nearly resemble the behavior of polyuronic acids, as in gum arabic.[48] This similarity probably resides in the lower charge density and the higher water solubility of the backbone structure of these two polymers. The viscosity data show that the shape of GP changes continuously with α to complete ionization. Polymethacrylic acid reaches its maximum extension at about 50% ionization[34] and gum arabic at 80%. Part of this difference is probably attributable to greater ion binding by the more densely charged acrylic acid polymer.[29]

Definitive interpretation of the electrical free energy term as determined from titration and electrophoresis will depend on more detailed information of ion binding and molecular shape changes as a function of the degree of ionization.

The value of $pK_0 = 3.75$ seems not unreasonable, though a somewhat smaller value which seems likely, in view of the foregoing discussion,[37] would be consistent with the known effects of an α-peptide group in lowering the pK_0 of monobasic aliphatic acids,[49] in addition to the presence of a second peptide group in the γ-position and of the two nearest neighbor carboxyl groups.

Acknowledgment.—The coöperation of Drs. C. B. Thorne and R. D. Housewright in furnishing materials for this research and information concerning them is gratefully acknowledged. The experimental part of the paper owes much to the excellent technical assistance of E. J. Weneck, J. H. Convey and Jean Godwin, and to the coöperation of A. Zellner. The measurements of electrophoresis by the microscope method were made by Roberta S. Hartman.

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The Denaturation of Pepsin. I. Macromolecular Changes[1]

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RECEIVED MAY 20, 1957

The molecular parameters of both native and denatured pepsin have been evaluated by velocity sedimentation, diffusion, viscosity, light scattering and electrophoresis. The experimental constants of native pepsin are in approximate accord with earlier values. Alkali-denatured pepsin has been shown to consist of at least three components, the major one comprising about 75% of the mass of the native molecule. The solution behavior of this component, based on the experimental data of solutions of denatured pepsin, probably may be described most realistically as those of a random-coil.

In 1933, Philpot and Eriksson-Quensel[3] examined the sedimentation properties of the enzyme pepsin, which had been crystallized four years earlier by Northrop.[4] They reported a sedimentation coefficient of 3.3 ± 0.15 S and also found a molecular weight of 35,500 by the equilibrium-sedimentation method. Two years later Philpot[5] studied the sedimentation characteristics of denatured pepsin. He reported that the sedimentation coefficient observed between pH 7 and 11 decreased rather uniformly from 3.3 S to a value near 2 S at the latter pH. Philpot was particularly impressed with the "homogeneity of the alkali-denatured pepsin" as deduced from the symmetrical appearance and the sharpness of the absorption boundary in the ultracentrifuge. The decrease in sedimentation rate was attributed to a change in frictional coefficient. This conclusion was based on unpublished results of Polson[6] which showed a decrease in the diffusion coefficient of pepsin when denatured by alkali.

The present report constitutes a more detailed investigation of the molecular composition and molecular-kinetic parameters of solutions of denatured pepsin, as well as some further measurements on native pepsin. Preliminary communications of some of the data presently reported have appeared.[7,8]

Materials and Methods

Preparation of Pepsin Solutions.—Twice recrystallized pepsin, obtained from Worthington Biochemical Corp. (Freehold, N. J.), was used in all experiments. When pepsin was dissolved in water or dilute NaCl, a pH near 3.6 resulted.

Solutions of native pepsin were prepared by dissolving crystalline pepsin in buffer with the aid of a magnetic stirrer. If the pH was altered, due to the buffering action of the pepsin, the solution was re-adjusted to the buffer value by the careful addition of NaOH (or HCl). Solutions of denatured pepsin were prepared by two different procedures. If an alkaline buffer was employed (pH > 7.0), pepsin was dissolved directly in the buffer and the pH was prevented from falling by the (continuous) addition of NaOH, by a manually operated "pH-Stat." In this manner pepsin was denatured immediately on solution and mixtures of native and denatured pepsin were avoided. It is well known that denatured pepsin is an excellent substrate for native pepsin.[4] This procedure was utilized in the sedimentation and viscosity determinations of denatured pepsin. Alternatively, pepsin could be dissolved in its native form in dilute salt or buffer below pH 6.0. When solution was complete it was rapidly denatured by increasing the pH to 8–10 by the addition of strong base. Solutions were then adjusted to experimental conditions by direct addition of acid or base or by dialysis against buffer.

(1) Supported in part by an Institutional Grant from the American Cancer Society and by grant No. C-1974 of the National Cancer Institute of the National Institutes of Health.

(2) National Institutes of Health, Bethesda, Maryland.

(3) J. St. Leger Philpot and I. Eriksson-Quensel, *Nature*, **132**, 932 (1933).

(4) J. H. Northrop, *J. Gen. Physiol.*, **13**, 739 (1930); *Science*, **69**, 580 (1929).

(5) J. St. Leger Philpot, *Biochem. J.*, **29**, 2458 (1935).

(6) A. Polson, cited by Philpot in ref. 5.

(7) Abstracts of Papers, 126th Meeting of the Am. Chem. Soc., New York, Sept. 12–17, 1954, 62c. 3rd International Congress of Biochemistry, Brussels, Belgium, Aug. 1–6 (1955), 2.-36.

(8) H. Edelhoch, THIS JOURNAL, **78**, 2644 (1956).

It should be recalled that pepsin is stable in acid solution and inactivated at neutrality.[9] Pepsin inactivation shows an unusually high dependence on *p*H, such that it is quite stable at *p*H 6.0 and extremely rapidly inactivated at *p*H 7.0 at room temperatures. Most measurements have been made at these two *p*H values in phosphate buffer in order to keep the charge of the two forms of pepsin as close as feasible and to permit the use of a single buffer, albeit a multiply charged anion.

The crystalline preparations of pepsin that were used contained about 20% split products. Polypeptide impurities of this kind always appear to be present in pepsin solutions and undoubtedly originate from autolytic processes. In native (or denatured) pepsin solutions, the concentration of split products may be reduced to about 4% by extensive dialysis. Split products were measured by denaturing native pepsin with alkali (*p*H $\sim$ 10) and then precipitating with 3 volumes of a solution containing 10% TCA, 0.2 *M* HCl and 1 *M* NaCl. The material not precipitated by this reagent is defined as split products, whose concentration was determined from the 280 mμ absorption. The ratio of this value to the 280 mμ absorption of native pepsin was considered to be a measure of the concentration of split products.

Concentrations of solutions were usually measured by their absorption at 280 mμ. The extinction coefficient of a 1.00% solution of crystalline pepsin (undialyzed) was 14.3 at 280 mμ. This value was based on dry weights, determined by vacuum drying at 100° to constant weight. The nitrogen content of this sample was 14.5% (performed by Dr. M. Berenbom), which is a slightly smaller value than reported by Northrop.[4] This probably results from the larger amount of Na^+ present. The *p*H of the stock solution, in the absence of neutral salt, was 4.27.

