functional groups on the fibrous structure. Such a change manifests itself in the increased affinity of the protein for dye ions. Further, the effective pK of some of the titratable groups is altered during the heating process. The structural reorientation of the fibrous network may be the first step in the solubilization of collagen in acid media.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE RESEARCH DIVISION, ARMOUR AND COMPANY]

The Degradation of Collagen. III. Characterization of Soluble Products of Mild Acid Degradation

BY ARTHUR VEIS, DELBERT N. EGGENBERGER AND JEROME COHEN

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The soluble protein obtained during the mild thermal and acid dissolution of purified bovine hide collagen which has had no previous pretreatment does not behave as a typical gelatin but is more closely related to native collagen and "procollagen." The unfractionated soluble collagen is exceedingly polydisperse and consists of molecules ranging in molecular weight from 20×10^6 to less than 1×10^6. The largest fragments are highly asymmetric and have radii of gyration on the order of 6000 Å., deduced from light scattering and viscosity measurements at 40°. The soluble material obtained under a variety of conditions has been fractionated and the molecular parameters of each fraction have been determined. These parameters have been related to the initial phases of the collagen–gelatin transformation process. The stability of collagen solutions at 40° is discussed

The mild thermal and acid degradation of previously untreated bovine hide collagen under conditions where only a limited portion of the protein is dissolved yields soluble fragments which do not behave as typical commercial gelatins. In the previous paper of this series[1] it was argued, from a consideration of the properties of the insoluble residues of each extraction, that these soluble fragments might be related more closely to collagen than to gelatin. The work discussed here was undertaken as a test of the validity of the above argument. Particular emphasis is placed upon the question of the existence of thermally solubilized collagen as opposed to the necessary immediate appearance of a "parent" gelatin of molecular weight near 100,000 to 200,000.[2]

The literature on collagen is filled with conflicting terminology relating to "native" collagen and its various forms and degradation products, ranging from Nageottes' early Collagen A and B through Scatchard's "parent gelatin," Orekhovitch's procollagen, the English School's "extracted skin" collagen, on to the new "parent gelatin" described by Gallop and Bear. This profusion of names, each with unfortunate semantic overtones, makes absolutely necessary some clarification of the particular language used in the preceding paragraph, and in those that follow.

The confusion and diversity of results reported is akin to a separate experiment, in itself testifying to the fact that collagens originating in different tissues are not identical in physical fine structure, degree of cross linking, or even in amino acid content. Indeed, collagenous proteins originating in the same tissue, *e.g.*, insoluble skin collagen and "extracted skin" collagen, are not identical on several counts, both physical and chemical. In this discussion we are concerned with the degradation of mature, insoluble and intact skin collagen only into water soluble or water dispersible fragments. Because of our previous conclusions[1] that extensive hydrolysis is not a factor in the mild acid extraction of this soluble protein and because of the experiments to be described below we have chosen to term these extracts "soluble collagen" with the implication that much of the chemical and physical structure characteristic of the three-dimensional network which comprises mature, intact skin collagen may be retained in at least part of the soluble protein. The term "acid-precursor gelatin" is not applicable, though of names now in common usage it is the closest, because it implies a longer history of pretreatment for the insoluble collagen than we have used. The term "parent gelatin" is discussed in the sense in which it was introduced by Scatchard and his co-workers[2] and not in the sense now used by Gallop and Bear. The relationships between "soluble collagen," "extracted skin collagen," Gallop's "parent gelatin" and the usual gelatins are to be the subjects for further research.

Experimental

Materials.—Bovine hide collagen was prepared as described previously[3] by a method similar to that of Highberger.[4] All of the low temperature acid-extractable collagen (procollagen) is removed by this treatment. The soluble collagens were obtained by thermal extraction of native collagen at 0.1 ionic strength and 60° for varying lengths of time at several pH's.[1] Each soluble extract was filtered and then dialyzed against cold tap water to remove the buffer salts and finally against cold distilled water until salt free. The collagen was recovered by lyophilization.

The gelatin used for occasional comparison studies was the Pharmaceutical Grade Gelatin of the Grayslake Gelatin Company, an acid pretreated pig skin gelatin.

Five times recrystallized bovine serum albumin, very generously supplied by Dr. K. C. Robbins of the Armour Laboratories Division Research Department, was used in light-scattering calibration check determinations.

Light Scattering.—The light-scattering photometer has been described previously.[5] Scattering measurements were taken at angles of 30 to 135° from the incident beam in 15° intervals. The cell temperature was constant at 40° for all

(1) A. Veis and J. Cohen, THIS JOURNAL, **77**, 2364 (1955).

(2) G. Scatchard, J. L. Oncley, J. W. Williams and A. Brown, *ibid.*, **66**, 1980 (1944).

(3) A. Veis and J. Cohen, *ibid.*, **76**, 2476 (1954).

(4) J. H. Highberger, *J. Am. Leather Chem. Assoc.*, **31**, 345 (1936).

