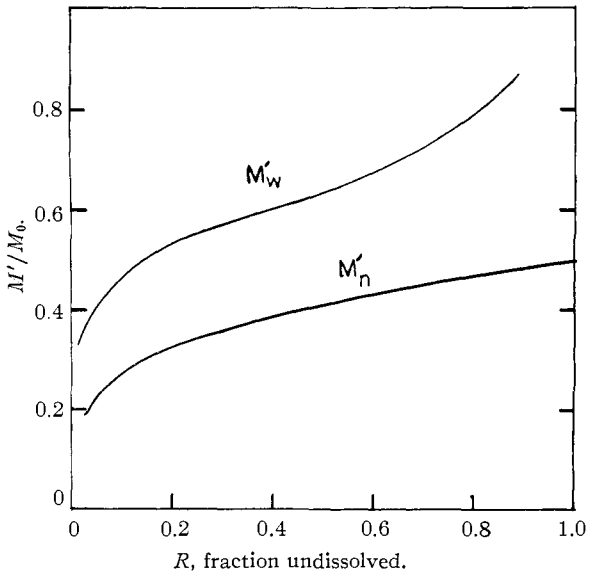


Fig. 5.—Molecular weight distribution of soluble fraction as predicted from eq. 4 and 5, relative to the particle weight, M_0, of the insoluble molecules. R = fraction of protein still intact.

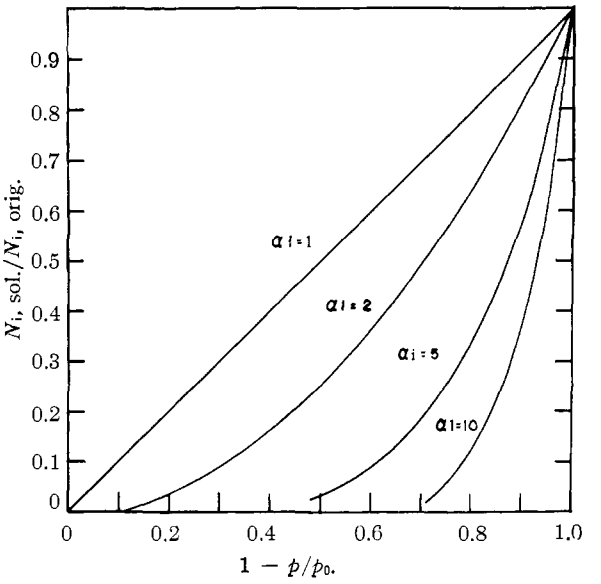


Fig. 6.—Fraction of each species N_i solubilized as a function of the fraction of transverse bonds broken, according to eq. 6.

where W is the initial weight of insoluble protein. Correlation of eq. 8 with the experimental data requires some knowledge of the original rod weight distribution. However, computations based on eq. 6 (Fig. 6) clearly show that in this system larger molecules are obtained only in the final stages of reaction, as $(1 - p/p_0)$ approaches unity.

Thus, one may predict that in a cross-linked network of filaments of varying length the larger molecules would appear in solution following the more drastic solubilization procedures. In the event that the transverse and longitudinal linkages are equally labile one would find a preponderance of smaller fragments being solubilized. However, if the degradation were carried out in a stepwise fashion so that many of the transverse linkages were ruptured first, then subsequent degradation of the long chain molecules in the intact structure would follow the course described by eq. 4 and 5 rather than by 6, 7 and 8.

A comparison of this reasoning with the extraction and fractionation data, particularly those at 80°, leads one to conclude that the second of the two alternative models is closest to reality.

This argument does not vitiate the supposition that collagens are composed of some fundamental asymmetric unit such as "tropocollagen."[17] The distribution of molecular weights (rod lengths) assumed for the cross-linked model could arise from the presence of a second type of cross-link (B), more stable in acid and in lower concentration than the primary set of labile transverse bonds (A). If N_p is the number of primary molecules and b_A and b_B the number of bonds of type A and B, respectively, then $b_A \geq N_p/2$ while $b_B < N_p/2$.

The required heterogeneity of the intact collagen could thus be ascribed to the presence of a relatively few acid stable B bonds, not necessarily distributed randomly throughout the structure but dependent upon the mechanism of tissue formation and maturation.