Pepsin concentrations reported in sedimentation, viscosity and light scattering experiments are for standard stock samples and have not been corrected for split products unless otherwise specified. The molecular coefficients obtained from sedimentation, diffusion and electrophoresis are not very sensitive to the absolute concentration of protein and consequently these data should not be affected very much by using the total concentration of protein (pepsin and split products) instead of the concentration of pepsin. The intrinsic viscosity and light scatter are directly dependent on the concentration of protein. Since we do not know the contribution of the split products to viscosity and light scatter (probably negligible in light scatter) no simple correction can be made. In experiments where dialysis was employed the final concentrations of split products are recorded in the protocol.

All listings of ionic strength or salt concentration *do not* include the contribution of the protein or its gegenions to the total ionic composition.

Sedimentation Velocity.—Sedimentation experiments were performed in the Model E Spinco Ultracentrifuge. Measurements were made at room temperatures. Sedimentation coefficients (s) were calculated by standard procedures[10] and corrected to the reference state of water at 20°. Sedimentation coefficients have been corrected for the decrease in rotor temperature that occurs on acceleration.[11] This correction leads to an increase in the observed value of s of 2.4%.

Viscosity.—Viscosities were measured in a conventional Ostwald viscometer, having flow times of about 90 seconds. Kinetic energy corrections were obtained by measuring the viscosity of water at several temperatures. Since these corrections were quite small and within experimental errors they were not applied. Constant temperature baths were controlled by a thermoregulator to a hundreth of a degree. The reduced kinematic viscosity $[\nu]$ is defined as $(t - t_0)/t_0c$, where t and t_0 are the flow times of the solution and solvent, respectively, and c is the concentration in g./100 ml. Intrinsic viscosities may be obtained from kinematic viscosities by the equation of Tanford[12] $[\eta] = [\nu] + (1 - v_{2\rho_0})/100\rho_0$. All viscosity data are in units of dl./g. Solutions used for viscosity measurements were centrifuged routinely for 30 minutes at 20,000 $\times$ g in the high-speed-head of the International Centrifuge, Model PR2.

Diffusion.—Diffusion measurements were conducted in the Model H, Spinco Electrophoresis-Diffusion instrument. The method of free diffusion from an initially sharp boundary between solvent and solution was employed. Boundaries were initially formed at the ground-glass surfaces of the Tiselius electrophoresis cell. The boundary was brought into view by the capillary siphoning technique of Kahn and Polson.[13] When the boundary reached the level of the tip of the syringe needle, siphoning was stopped. After about an hour the total volume of solution in the diffusion limb was replaced with more solution by further siphoning. This process was usually repeated a third time to rinse completely the walls of the vessel where solution had displaced solvent. Zero time was set at the time when the final siphoning was stopped. The bottom section of the cell was then displaced, thereby isolating the diffusion limb from the rest of the electrophoretic channel.

The Rayleigh interference fringe method was used to follow the diffusion process. For this purpose the electrophoretic cell was equipped with extensions to the channel windows which served as a reference path. The procedure of analyzing interference fringes in diffusion experiments has been described in detail by Longsworth.[14] Since his procedures have been followed in most essentials only the application of the basic theory will be discussed.

The apparent diffusion coefficient is defined by $D' = (\Delta H/\Delta Z)^2/4t$ where ΔH is the measured distance between fringes j_k and j_1; ΔZ is the difference in "reduced cell height" and is evaluated from the probability integral which was

$$f(Z) = \frac{2j - J}{J} = \frac{2}{\sqrt{\pi}} \int_0^Z e^{-\beta^2}\, d\beta$$

obtained from published tables of this function.[15] J is the total number of fringes and is related to the refractive index increment of the protein $(\Delta n/\Delta c)$, the wave length of light, λ (5460.7 A.) and the cell width, a, by $J = 2a(\Delta n/\Delta c)c/\lambda$ where c is the concentration of protein.

Usually six or more photographs were taken during a two-day period. A series of reference fringes of the diffusion channel were obtained before the start of the siphoning procedure for purposes of fringe alignment. Several pictures were taken immediately after the start of the experiment for determination of the fractional fringe. Fringe patterns were enlarged about 40-fold and the intersection of each fringe minima with the minima of a well-defined vertical fringe was plotted on graph paper. This procedure resulted in fractional fringe values with a precision of $\sim$0.1 fringe.

Average values of D' were calculated for each exposure from ratios of $\Delta H/\Delta Z$ for 10 or more pairs of fringes, choosing pairs such that Δj was constant and approximately equal to $^1/_2$ J. D' was plotted against $1/t$ and extrapolated to infinite time to give the true diffusion coefficient. Diffusion experiments were performed at 1.4° and expressed with reference to water at 20° by the Stokes-Einstein relation

$$D_{20,w} = D_t\, \eta_t T_{20}/\eta_{20,w} T_t$$

Experimental methods were checked by determining the diffusion coefficients of two materials which have been studied extensively in other laboratories. A $D_{20,w}$ value of 5.73×10^{-7} unit was observed for a 0.27% solution of crystalline bovine serum albumin at *p*H 6.5 in 0.10 $\Gamma/2$ phosphate buffer and a $D_{1.4} = 2.42_5 \times 10^{-6}$ unit for a 0.5% sucrose solution. These values are in good agreement with published results.[16,17]

Light Scattering.—The Brice-Phoenix Photometer[18] was employed to measure light scatter. The principal modifi-

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(10) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, 1940.

(11) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instru.*, **23**, 609 (1952); A. Biancheria and G. Kegeles, THIS JOURNAL, **76**, 3737 (1954).

(12) C. Tanford, *J. Phys. Chem.*, **59**, 798 (1955).

(13) D. S. Kahn and A. Polson, *ibid.*, **51**, 816 (1947).

(14) L. G. Longsworth, THIS JOURNAL, **74**, 4155 (1952); **75**, 5705 (1953).

(15) "Tables of Probability Functions," Vol. 1, Federal Works Projects Administration, U. S. Government Printing Office, 1941.

(16) M. L. Wagner and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1066 (1956).

(17) L. J. Gosting and M. S. Morris, THIS JOURNAL, **71**, 1998 (1949).

(18) Brice-Phoenix Light Scattering Photometer: Phoenix Precision Instrument Co., Phila., Penn., Bulletin B-P1000; see also, B. A. Brice, M. Halwer and B. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

cation in instrumentation consisted of reducing the width of the light beam by suitable masks to permit the use of Beckman 1.00 cm.[2] cuvettes. The latter had all sides ground and polished. A special stand was used to fix the position of the cell and permit reproducible alignment.

Water was obtained from an all-glass distillation unit. Solutions were clarified routinely by centrifugation at 20,000 × *g* for at least a half hour. All light scatter observations were made at 90° to the incident beam and are reported in terms R_{90}/c (where $R_{90} = i_{90}r^2/I_0$). The instrument was calibrated with solutions of bovine serum albumin. This material has been characterized extensively and is generally of reproducible quality.[19] It can therefore serve as a convenient standard, though it should be supplemented by other methods for critical work.