(5) A. Veis and D. N. Eggenberger, THIS JOURNAL, **76**, 1560 (1954).

measurements. The primary calibration of the reduced intensity of scattering was made with Ludox (supplied by the E. I. du Pont de Nemours Company) and checked with clean benzene solutions. A further standardization was made by determining the molecular weight of bovine serum albumin, for which a molecular weight of 72,000 ± 2,000 was obtained. The symmetry of scattering from the cell was checked with fluorescein solutions of varying scattering intensity. All solutions were cleaned by centrifugation for 4 or more hours (as required) at 26,000 × g. Refractive index increments were measured directly in a Rayleigh Interferometer. $(dn/dc)_{\lambda=4360}$ was found to be 0.192. Light-scattering terms used in the following discussion are: $R(\theta)$, the reduced intensity of scattering at angle θ from the incident beam; λ, the wave length of incident light in air; $P(\theta)$, the diminution of the intensity of scattered light at angle θ because of internal interference; E, the initial slope in a plot of $1/P(\theta)$ *vs.* $\sin^2(\theta/2)$; and v, $3E \sin^2(\theta/2)$.

Viscosity Measurements.—All viscosity measurements were made in Cannon–Fenske U-tube capillary viscometers. Several viscometers of different flow time were used; pertinent information about each viscometer is tabulated with the appropriate data. All measurements were made in the bath held at 40.00 ± 0.02°.

Solutions.—The properties of collagen solutions depend, as will be shown, upon the past history of the solution, *e.g.*, the temperature at which it is stored and the time it is held at a given temperature. Except for the preliminary examination of this time and temperature sensitivity, all solutions were made up at 40° and allowed to stand at that temperature for at least two hours at their isoelectric *p*H. Centrifugations of the solutions for light scattering were made in an air-conditioned room under conditions where the rotor temperature at 26,000 × g was between 38 and 40°. The rotor temperature never exceeded 40°. Solutions were transferred directly from the centrifuge to the light-scattering cell or to clean storage vessels also held at 40°. To standardize light-scattering and viscosity measurements all viscosity determinations were made on solutions which had received the same 4 to 6 hours heating treatment at 40°. There was no appreciable breakdown of the collagen in this period.

Fractionation.—Two fractionation methods have been used. The first was the alcohol coacervation method of Pouradier and Venet.[6] Absolute ethanol was added to a known volume of a 2% solution of collagen at 40°. The volume of ethanol at which turbidity appeared was noted and the first coacervate allowed to settle out. Ethanol was then added to make solutions with ethanol–water ratios (by volume) equal to 1.6, 2, 3, 4, 5 and 10. The coacervate layer was separated after each addition of ethanol. The coacervates were diluted with water and then dried by lyophilization.

The sodium dodecyl sulfate–sodium chloride coacervation technique described by Stainsby, Saunders and Ward[7] was chosen as the second fractionation method. The general plan of these workers was employed but some changes were introduced since under their conditions all of the collagen fractions studied, as well as the Grayslake gelatin, were precipitated during the first fractionation step. This difference in behavior might be due to the fact that all of the present work is with "acid-process" gelatins whereas all of the work of Stainsby, *et al.*, was with alkali-treated hide gelatin. In the modified method all equilibrations and separations were made at 40°. The first fraction was obtained from the coacervate formed in an 0.8% protein solution which contained 9.4 millimoles of sodium dodecyl sulfate per gram of protein, and which was 0.378 N in NaCl. The coacervate was allowed to settle for 18 hours. The clear supernatant layer was then separated from the oily coacervate and made 0.415 N in NaCl. Succeeding supernatants were made, respectively, 0.44, 0.65 and 1.00 N in NaCl after separation. The protein was precipitated from each coacervate by the addition of cold acetone to give a final acetone–water ratio of 2 to 1. Sodium dodecyl sulfate and sodium chloride are soluble in this mixture whereas the protein is not. The precipitated protein was washed in ice water to extract additional salt and was then redissolved in warm water and reprecipitated with acetone. Titration with trimethyloctadecylammonium chloride in a carbon tetrachloride–dichlorofluorescein indicator system showed that a trace of sodium dodecyl sulfate remained. It was not practical to reprecipitate the protein a third time since, in the complete absence of salt, some of the fractions formed colloidal sols which were difficult to break. The final precipitates were dispersed in fresh distilled water and dried by lyophilization. Stainsby[7] found that traces of sodium dodecyl sulfate did not interfere significantly with physical measurements.

(6) J. Pouradier and A. M. Venet, *J. chim. phys.*, **47**, 11 (1950).

(7) G. Stainsby, P. R. Saunders and A. G. Ward, *J. Polymer Sci.*, **12**, 325 (1954).

Results and Discussion

I. The Stability of Soluble Collagens.—Dry soluble collagen imbibes water readily at room temperature to form difficultly dispersible clumps of transparent gel. These clumps disperse at 40° but the hydrodynamic properties of the solutions depend upon the length of time which they are held at this temperature. The viscosities of unfractionated, salt-free collagen solutions were determined at 40° at a function of the age of solution, after a common one-hour aging period, Fig. 1. The collagen used was that obtained by extraction at the isoelectric point for one hour at 60°. Two viscometers were used, with flow times of 50.5 and 196.0 sec., respectively, for water at 40°. Portions of the same solution were transferred to each viscometer and held at 40.0 ± 0.02°. The efflux time was measured every 20 to 30 minutes. The efflux time falls rapidly and then levels out so that it becomes constant within four hours for any given concentration of protein. Stability is attained most quickly in the most dilute solutions and where the rate of shear is highest. Light-scattering measurements could not be used to examine the initial stages of the solution process since the dissociating aggregates were removed during the cleaning process. Centrifugation, as in cleaning solutions for light scattering, materially lowers the viscosity. However, only a small fraction of the total solute is involved since, on the average, less than 5% of the collagen is removed from solution by prolonged centrifugation. After one hour centrifugation at 40°, solutions were stable with respect to light scattering for several hours. Boedtker and Doty[8] showed that gelatin solutions