The characterization data of Table III show that the soluble fragments of higher molecular weight (fractions 2 and 3) are stiffly extended coils or rods which are composed of laterally ordered peptide chains. As degradation proceeds the lateral bonds are ruptured; then, with peptide bond hydrolysis, shorter single chain random coils are to be found. The stable transverse bonds which persist after solubilization are further evidence of the existence of such stable linkages in the intact tissue.

The discussions of the molecular parameters of gelatin reported in the literature[14,15,18] infer that all gelatins are random coil single polypeptide chains. These data, however, invariably refer to the usual alkali precursor gelatins. In the instances where acid precursor gelatins have been examined[19,20] anomalous behavior has been observed in correlating both viscosity and light scattering data in terms of random coils. For example, Courts showed[19] that while end-group analysis could be correlated with molecular weights determined from the viscosity of alkali-precursor gelatins, the acid-precursor gelatins had higher end-group determined molecular weights but lower viscosities. Similarly, McEwen and Pratt[20] showed that tendon collagen dissolved in HCl was most probably composed of lateral aggregates of stiffly extended peptide chains. Ames[1] has pointed out that if transverse linkages exist in native collagen, those which are stable in acid must be labile in alkali. From the characterization data[14,18] and the degradation experiments of Pouradier and Venet[15] one might predict that the thermal extraction of alkali-precursor collagen would follow the course described by eq. 4 and 5.

(17) J. Gross, J. H. Highberger and F. O. Schmitt, *Proc. Natl. Acad. Sci., U. S.*, **40**, 679 (1954).

(18) H. Boedtker and P. Doty, *J. Phys. Chem.*, **58**, 968 (1954).

(19) A. Courts, *Biochem. J.*, **58**, 70 (1954).

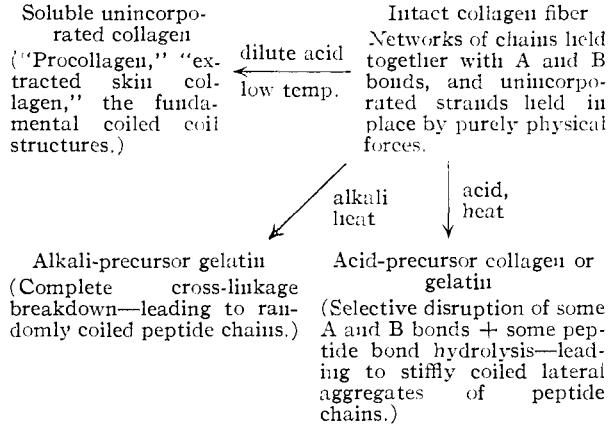
(20) M. B. McEwen and M. I. Pratt, "Nature and Structure of Collagen," Academic Press, New York, N. Y., 1953, pp. 158–168.

Conclusion

These data and computations bear out Gustavson's[4] conclusions, based on chemical evidence, that acid stable cross-linkages of at least two varieties are present in varying degree in different collagens. Beyond this, however, these data show that the inhomogeneities which are noted in a single specimen of intact tissue collagen may arise from the fact that neither class of these semi-labile cross-linkages is distributed uniformly (at random) throughout the structure.

A collagen fibril may thus be pictured as follows. The fibril is composed of segments varying in length and cross-section area due to differences in the cross-link distributions and the lateral ordering of the polypeptide chains. (The polypeptide chains are undoubtedly several times longer than the 640 Å. periodic spacing characteristic of intact, undegraded collagen. The chains do not have to be exactly matched but may be shifted by several periods with respect to each other giving continuity to the macrostructure.) The segments are chain networks held together by sets of the acid stable or B bonds. These segments contain, and are held in the gross structure by, the acid-labile A bonds and physical forces.

The disaggregation of collagen consists of various combinations of A- and B-bond breakage and disruption of physical forces to give "procollagen," acid-precursor collagen or gelatin and alkali-precursor gelatin, as illustrated in the following schematic outline



These models provide the basis for understanding the well-known differences in titration curves, and the viscosity–*p*H and viscosity–molecular weight relationships in acid- and alkali-precursor gelatins. One of the most important questions yet to be answered is the chemical identity of the polypeptide chains themselves. It does appear[5,18,21] that even these are not all identical in composition.

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(21) D. Burton, D. A. Hall, M. K. Keech, R. Reed, H. Saxl, R. E. Tunbridge and M. J. Wood, *Nature*, **176**, 966 (1955).