The extrapolated reduced scattering intensity $(R_{90}/c)_{c\to 0}$ of pepsin solutions was about 0.45–0.50 of that of serum albumin. On this basis the observed molecular weight of pepsin is about 33–37 × 10^3 if the scatter of the two protein solutions are proportional to their molecular weights, and we accept a value of 75,000[19] for the albumin sample. Since several careful light scattering investigations of the molecular weight of pepsin have appeared recently,[20,21] this aspect of the problem was only incidentally touched on.

Turbidimetric titration curves were obtained by titrating pepsin solutions with glycine buffer (0.10 *M*, *p*H 10.2). The latter was used to avoid the addition of free alkali. Solutions were mixed directly in the 1 cm.[2] cuvette by magnetic stirring using small glass-encased iron stirrers. In this way a turbidimetric *p*H curve could be obtained with a single solution. Each change in *p*H was accomplished by the addition of 0.10 cc. of glycine buffer.

Electrophoresis.—Electrophoretic mobilities were measured in the Spinco Electrophoresis-Diffusion instrument, Model H. Mobilities were determined by standard procedures. Rayleigh fringes were obtained simultaneously with schlieren patterns. Where boundaries were analyzed for relative amounts of components, the Rayleigh fringes were used for this purpose. In the electrophoresis of denatured pepsin solutions at *p*H 6.5 and above, the mobilities of the descending boundaries of the principal component were 0.90 ± 0.20 unit smaller than that determined from the ascending boundaries. In solutions of native pepsin having similar mobilities this discrepancy was much smaller. Polyelectrolytes tend to show this type of behavior.[22]

Results

Sedimentation Velocity.—The effect of *p*H on the sedimentation coefficient (*s*) of pepsin has been measured from *p*H 1.2 to 10.2 in a variety of buffers at 0.100 Γ/2 and ~0.3% pepsin concentration. From *p*H 1.2 to 6.0 the sedimentation coefficient was independent of *p*H. The schlieren patterns in acid media showed a single, symmetrical boundary. *s* varied linearly with concentration (Fig. 1) and had a small negative dependence which increased somewhat as the ionic strength was decreased. The extrapolated value of *s* at zero concentration was independent of ionic strength and equal to 3.20*S*.

Solutions of alkali denatured pepsin also showed only a single boundary in the ultracentrifuge. The most noteworthy feature of this boundary was its high degree of symmetry and sharpness, as was observed by Philpot.[5] In fact, the contours of the refractive index gradient boundaries of native and denatured pepsin were remarkably similar; the principal difference was in their sedimentation rates. No effect of *p*H on sedimentation coefficient was observed from 7.0 to 10.2.

In 0.100 Γ/2 solutions of denatured pepsin *s* decreased linearly with concentration whereas at 0.010 Γ/2 the data were better represented by the equation $s = s_0/(1 + k_s c)$, where $k_s = 0.75$ (Fig. 1). The intrinsic sedimentation constant ($s^0_{20,w}$) of denatured pepsin was dependent on ionic strength and was equal to 2.17 and 1.84 at the higher and lower values, respectively.

Acidification of a solution of denatured pepsin to *p*H 6.0 had no influence on the sedimentation characteristics that were observed at *p*H 7.0. The changes in *s* produced on denaturation are therefore not reversed with *p*H. Northrop found less than one per cent. recovery of enzyme activity after acidification of denatured pepsin solutions to *p*H 5.4, which appeared only after several days.[23,24]

Viscosity. A. Intermediate Ionic Strengths.—A decrease in sedimentation coefficient may originate from either a decrease in molecular weight or an increase in frictional coefficient or by a combination of both factors. To distinguish between these possibilities viscosity and diffusion experiments were performed.

In Fig. 2, the viscometric behavior of denatured pepsin, measured in the same buffers as the sedimentation experiments, is reported. Since ionic strength was observed to have a significant effect on the sedimentation data, viscosity measurements were made at comparable ionic strengths. Similar data were obtained with native pepsin solutions (Fig. 3) to indicate the magnitude of salt effects on the viscosity of the native form of pepsin where molecular deformations are unlikely.

The variations in viscosity of native pepsin solutions are small and in accord with expectations of the behavior of non-deformable globular proteins.[25] The reduced specific viscosities (η_{sp}/c) vary linearly with concentration at 0.100 and 0.010 Γ/2 and almost have a common intercept at zero concentration. The small increase in slope in 0.010 Γ/2 results from the fact that the protein particle has an appreciable charge which gives rise to the so-called "electroviscous effect." Our results are similar in magnitude as observed with ribonuclease.[25] In solutions of 0.001 Γ/2 the contribution of the protein and its gegenions to the total ionic concentration becomes comparable to that of the added neutral salt. Thus on dilution of the more concentrated protein solution with (a fixed) salt solution we are reducing the total ionic concentration and thereby increasing the "electroviscous effect." The method of Pals and Hermans[26] of "isoionic dilu-

(19) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950); P. Doty and R. F. Steiner, *J. Chem. Phys.*, **20**, 85 (1952).

(20) M. J. Kronman and M. D. Stern, *J. Phys. Chem.*, **59**, 969 (1955).

(21) D. S. Yasnoff and H. B. Bull, *J. Biol. Chem.*, **200**, 619 (1953).

(22) E. Goldwasser and F. W. Putnam, *J. Phys. Colloid Chem.*, **54**, 79 (1950); H. Edelhoch and J. B. Bateman, THIS JOURNAL, **79**, 6093 (1957).

(23) J. H. Northrop, *J. Gen. Physiol.*, **14**, 713 (1931).

(24) On acidification of denatured pepsin solutions to *p*H 5.4, a broader sedimenting boundary was observed, though the *s* value, calculated from the maximum ordinate, was the same as at 7.0. At *p*H 4.5 (0.10 Γ/2 acetate), a very diffuse boundary, with an *s* value of ~17 appeared, as well as a much smaller boundary, which moved at the lower rate (~2). The major peak observed at *p*H 4.5 undoubtedly represents aggregated denatured pepsin molecules similar to that reported in more detail by Philpot.[5] New peaks of this kind are observed also in electrophoresis of acidified solutions of denatured pepsin (see below).

(25) J. G. Buzzell and C. Tanford, *J. Phys. Chem.*, **60**, 1204 (1956).

(26) D. T. F. Pals and J. J. Hermans, *Rec. trav. chim.*, **71**, 433 (1952).

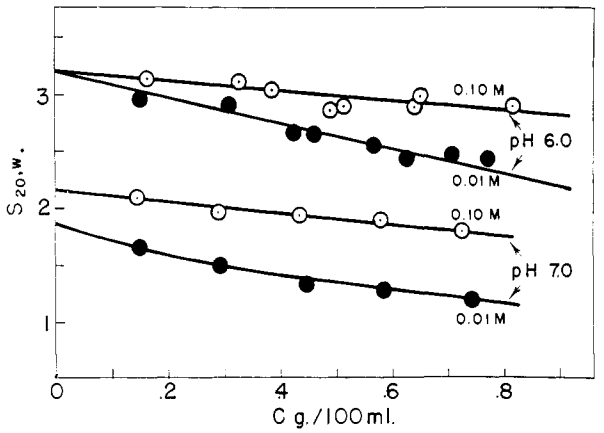

Fig. 1.—Variation of sedimentation coefficients with concentration of native (*p*H 6.0) and denatured pepsin (*p*H 7.0).