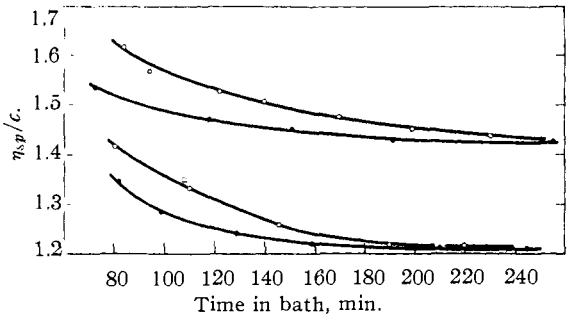

Fig. 1.—The reduced viscosity of aqueous, salt-free solutions of unfractionated M as a function of the time held at 40°. Upper points O, from viscometer with flow time for water = 196.0 sec. at c = 0.0894 g./100 cc.; lower points O, flow time for water = 50.5 sec.; c = 0.0894 g./100 cc. Upper points ●, flow time for water = 196.0 sec.; c = 0.2123 g./100 cc. Lower points ●, flow time for water = 50.5 sec.; c = 0.2123 g./100 cc.

(8) H. Boedtker and P. Doty, ONR Technical Report NR-330-025, Dec. 15, 1952.

also exhibit similar time–temperature sensitivity without undergoing hydrolytic degradation.[9]

II. Comparative Fractionation.—The soluble products of a one hour, 60°, *p*H 5.75 (isoelectric), degradation (designated subsequently as M) and of a 4 hour, 60°, *p*H 2.0, degradation (designated subsequently as D) were chosen for fractionation. These samples represent the extremes of mild and drastic degradation and should have the greatest variation in properties. The fractionations were carried out at 40° after the stock protein solutions had been held for 4 hours at that temperature.

The ethanol fractionation differentiated M and D immediately. With M the first noticeable turbidity occurred when the alcohol–water ratio (A/W) was 1.48. At A/W = 1.6 an oily coacervate layer of M (fraction M-A-I) was formed which contained almost all of M. There was no corresponding fraction D-A-I, the solution of D was still clear at this point. The first turbidity was noted in D at A/W = 2.70. The solutions of D became progressively more turbid as A/W was increased but phase separation did not occur to any extent even after centrifugation. The fractionation data are compared in Table I. Grayslake gelatin was run through the fractionation process and yielded turbid sols comparable to those of D. Due to the formation of stable sols it appears that a salt-free ethanol–water system is not as satisfactory for the fractionation of acid-precursor collagen or gelatin as for alkali-precursor gelatin.

TABLE I

COMPARISON OF THE PRODUCTS OF MILD AND DRASTIC DEGRADATION IN AN ETHANOL FRACTIONATION SCHEME AT 40°

Fraction	Alcohol-water	% M recovered (settled out as oily coacervates)	% D recovered (by centrifugation for 1/2 hour)
A-I	1.6	76.71	...
A-II	2.0	9.94	...
A-III	3.0	8.13	9.92
A-IV	4.0	0.56	5.98
A-V	5.0	...	2.32
A-VI	10.0	1.99	2.58
Recovery from final supernatant		...	78.17
Total recovery		97.32	98.97

Trial fractionations by the sodium dodecyl sulfate–sodium chloride complex coacervate method indicated that within the salt concentration range necessary to cause initial flocculation and complete flocculation the amount of protein recovered is roughly proportional to the amount of salt added. Since only relatively small amounts of collagen were to be fractionated, the fractions were arranged so that out of five fractions the first and fourth would each contain about 30% of the total protein and be separated by two sharp fractions. These latter should remove the very largest molecules not precipitated in the first fraction so that fractions I and IV would be representative of the range in properties to be expected. The data are shown in Table II.

TABLE II

COMPARISON OF THE PRODUCTS OF MILD AND DRASTIC DEGRADATION BY THE SODIUM DODECYL SULFATE. SODIUM CHLORIDE CONCENTRATION METHOD AT 40°

Fraction	Salt concn., range	M, % Recovered	M, % 100% Basis	D, % Recovered	D, % 100% Basis
B-I	-0.378	26.95	27.31	32.93	35.07
B-II	0.378–0.415	15.56	15.77	16.06	17.11
B-III	.415–0.440	12.27	12.43	7.73	8.23
B-IV	.440–0.650	29.00	29.38	32.44	34.55
B-V	.650	14.69	14.88	4.71	5.03
Total recovery		98.47		93.88	

The lower recovery of protein from D is not surprising since in the final acetone precipitation step of each fractionation D tended to form stable sols as it did in ethanol whereas M flocculated readily into a network of fine filaments.

Grayslake gelatin gives a weight distribution similar to those shown in Table II when carried through the same fractionation scheme, though light scattering data place its highest molecular weight fraction at a considerably lower molecular weight than the lowest weight fraction of D [$(2\text{-}3) \times 10^5$ as compared with 2×10^6]. Thus, the detergent complex method does not fractionate collagen strictly by molecular weight but must be sensitive as well to the internal structure of the protein. Similar factors complicate the alcohol fractionation method.[10]

III. Characterization by Light Scattering.—The light-scattering patterns of solutions of unfractionated M were examined as a function of concentration in a variety of salts and at various *p*H's. Figure 2 shows typical Zimm-type plots for M in water at its isoelectric point and for M in 1.0 *M* KCl at the same *p*H. The plots are typical of polydisperse systems of highly asymmetric molecules. In addition they indicate that charge interactions cannot be disregarded at the isoionic *p*H. The plots are superposed in the figure to demonstrate that the salt does not cause any aggregation or dissociation of the protein and that the limiting slopes of the $C = 0$ and $\theta = 0$ lines (characterizing the weight-average molecular weight (M_w) and the radius of gyration (ρ)) may be determined independently on the salt at sufficiently low dilutions. Thus, values for R_θ need be determined at only one low concentration of collagen; this greatly simplifies the experimental work. M_w is $(20 \pm 10) \times 10^6$ for unfractionated M. McEwen and Pratt[11] have shown Zimm plots for unfractionated low temperature acid extracts of rat-skin and rat-tendon collagen in acid solutions which indicate comparable molecular weights, extension, and polydispersity.[12] The scattering measurements were made by these authors at 20° but if run at 40°

(9) Disaggregation is not the only facet to the problem of collagen solution stability. Under certain circumstances macroscopic collagen threads are formed from apparently homogeneous solutions at 40°. These fibrils are reversibly formed and may be redispersed. An equilibrium of the type Gel → Solution → Fibril thus seems to be in operation. A fuller discussion of this phenomena will be made elsewhere.

(10) J. Pouradier and A. M. Venet, *J. chim. phys.*, **49**, 85 (1952).

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

(12) We have obtained nearly identical light-scattering results with citrate extracts of rabbit skin in citrate buffer, run at 25°. See M. B. Mathews, *et al.*, *Arch. Biochem. Biophys.*, **52**, 247 (1954).

TABLE III

ANALYSIS OF LIGHT-SCATTERING DATA ON COLLAGEN FRACTIONS

$T = 40°$; $\Gamma/2 = 0.25$ N NaCl; $\lambda = 4360$ Å.; $(dn/dc)_{4360} = 0.192$.

Fraction	$M_w \times 10^{-6}$	ρ, Å., radius of gyration	R, Å., root-mean-square end-to-end extension of polydisperse random coil	X, Peterlin's stiffness parameter	L, Å., total chain extension	M/L mol. wt., units (m.u.)/Å.		
M-B-I	20[a]	6840	13000	75	74000	270		
D-B-I	5.75	3880	7370	56	20500	280		
M-B-IV	1.25	1365	2590(4740)[b]	~0	n(4740)	n[c]		
						1	2	3
						264	132	89
D-B-IV	2.00	1480	2800(5130)[b]	~0	n(5130)	390	195	130

[a] The kc/R_θ *vs.* $\sin^2(\theta/2)$ data may be represented by an equation of the form $kc/R_\theta = a + b\sin^2(\theta/2) + c\sin^4(\theta/2)$. The constants have the values ($\times 10^{+6}$): M-B-I; $a = 0.05$, $b = 11.32$, $c = -4.81$; D-B-I, $a = 0.174$, $b = 12.71$, $c = -5.81$; M-B-IV, $a = 0.796$, $b = 7.31$, $c = -4.90$; D-B-IV, $a = 0.483$, $b = 5.12$, $c = -2.74$. [b] The numbers in parentheses are the lengths of stiff rods with the given radii of gyration. [c] n = number of chains laterally aggregated in a rod; $n - 1$ = the number of folds in a very stiff continuous chain.

probably would not be similar to those presented here.[13]

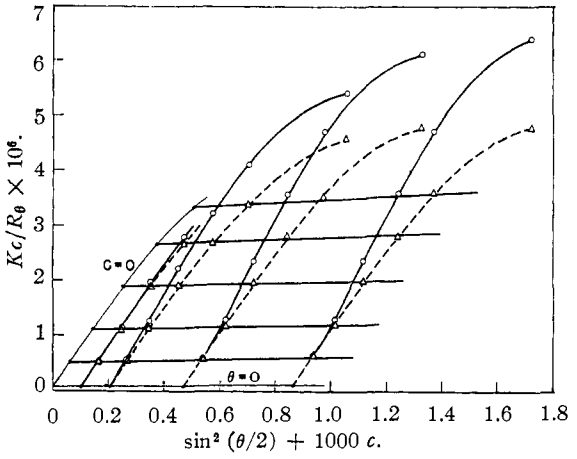

Fig. 2.—Zimm plots of light-scattering data on unfractionated M at 40°: O, in salt-free solution at *p*H 5.75; △, in 1.0 N KCl at *p*H 5.75.