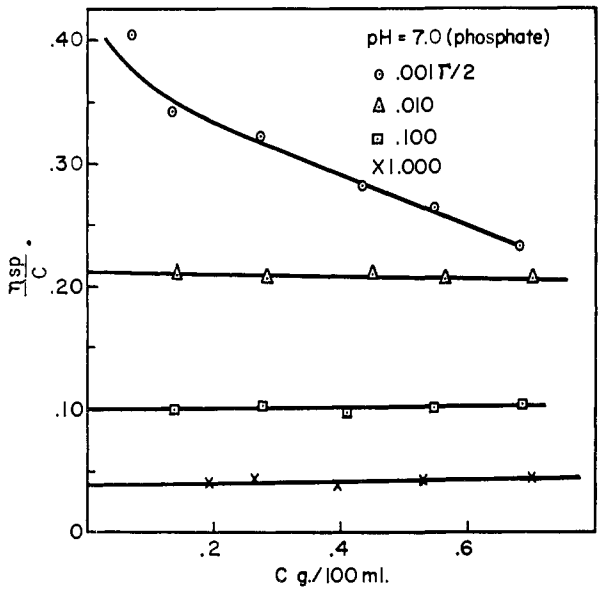

Fig. 2.—The effect of ionic strength on the reduced specific viscosity of denatured pepsin in phosphate buffer. Data at 1.00 Γ/2 have NaCl instead of phosphate buffer; T = 28.0°.

tion" would probably lead to lines with positive slopes at very low salt concentrations.

In solutions of denatured pepsin, the effects of ionic strength are of a different order of magnitude and cannot possibly be explained on the basis of electrical effects on the viscosity of a charged rigid particle. In 0.10 Γ/2 the intrinsic viscosity increased from 0.0335 in native pepsin to 0.102 in denatured pepsin. This change proved to be irreversible since the viscosity remained the same on decreasing the *p*H from 7.0 to 6.0. The intrinsic viscosity increased more than twofold on decreasing the ionic strength to 0.010 of denatured pepsin solutions. In 0.001 Γ/2 the negative slope of the concentration curve was more than twenty-fold greater than observed in native pepsin solutions. The variations in viscosity with ionic strength were found to be fully reversible in solutions of denatured pepsin.

The influence of *p*H on the viscosity was evaluated by experiments conducted in pyrophosphate

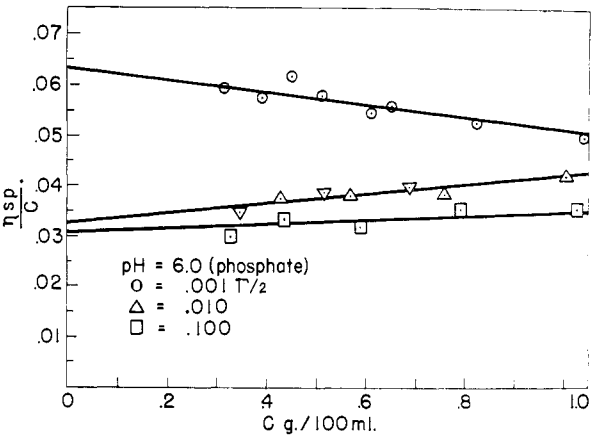

Fig. 3.—The effect of ionic strength on the reduced specific viscosity of native pepsin in phosphate buffer. The inverted triangles (▽) have cacodylic buffer instead of phosphate; T = 25.0°.

and in glycine–NaCl buffers at *p*H 10.0. The data were very similar in magnitude and concentration dependence to that observed with phosphate at *p*H 7.0. The intrinsic viscosity in 0.05 and 0.005 M pyrophosphate was 0.121 and 0.216, respectively.

B. High Ionic Strengths.—When the ionic concentration of a solution of denatured pepsin at *p*H 7.0 in 0.010 Γ/2 phosphate buffer was increased gradually by the addition of small amounts of a concentrated solution of phosphate buffer or NaCl the reduced specific viscosity decreased monotonically, as seen in Fig. 4. There was some dif-

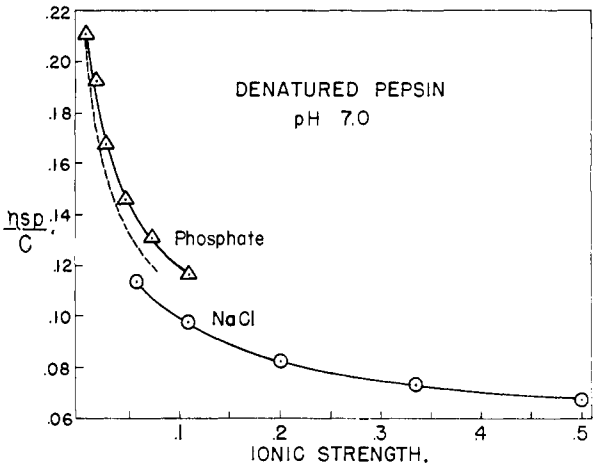

Fig. 4.—The monotonic decline in the reduced specific viscosity of denatured pepsin produced by the ionic atmosphere. The dashed line represents the phosphate data when plotted on a molar basis. Pepsin concentration ≅ 1.00%; T = 25.0°.

ference between phosphate and NaCl in viscosities but these were less when based on concentration rather than ionic strength (dashed line in Fig. 4). The reduced specific viscosity (which is approximately equivalent to the intrinsic viscosity in these solutions, *cf.* Fig. 2), decreased from 0.212 to 0.067 as the ionic strength was increased to 0.50. In 1.00 M NaCl, a small positive dependence on concentration was observed (Fig. 2).

The intrinsic viscosity was 0.039, which is only slightly greater than that of native pepsin.

This uniform change of viscosity with ionic composition is clearly not the behavior of a rigid particle of any shape. It more nearly resembles the behavior pattern of solutions of linear polyelectrolytes.[27-28]

C. Absence of Added Salts.—The polyelectrolyte concept of the structure of denatured pepsin may be tested by its conformity to the Fuoss equation.[28]

$$\eta_{sp}/c = D + \frac{A}{1 + B\sqrt{c}}$$

This equation has been shown to represent the viscometric behavior of a large number of charged linear polymers when depleted of all ions, except those necessary for electrical neutrality, and where dilutions of polymer concentrations are made with water. This equation very adequately represents the viscosity changes in denatured pepsin as it is diluted in this manner, as indicated in Fig. 5. The value of D was taken as 0.039, the intrinsic viscosity of denatured pepsin in 1.00 M NaCl.