The molecular parameters for fractions M-B-I, M-B-IV, D-B-I and D-B-IV, as determined by light scattering, are given in Table III; the scattering envelopes are illustrated in Fig. 3. The scattering data are amenable to treatment in terms of Peterlin's equations[14] for stiff chain molecules. The stiffness parameter, X, varies from 0 to ∞ as a rigid rod-like molecule takes on flexibility and approaches the completely random coil configuration. It is apparent from both X and the variation of ρ with M_w that the stiffness and relative extension of the chain increases as M_w decreases, with the smallest particles approaching the rigid rod. The mass per unit length (M/L) of fractions M-B-I and D-B-I suggests that there are about three amino acid residues (average weight ~ 98) per ångström along the main fiber axis. The 3-chain helix of Pauling and Corey[15] and the 1-chain helix of Bear[16] both have linear densities of 1 residue per Å. (90 m.u./Å.). Thus, the thermally solubilized

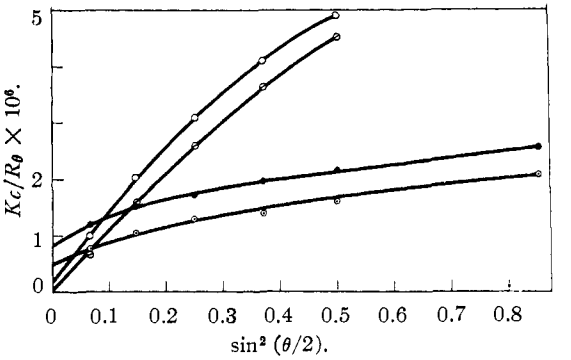

Fig. 3.—Scattering envelopes for fractionated collagens at $C = \sim 0.04$ g./100 cc. in 0.1 N KCl at 40°; ⊘, M-B-I; O, D-B-I; ●, M-B-IV; ⊙, D-B-IV.

collagen fragments may be three-unit lateral aggregates of these structures. The low temperature acid extracts of Gallop[13] and McEwen and Pratt[11] give M/L values ranging from 54 to 120 m.u./Å. which correspond to either helix model. It might have been expected that the collagen solubilized under the more drastic thermal conditions (hydrolysis kept to a minimum) should have a more complex structure than that readily extracted. The stability of the structure of the thermally solubilized material at 40° is pointedly in contrast to the rapid degradation of the low temperature extracts at 40°[13] where, in a matter of minutes, they reach molecular weights on the order of 70,000.

The low weight fractions, M-B-IV and D-B-IV, appear to be stiff rods composed of two or three polypeptide chains or of a single chain folded only once or twice. These fractions are obviously polydisperse since the $1/P(\theta)$ *vs.* v plots (Fig. 4) fall below the calculated $X = 0$ curve for rigid rods, as predicted by Benoit.[17] Since stiffness and polydispersity effect $P(\theta)$ in the same sense, the apparent values for X in polydisperse systems of stiff-chain coils will be too low and the M/L values too high. McEwen and Pratt[11] estimate the maximum error due to polydispersity to be about 50%. Even at this extreme the soluble collagens would approach the rigid rod in stiffness. Further fractionations of the rough fractions are being made to clarify this point.

(13) Paul M. Gallop, Ph.D. Thesis, Department of Biology, Massachusetts Institute of Technology, Sept., 1953.

(14) A. Peterlin, *J. Polymer Sci.*, **10**, 426 (1953).

(15) L. Pauling and R. B. Corey, *Proc. Natl. Acad. Sci. U. S.*, **37**, 272 (1951).

(16) R. S. Bear, *Advances Protein Chem.*, **7**, 69 (1952).

(17) H. Benoit, *J. Polymer Sci.*, **11**, 507 (1953).

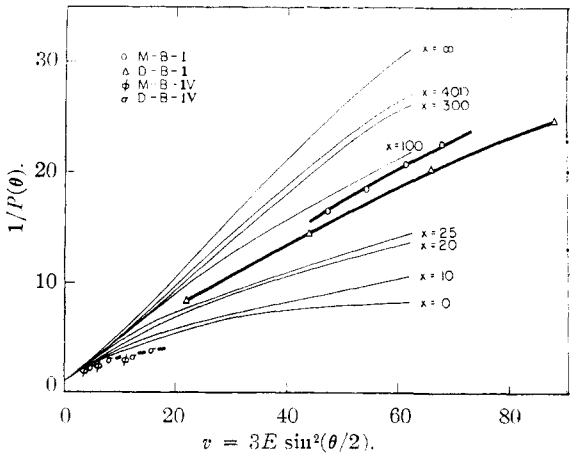

Fig. 4.—Reciprocal particle scattering factors, $1/P(\theta)$, *vs.* v for analysis according to Peterlin.

The values obtained for the viscosities of fractionated and unfractionated soluble collagens are only very roughly interpretable because of the heterodispersities in size and shape of each of the systems examined. Further, by comparing the viscosities obtained in viscometers of equal head but different capillary radii, it is readily seen that the viscosities are dependent upon the shear gradient in the viscometer. An extrapolation of the data in terms of the theory of Katchalsky and Sternberg[18] indicates that the specific viscosities at zero shear are nearly double the values obtained in the slowest viscometer available to us. Reliable extrapolations are not obtainable in Ostwald viscometers. In a viscometer with shear gradient ~1000 sec.$^{-1}$, $[\eta]_{40°}$ = 0.55 (c in g./100 cc.) for centrifuged, unfractionated M; 0.85 for M-B-I and 0.46 for M-B-IV. For all samples the viscosity increments, ν, range from 60 to 110. These data, and the light-scattering molecular weight values from Zimm plots (independent of shape), confirm the analysis of the angular dependence of scattered light at zero concentration. If the collagens were nearly completely flexible random coils, at these very high molecular weights, $[\eta]$ would be 10 to 20, as is the case for the low temperature acid collagen extracts[11,12] and many linear high polymers. On the other hand, if the collagens are stiff, nearly rod-like structures their viscosities should be similar to those of other asymmetric proteins in this weight range such as tobacco mosaic virus or gliadin. For tobacco mosaic virus of $M_w = 33 \times 10^6$, $[\eta] = 0.285$, $\nu = 39$[19], while for gliadin, $M_w = 0.03 \times 10^6$, $[\eta] = 0.105$, $\nu = 14.6$.[20] Thus, the soluble collagens obtained by thermal extractions give viscosities in the range to be expected for stiffly coiled lateral aggregates of polypeptide chains and not for single chain random coils.

IV. Ionic Properties of Thermally Solubilized Collagen.—Unfractionated soluble collagens M and D in 0.10 *N* KCl were titrated with acid and base. The data, Fig. 5, plotted in terms of equivalents of acid and base bound per gram of protein are reproduced from the preceding paper of this series.[1] The shift in isoelectric point from *p*H 5.75 for M to *p*H 6.7 for D is similar to that described by Ames[21] in his comparison of the titration curves of acid- and alkali-precursor gelatins but, as explained earlier,[1] cannot be due entirely to differences in the loss of amide nitrogen since the original insoluble exhibits a titration curve similar to that for curve B. Interpreted in terms of amide nitrogen loss (following Bowes and Kenton[22]) this means that the amide nitrogen is removed during the preparation of the insoluble collagen and not subsequently during the heat degradations. Soluble collagens M and D are both derived from this insoluble collagen.

Viscosity and light-scattering data, Fig. 6, establish a very broad isoelectric range for M between the titration I.E.P., 5.75 and the end of the base binding plateau at *p*H 10. The titration I.E.P.[6,7] is somewhat more closely reproduced by the viscosity–*p*H curves for unfractionated D but the increase of $\eta_{sp.}/c$ in the basic range is still slight. The net charge on the collagen molecule, governing *inter*molecular interactions, and for the coiled systems the configurational changes, must on the average vary only slightly in this range. The broad isoelectric range, typical also of icthyocoll[23] but not alkali-precursor gelatins, may be a consequence of a system heterodisperse with respect to molecular functional group distribution as well as to molecular size. Many years ago, in commenting upon the extended minimum in collagen swelling in the *p*H 7–10 range, Highberger[4] pointed out that several distinct molecular species must be present, either initially in native collagen or as degradation products. Shifts in the isoelectric points due to various denaturing processes have been noted frequently for collagens and gelatins prepared under conditions where all amide nitrogen would be lost prior to the first denaturing treatment.[24]

The difference in M (10% by weight of the original collagen) and D (70% by weight of the original collagen) titration curves is further evidence that intact collagen structures may be composed of a series of related but not identical proteins of different internal structure and slightly different amino acid composition.

Summary and Conclusions

The soluble protein fragments obtained during the mild thermal (60°) extraction of aqueous suspensions of purified, but otherwise untreated, insoluble bovine hide collagen at the I.E.P. and below, may have weight-average molecular weights as high as 20 × 10^6. These fragments, which appear to be molecularly dispersed at 40°, are stable with respect to hydrolytic degradation at that temperature for at least 18 hours at *p*H's near the I.E.P. The soluble protein obtained is polydisperse regardless of the extraction *p*H.

(18) A. Katchalsky and N. Sternberg, *J. Polymer Sci.*, **10**, 253 (1953).

(19) M. A. Lauffer, THIS JOURNAL, **66**, 1188 (1944).

(20) A. Polson, *Kolloid Z.*, **88**, 51 (1939).

(21) W. M. Ames, *J. Sci. Food & Agr.*, **3**, 579 (1952).

(22) J. H. Bowes and R. H. Kenton, *Biochem. J.*, **43**, 365 (1948).

(23) H. G. Bungenberg de Jong and N. F. de Vries, *Rec. trav. chim.*, **50**, 238 (1931).

(24) G. D. McLaughlin and E. R. Theis, "The Chemistry of Leather Manufacture," Reinhold Publ. Corp., New York, N. Y., 1945, pp. 109-112.