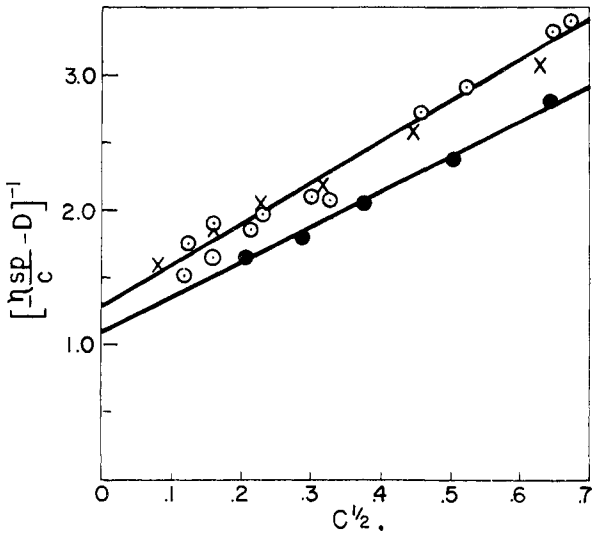

Fig. 5.—Fuoss plots of viscosity of denatured pepsin in the absence of added salt in several solvents: X, water at pH 10.2; O, water at pH 8.5; ●, 50–50 (v./v.) water-ethyl alcohol mixtures. D was taken as 0.039 from intrinsic viscosity in 1.00 M NaCl.

D. Ethyl Alcohol–Water Mixtures.—The influence of intrapolymer interactions between nonpolar residues of the polypeptide backbone in maintaining a greater degree of internal cohesion (and reduced molecular size) may be tested by working with solvents of smaller polarity than water. This was afforded by using a 50–50 mixture (v./v.) of ethyl alcohol and water. In this solvent at pH 7.0 in 0.10 M NaCl, the intrinsic viscosity was 0.20 with no concentration dependence. This result was about twice as large as observed in aqueous 0.10 M NaCl.

(27) A. Katchalsky, *J. Polymer Sci.*, **VII**, 393 (1951); S. Basu, P. Ch. Dasgupta and A. K. Sircar, *J. Colloid Sci.*, **6**, 539 (1951).

(28) R. M. Fuoss, *J. Polymer Sci.*, **3**, 603 (1948); W. N. Maclay and R. M. Fuoss, *ibid.*, **6**, 511 (1951); R. M. Fuoss and D. Edelson, *ibid.*, **6**, 523 (1951).

TABLE I

MOLECULAR PARAMETERS OF PEPSIN FROM SEDIMENTATION AND DIFFUSION

	Native (pH 6.0, 0.10 Γ/2)	Denatured (pH 7.0, 0.10 Γ/2)
$s^0_{20,w} \times 10^{13}$	3.20	2.17
$D_{20,w} \times 10^7$	8.71	6.9[a]
M[b]	35.8	30.7
f/f_0	1.11	1.48
a/b[c]	3.0	8.8

[a] The ionic strength of the diffusion experiment was 0.18. [b] Calculated from Svedberg equation; assume $\bar{v}$ = 0.75. [c] Obtained from Perrin equation, neglecting hydration effects.

In the absence of neutral salt a Fuoss plot was found to fit the data (Fig. 5). The increase in viscosity from that observed in aqueous media was greater at higher concentrations of pepsin and became smaller in more dilute solutions. The extrapolated value at infinite dilution was about 15% larger than in aqueous media.

Diffusion.—Two diffusion experiments were performed with native pepsin. The experimental details appear in Table II. Values of D', and their

TABLE II

DIFFUSION COEFFICIENTS OF NATIVE AND DENATURED PEPSIN SOLUTIONS

No.	pH phosphate buffer	Γ/2	Protein concn., %	Hr. dialyzed	% Split products	$D_{20,w} \times 10^7$
1	6.00	0.10	0.26	138	4.6	8.71 ± 0.05
2	6.15	.20	.34	36	12.1	8.93 ± 0.06
3	7.00	.18	.26	186	6.4	6.9

average deviations, during a 48-hour experiment are shown in Fig. 6. The diffusion coefficient, D, is obtained from the value of D' at $1/t = 0$. The first sample was extensively dialyzed and contained only 4.6% split products. The second contained a larger percentage of split products and had a value of D about 2% larger than the first.

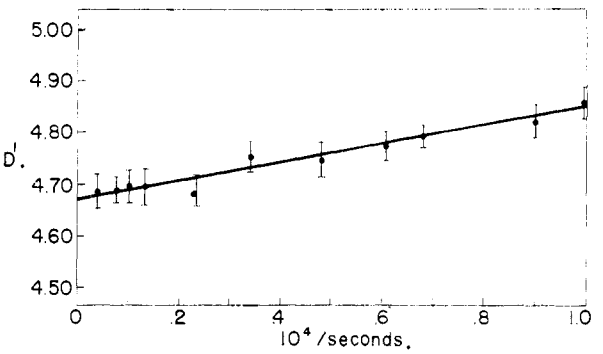

Fig. 6.—Plot of diffusion data as determined by the Rayleigh interference fringe method. Each point is the average of 14 values of $\Delta H/\Delta Z$. The vertical lines through the points represent the average deviations. Experimental details are listed in Table II, no. 2. The ordinate values are those obtained at 1.4°, the experimental temperature.

In the relatively dilute solutions of pepsin studied, the observed values of the diffusion coefficient would not be expected to be much different from its value at infinite dilution, since Donnan and frictional effects are small and tend to cancel each

other. The principal source of error in the observed values of D is the presence of impurities in the form of so-called split products. These are probably larger fragments of autolyzed pepsin that are not readily dialyzable. Judging from the small difference in D between the two preparations it would appear that their effect is relatively small. The value found at the lower level of split products (8.71×10^{-7}) is considered to be the better representation and is used for computations involving the diffusion constant.

This value is in approximate accord with values found by the older scale method of boundary analysis. Neurath,[29] *et al.*, found a value of 8.15×10^{-7} and 8.86 (when corrected to 20°) for a 1% solution of a "more insoluble and a more soluble fraction," respectively, of pepsin. A value of 9.0 is quoted as due to Polson.[30]

In experiment 3 (Table II) the denatured pepsin solution was dialyzed extensively to remove split products. A value of 6.9×10^{-7} was obtained for the diffusion coefficient of denatured pepsin. Since solutions of denatured pepsin represent a mixture of several molecular entities (see following sections) the usefulness of this diffusion value in deriving molecular dimensions is restricted.

Light Scattering.—Light scattering experiments were initiated to ascertain whether the denaturation of pepsin involved a change in the molecular weight of pepsin. Light scatter does not directly provide information of the molecular composition of a mixture of macromolecules. A decrease in intensity of light scattering, in the presence of ample amounts of salt to suppress Donnan effects, is normally associated with a decrease in weight-average molecular weight.

In the turbidimetric–*p*H curve shown in Fig. 7 practically the total decrease in light scatter occurred between *p*H 4.89 and 6.95. The final value of R_{90}/c was about 60% of the initial value. The fall in light scatter at 6.95 was complete when measured.

In a second turbidimetric titration experiment, in 0.15 M NaCl as solvent, no change in light scatter was observed between *p*H 3.65 and 4.89. Between 4.89 and 6.95 a decrease of 42% in R_{90}/c occurred. On mixing equal volumes of solutions at *p*H 3.65 (stock) and 6.95, which resulted in a *p*H of 4.89, the scatter was close to the average of the two separate solutions. When the *p*H of this solution was increased to 7.50 with glycine, the R_{90}/c value fell to that found previously at 6.95, *i.e.*, 58% of that observed initially at *p*H 3.65. This experiment provides further confirmation that the molecular changes induced by alkali denaturation are essentially irreversible.