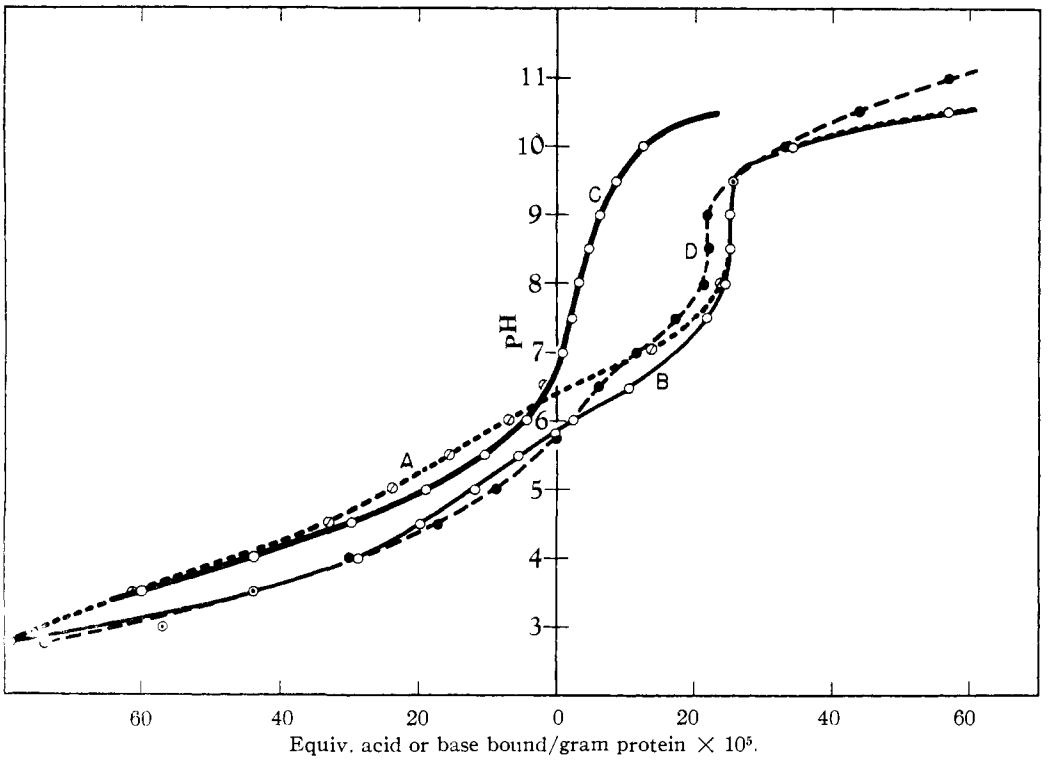

Fig. 5.—Acid–base titration curves of unfractionated extracted collagens and their insoluble residues: curve D, soluble collagen M; curve B, residue from extraction of M; curve C, soluble collagen D; curve A, residue from extraction of collagen D.

The proteins extracted during the most drastic acid degradations (D) are distinguished from the first products of mild degradation (M) by their higher isoelectric points, over-all acid–base titration curves, lower average molecular weights, and by their behavior in mixed solvents (alcohol–water, acetone–water). The same fractionation scheme, based on solubilities in such mixed solvents, does not work equally well with M and D. Low molecular weight fractions of M will precipitate out under conditions where higher weight fractions of D remain in solution. In the sodium dodecyl sulfate–sodium chloride fractionation procedure, the acid-precursor collagens and gelatins all precipitate out in the same salt concentration range, the amount precipitated being roughly proportional to the amount of salt added and independent of the molecular weight of the protein. However, the higher molecular weight molecules are precipitated in preference to the lower weight molecules.

Light-scattering and viscosity measurements show that physically all fractions have the same gross structure. The larger molecules appear to be stiff coils of three laterally aggregated fundamental helix structures. The smaller molecules (M_w = 1 − 2 × 10^6) are stiffer, possibly segments of dissociated larger chains, but are also lateral aggregates of the fundamental polypeptide helices. Low temperature acid extracts are not similar in structure, being randomly coiled, flexible threads of the single polypeptide helix unit, unstable at 40°. The stability of the bonds which are responsible for lateral aggregation is emphasized by the fact that the drastic acid degradation results in shorter chain

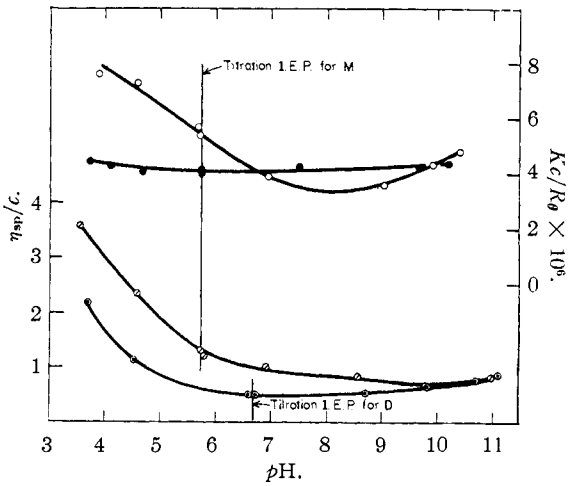

Fig. 6.—Viscosity and turbidity of unfractionated collagens as a function of *p*H: O, turbidity, collagen M; ●, turbidity, collagen M in 0.1 *M* KCl; ⊘, viscosity, collagen M; ⊙, viscosity, collagen D.

lengths but not in more flexible, single chain molecules. The lateral bonds are probably sensitive to alkali and their presence or absence may account for part of the difference between acid- and alkali-precursor gelatins. Ames[25] has shown that acid and alkali treated collagens yielded gelatins of different properties which, by subsequent alkali and acid treatment, respectively, still do not yield identical gelatins. The data described here favor his multi-chain model for intact collagen.

(25) W. M. Ames, *J. Sci. Food and Agriculture*, **3**, 454 (1952).

Two further conclusions may be stated more positively. First, the high molecular weights found for a very considerable portion of the solubilized protein, in conjunction with the functional group analyses previously reported,[1] show that extensive hydrolysis is not a requirement in the initial steps of the collagen–(acid) gelatin transition. Second, stable thermally solubilized proteins may be obtained which are similar to intact collagen and which might properly be called solubilized collagens. The term "parent gelatin," as applied to low molecular weight gelatins, lacks any real significance as applied in the degradation scheme.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Intrinsic Viscosity and Optical Rotation of Proteins in Acid Media[1]

BY JEN TSI YANG AND JOSEPH F. FOSTER[2]

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Bovine plasma albumin is subject to gross, reversible structural alterations upon reduction of the *p*H of its solutions. Ovalbumin, on the other hand, does not show this structural versatility. A preliminary survey has now been made of the behavior of ten additional proteins under similar conditions. Of the proteins studied only one, namely, γ-globulin (human fract. II) shows definite evidence of a pronounced structural expansion in acid. Some possible implications of this fact in immunochemistry are discussed. The absence of evidence for structural variations at low *p*H in the case of the enzymes is discussed from the viewpoint of a current theory of enzyme activation based on the concept that enzymes must be capable of structural fluctuations. It is concluded that the results cannot be taken as evidence against this theory and the possibility that enzymes may show such effects under other conditions, particularly at alkaline *p*H, is raised.

There has been a recent upsurge in interest in the effect of *p*H on the optical activity and intrinsic viscosity of proteins. The specific rotations of a number of proteins as a function of *p*H and of denaturation have been reported in a sequence of papers by Jirgensons and co-workers.[3] Macheboeuf, *et al.*,[4] have demonstrated the pronounced *p*H dependence of the specific viscosity of horse serum albumin solutions. Golub and Pickett[5] have recently reported data on the *p*H dependence of the optical rotation of a number of common proteins.

The authors have recently shown that in the case of bovine plasma albumin there are marked increases in both specific rotation and intrinsic viscosity upon acidification.[6] The alterations appeared to be instantaneous, or nearly so, and fully reversible. The absence of any measurable streaming birefringence at low *p*H was taken as evidence that no pronounced unfolding of the protein molecule is involved but rather a molecular expansion. The closely parallel character of the shifts in the two properties was taken as evidence that the expansion is not gradual or stepwise, but rather an all-or-none transition from a condensed (native) to an expanded form. The viscosity increase, when extrapolated to zero protein concentration and zero ionic strength, is of such magnitude as to suggest an expansion in volume of the order twenty-fold.

Under the same conditions, it was shown that ovalbumin does not undergo a comparable alteration. Similar contrasts in the behavior of these two proteins have been demonstrated in urea denaturation by Kauzmann and co-workers.[7] Bovine plasma albumin and ovalbumin might thus be pictured as representing two extreme types of protein, the one being pliable and readily subject to reversible alterations in configuration, the other being tightly cross-linked so that any structural alterations take place only explosively and irreversibly.

It appeared of interest to survey the behavior of a number of proteins under the same experimental conditions employed in our earlier studies on plasma albumin and ovalbumin. It seemed particularly desirable to examine as many crystalline enzymes as possible in view of suggestions that pronounced reversible alterations in protein structure might be an inherent part of enzymic activation.[8]

Materials and Methods

Human γ-globulin, in the form of whole plasma fraction II, was supplied through the courtesy of Dr. J. N. Ashworth of the American Red Cross (sample designated as Squibb Lot No. 1201). Crystallized zinc insulin, pepsin, trypsin, chymotrypsin, chymotrypsinogen and ribonuclease were obtained from Armour and Company, and crystallized trypsinogen from Mann Research Laboratories, Inc. Three-times crystallized β-lactoglobulin was kindly donated by Drs. T. L. McMeekin and William G. Gordon of the Eastern Regional Research Laboratory. Three-times crystallized lysozyme was prepared earlier in this Laboratory by Dr. E. G. Samsa according to the procedure of Alderton and Fevold.[9] It has been stored for approximately two years at 2° and appeared to be approximately 98% pure by electrophoresis at *p*H 7.8.

All other chemicals employed were of reagent grade.

Protein samples were dissolved in distilled water, dilute HCl or dilute NaCl solution and dialyzed two or three times against large volumes of the same solvent. Prior to use,

(1) Journal Paper Number J-2598 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 1223. This research was carried out under contract Nonr-803 (00) between the Office of Naval Research and Iowa State College.

(2) Department of Chemistry, Purdue University, Lafayette, Indiana.

(3) B. Jirgensons, *Arch. Biochem. Biophys.*, **39**, 261 (1952); *ibid.*, **41**, 333 (1952); B. Jirgensons and S. Sirotzky, THIS JOURNAL, **76**, 1367 (1954).

(4) E. Gavrilesco, E. Barbu and M. Macheboeuf, *Bull. soc. chim. biol.*, **32**, 924 (1950); S. Bjornholm, E. Barbu and M. Macheboeuf, *ibid.*, **34**, 1083 (1952).

(5) M. Golub and E. Pickett, *J. Polymer Sci.*, **13**, 427 (1954).

(6) J. Yang and J. Foster, THIS JOURNAL, **76**, 1588 (1954).

(7) R. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953); J. Schellman, R. Simpson and W. Kauzmann, *ibid.*, **75**, 5152 (1953); W. Kauzmann and R. Simpson, *ibid.*, **75**, 5154 (1953); H. Frensdorff, M. Watson and W. Kauzmann, *ibid.*, **75**, 5157 (1953).

(8) K. Laidler and J. Hoare, *ibid.*, **72**, 2489 (1950).

(9) G. Alderton and H. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).