The decrease in R_{90}/c could be followed kinetically by using cacodylic acid as the titrating buffer. A preliminary report of these results has appeared.[8] The rate of decrease in scatter (*p*H 6.42 in 0.136 M KNO_3–0.023 M cacodylic acid) was identical to the rate of formation of acid-insoluble pepsin and the net change in R_{90}/c was in close agreement with the values reported above.[31]

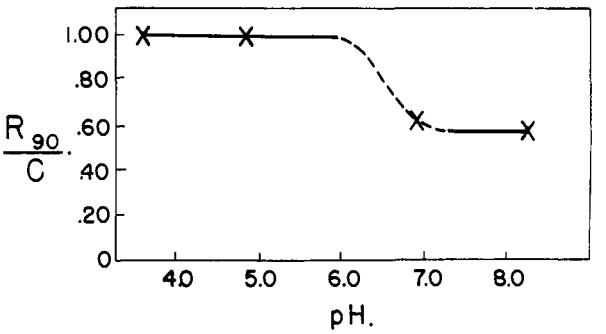

Fig. 7.—Turbidimetric–*p*H plot. The ordinate is the light scattering intensity of pepsin solutions relative to that of native pepsin. Glycine buffer (*p*H 10) was employed to adjust the *p*H. The dashed line indicates the approximate *p*H range of pepsin instability. Pepsin concentration ~0.35%; 0.05 M NaCl; $T \cong 25°$.

Electrophoresis.—Electrophoretic analysis was undertaken to provide additional information of the molecular composition of denatured pepsin. To explain the single boundary observed in ultracentrifugation required that the native molecule split either into 2 fragments of about the same mass or into a major residue and low molecular weight polypeptides fractions. If the former occurred the frictional ratios of the products would have to be close to 1.00 to account for the observed value of s. Both the diffusion and viscosity data refute this possibility, since they indicate a substantial increase in frictional coefficient in solutions of denatured pepsin.

If non-sedimentable low molecular weight polypeptides were produced on denaturation, then the light scattering data would require that 25–30% of the weight of native pepsin be liberated in this manner. The residue or core would then have to be highly asymmetric or swollen to be compatible with the viscosity and diffusion data.

In Fig. 8 an electrophoretic diagram of denatured pepsin is reproduced. At least three (and probably more) boundaries are formed on alkali denaturation of pepsin. The electrophoretic patterns of denatured pepsin solutions, from *p*H 6.5 to 9.6, are similar in appearance to those shown in Fig. 8. Analysis of the Rayleigh fringes pertaining to the various schlieren peaks indicated that the largest component (core) constituted about 75% of the total composition, with the faster moving com-

(29) H. Neurath, G. R. Cooper and J. O. Erickson, *J. Biol. Chem.*, **138**, 411 (1941).

(30) Quoted by Edsall in Table VIII, from chapter in, "The Proteins," by H. Neurath and K. Bailey, Vol. I, Part B, Academic Press, Inc., New York, N. Y., 1953.

(31) Considerable caution must be exercised when interpreting kinetic denaturation experiments with pepsin when any property other than enzyme activity is measured. It is well known that denatured pepsin is a very good substrate for native pepsin.[4] Thus in a kinetic experiment similar to the above, performed at *p*H 6.21 (0.136 M KNO_3–0.012 M cacodylic acid) the light scatter decreased regularly during a period of five hours. Simultaneous experiments failed to show any formation of denatured pepsin by the criterion of the formation of acid insoluble protein. Evaluation of the free amino groups by the ninhydrin method showed a sizable increase over control experiments. The rate of denaturation as determined from enzyme inactivation experiments was much smaller than indicated by light scattering. The light scattering effects therefore probably result from the proteolysis of denatured pepsin by the native enzyme which occurred as fast as the former was formed. This would account for the increase in ninhydrin value and the failure to detect acid insoluble denatured pepsin.

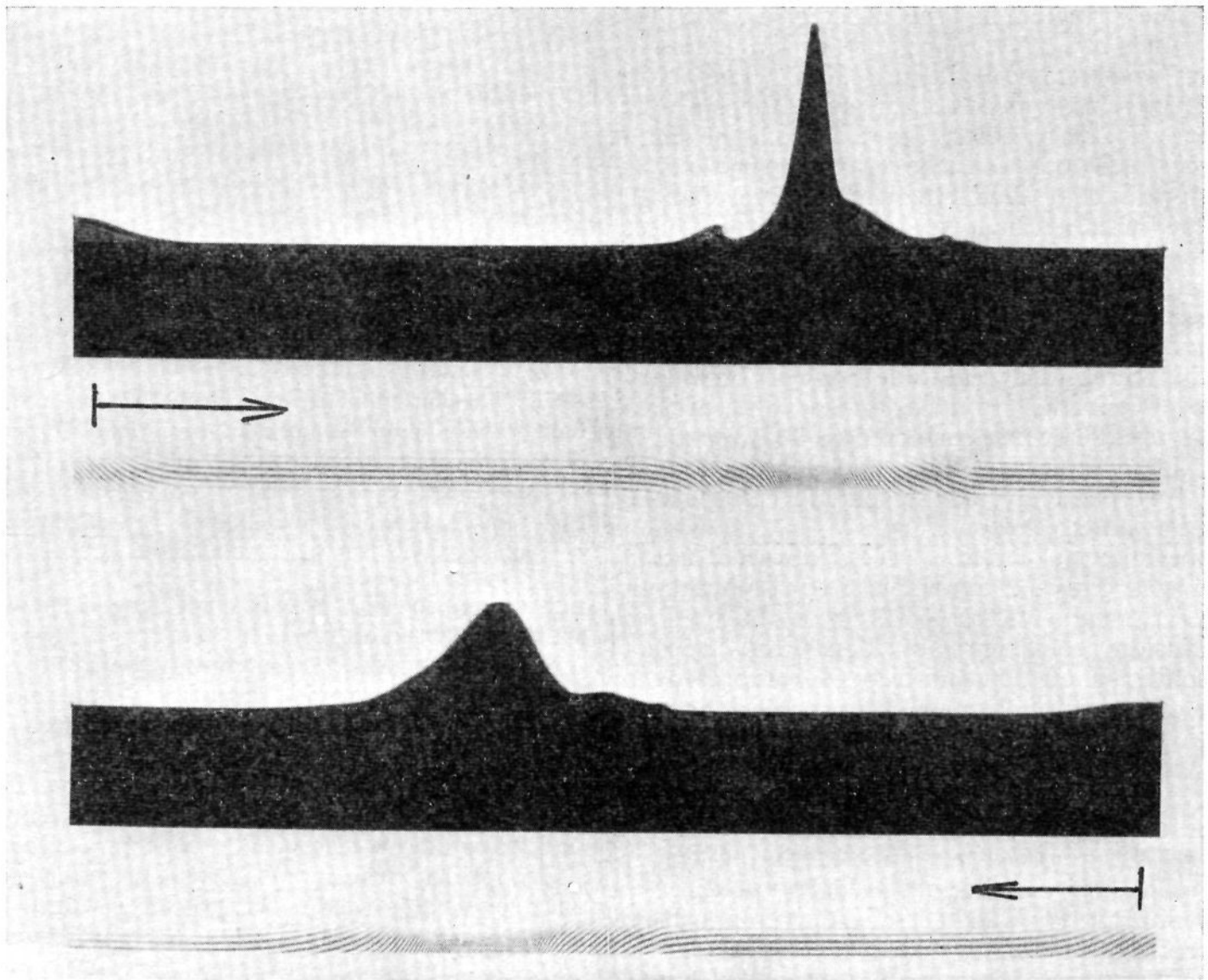

Fig. 8.—Electrophoretic schlieren and Rayleigh patterns of denatured pepsin. This preparation was isolated by precipitation with trichloroacetic acid mixture. It was dissolved in and then dialyzed against phosphate buffer *p*H 6.50, 0.10 Γ/2; pepsin concentration ≅ 1.00%; photographed at 158 minutes after start of experiment; $E = 5.8$ volts/cm. Descending mobility (lower section) of major peak was 8.12×10^{-4}.

ponent(s) containing about twice as much polypeptide as the slower moving component(s).

Native pepsin possessed only a single symmetrical boundary at all *p*H values observed. The mobilities of native pepsin and the principal component of denatured pepsin are plotted against *p*H in Fig. 9. The solid curve represents mobility data of native pepsin observed by Perlmann.[32] The open circles were obtained for native pepsin in the present investigation. The filled circles are the mobilities of the principal peak in solutions of denatured pepsin. At *p*H 5.5 (and 4.4) the electrophoretic diagrams showed a second diffuse boundary, comprising about 25% of the total composition, which moved at about $^2/_3$ of the rate of the main peak. This new boundary was not observed at *p*H values above 6.0 and presumably represents aggregated denatured pepsin.

Denatured pepsin at *p*H 8.5 in tris-(tris-hydroxymethyl-aminomethane) buffer migrated slower than at *p*H 7.5, in phosphate (Fig. 9). This reversal can be rationalized by assuming a small amount of binding of buffer cations at 8.5. The fall in mobility at *p*H 7.0 could reflect the neutralization of the 2 moles of histidine present in native pepsin.

The pronounced difference in slope in the mobility–*p*H curve, between native and denatured pepsin, in the *p*H range 5.0 to 6.0 is noteworthy and may be correlated with similar data from titration experiments.[33] The latter showed a shift to lower *pK* values of about 5–6 carboxyl groups which are hydrogen-bonded in the native molecule. In addition the *pK* values of all titratable groups are decreased due to a substantial reduction in the electrostatic free energy of ionization in denatured pepsin relative to the native form. The latter effect is a result of the large increase in frictional coefficient or the greater separation of charged centers in denatured pepsin.

Discussion

Molecular Properties of Native Pepsin.—The sedimentation and electrophoretic diagrams showed only a single symmetrical boundary with solutions of native pepsin. The electrophoretic mobilities agreed closely with the more extensive data reported by Perlmann.[32] The sedimentation data are in accord with earlier observations by Philpot and Eriksson-Quensel[3] and by Steinhardt.[34] A somewhat larger negative dependence of sedimentation on concentration was observed in 0.010 Γ/2 than in 0.100 Γ/2 buffer. Linear relationships were observed in the concentration range studied

(32) G. E. Perlmann, chapter in "Advances in Protein Chemistry," Vol. 10, Academic Press, Inc., New York, N. Y., 1955.

(33) Paper II in this series; to be published.

(34) J. Steinhardt, *J. Biol. Chem.*, **122**, 371 (1938).

and both lines extrapolated to a common intercept at infinite dilution, *i.e.*, $s^0_{20,w} = 3.20 \times 10^{-13}$ unit.

In the diffusion measurements the concentration of pepsin used was smaller than heretofore practicable with the older methods of boundary analysis. The Rayleigh interference fringe method of analysis of concentration gradients permits considerably greater precision in evaluating diffusion coefficients.[14] Full advantage of the precision inherent in this method could not be taken with pepsin preparations, since solutions of native pepsin could not be obtained free of split products. In addition, a small amount of autolysis probably occurs, during the long period of diffusion, in native pepsin solutions in which the split product content has been reduced substantially by dialysis. These factors tend to reduce the precision of the measurements. The value of 8.71×10^{-7} for $D_{20,w}$, observed in experiment 1 (Table II) is in agreement with earlier reports and will be used for characterization of the molecular properties of native pepsin.

Earlier estimates of the molecular weight from sedimentation and diffusion data were based on an assumed value of $\bar{v}$ of 0.75. In this way a value of 35,500 may be calculated by using $s = 3.3 \times 10^{-13}$, and Polson's value of 9.0×10^{-7} for the diffusion coefficient. With the present data a value of 35.7×10^3 was obtained for the molecular weight from the Svedberg equation. This value is well in the range of values obtained by light scattering (37,600,[21] 34,800[20]) and osmotic pressure (35,000) methods[35] and from the amino acid composition (34,400).[36] Due to the limitations inherent in pepsin preparations, the most reliable value of molecular weight is probably that obtained from the amino acid composition (or phosphorus content).

The combination of sedimentation and diffusion values may be employed to give information of the deviation of the shape of native pepsin from that of an anhydrous sphere. A value of the frictional ratio (f/f_0)[10] of 1.11 was obtained. This leads to an axial ratio of 3.0 from the Perrin equation[38] if the molecule is considered as an unhydrated prolate ellipsoid of revolution. Smaller values of the axial ratio result if hydration effects are assumed.

The viscosity data may be interpreted in an analogous manner by use of the Simha equation[39] (for prolate ellipsoidal particles). Again, if we attribute values of ν, $(100[\eta]/\bar{v})$, larger than the Einstein coefficient, 2.5, to asymmetry in shape only, a value of 3.9 for the axial ratio was obtained. A molecular weight of 36.7×10^3 may be calculated from combined sedimentation and viscosity data if we utilize the Perrin equation for evaluation of the frictional ratio from the axial ratio cited above (*cf.* eq. 2 that follows). This method of evaluating M is relatively independent of assumed hydration effects since the frictional ratio does not change very much with reasonable values for the degree of hydration.

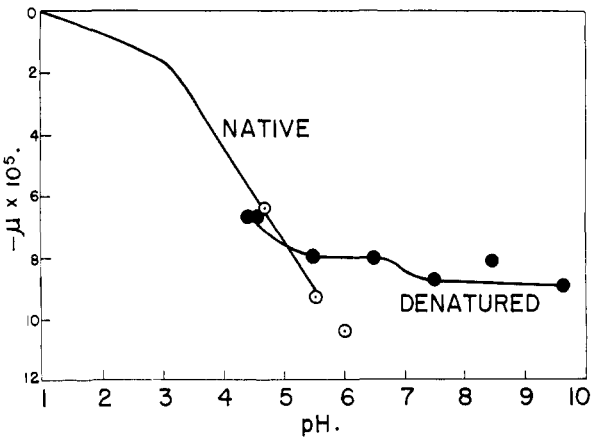

Fig. 9.—Electrophoretic mobilities of native and denatured pepsin. Line without points reproduced from the data of Perlmann.[33] ⊙, Descending mobilities of native pepsin at 0.10 ionic strength: buffers; *p*H 4.70 and 5.52, acetate; *p*H 6.0, phosphate. ●, Descending mobilities of the main component of denatured pepsin at 0.10 ionic strength: buffers; *p*H 4.42, 4.70 and 5.47, acetate; *p*H 6.50 and 7.50, phosphate; *p*H 8.47, 0.02 *M* tris–0.08 *M* NaCl; *p*H 9.64, 0.02 *M* glycine–0.08 *M* NaCl.

Molecular Changes Involved in Pepsin Denaturation.—It is apparent from Fig. 8 that the electrophoretic pattern of denatured pepsin is considerably more complex than that of native pepsin. At least three components are distinctly evident. The middle boundary, which forms the principal component in the diagram, represents about 75% of the total composition. This figure was arrived at from the relative number of interference fringes under the various schlieren boundaries. If this component results from the molecular dissolution of native pepsin and is unencumbered by further reactivity, it should have a molecular weight of about 0.75 (35,700) = 26,800. The molecular weights of the faster and slower moving components would be about 6000 and 3000, or smaller if a larger number of boundaries were formed. The appearance of only a single boundary in the ultracentrifuge in solutions of denatured pepsin can now be explained on the basis that the low molecular weight components are not readily sedimentable and do not form an observable boundary. The light scattering results are also quite compatible with the electrophoretic diagnosis of molecular fragmentation and indicate that no time-dependent aggregation occurs after the molecular breakdown.

Herriott and Van Vunakis[37] have postulated that pepsin is a single polypeptide chain, based on evidence that pepsin possesses only a single terminal amino and carboxyl group. Williamson and Passman[40] are in minor disagreement and find one free amino and two free carboxyl groups. If the pepsin preparations used by these investigators

(35) J. H. Northrop, *J. Gen. Physiol.*, **13**, 767 (1930).

(36) E. Brand, quoted in "Crystalline Enzymes," by J. H. Northrop, M. Kunitz and R. M. Herriott, Columbia Univ. Press, New York, N. Y., 1948, see also R. M. Herriott, ref. 37.

(37) R. M. Herriott, in "A Symposium on the Mechanism of Enzyme Action," edited by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md., 1954.

(38) F. Perrin, *J. phys. radium* [7], **7**, 1 (1936).

(39) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(40) M. B. Williamson and J. M. Passman, *J. Biol. Chem.*, **199**, 121 (1952); *Biochim. Biophys. Acta*, **15**, 246 (1954). These results are undoubtedly for denatured pepsin, though their method of inactivation depended on thier subsequent method of terminal group analysis.

coincide with our denatured solutions then all the molecular species observed by electrophoresis, save one, are cyclic polypeptides without free terminal groups, or with one free carboxyl group, which would explain Williamson and Passman's data.

Reversibility.—The denaturation of pepsin, as disclosed by the physical chemical procedures presently employed, is an irreversible process, within the time limits investigated. This is not surprising if we consider the extensive changes in composition and configuration that occur. It is of interest to find that a small percentage of activity (<1%) may be recovered after denatured solutions were acidified to *p*H 5.4 and allowed to stand for 24–48 hours.[23]

Molecular Properties of Denatured Pepsin.—In view of the molecular inhomogeneity of solutions of denatured pepsin, the data are not amenable to quantitative evaluation. However, the experimental results probably reflect to a large extent the properties of the principal component and are therefore worth examining in some detail.

The molecular weight of the main component of denatured pepsin may be estimated from the sedimentation and diffusion data listed in Table I. The computed value of 30,700 is approximate since the measured diffusion coefficient is an average value for all the non-dialyzable fractions present. Moreover, the concentration dependence of diffusion may not be negligible for a molecule with the moderately large frictional ratio found for denatured pepsin although the diffusion boundary did not show any evidence of being non-symmetrical. However, it is not without significance that this value of M is only slightly greater than that deduced from measurements obtained by the totally unrelated procedures of electrophoresis and light scattering.

The most interesting feature of denatured pepsin was the marked flexibility of its molecular shape, noticeably resembling the behavior of many linear polyelectrolytes.[26-28] This effect was most apparent in the large (and reversible) variations in viscosity produced by concentration changes in the ionic atmosphere of the denatured protein. At very low salt levels the molecular domain of denatured pepsin was significantly enlarged by strong electrostatic repulsions between charged loci along the polypeptide chain; increasing the salt concentration reduced the electric field about the charged groups and resulted in a uniform decline in molecular dimensions, as revealed by the viscosity changes.

In the absence of extraneous salt, the intrinsic viscosity of denatured pepsin at *p*H 8.5 was $\sim$ 0.9 dl./g., as determined from a Fuoss type of plot. This result may be compared with the data reported by Oth and Doty[41] on a sample of polymethacrylic acid of M = 43,100. They found the intrinsic viscosity equal to 0.465 and 2.27 for 8 and 20% neutralized polymer. Native pepsin contains 37 acidic and 6 basic amino acid residues (as well as 1 terminal group of each kind) out of a total of 306 amino acids.[36,37] Practically all of the dissociable groups are ionized under the experimental conditions cited above. If denatured pepsin (*i.e.*, the principal component) possesses the same ratio of charged to total groups then about one group in ten is ionized. From this analogy we are tempted to conclude that the viscometric behavior of denatured pepsin is indeed similar to that of linear polyelectrolytes and is to be differentiated from the swelling that characterizes the molecular inflation that occurs in acid solutions of serum albumin[42,43] or urea solutions of serum albumin[44,45] or fibrinogen.[46]

Most macromolecular studies on denatured proteins have shown them to be characterized by increased viscosity and decreased frictional coefficient when compared to their native forms.[47,48] Generally, these observations were explained by postulating an increase in molecular asymmetry or axial ratio of a hypothetical rigid ellipsoid of revolution which was presumed to represent the dominant configurational features of the protein molecule in either its compact native state or its disorganized denatured state(s).[49]

Intrinsic viscosity and sedimentation data have been obtained for denatured pepsin solutions in phosphate buffer at 0.010 $\Gamma/2$ as well as at 0.100 $\Gamma/2$. Variation in ionic strength in solutions of polyampholytes furnishes the same possibilities of evaluating inter- and intra-molecular interactions as employing "good" or "poor" organic solvents to assess the behavior of synthetic organic polymers.

Scheraga and Mandelkern[44] have shown that the data of Neurath and Saum[47] on albumin in solutions of urea may be interpreted in terms of molecular swelling, with the albumin molecule retaining an effective spherical shape throughout a series of inflated stages. A similar interpretation has been arrived at from detailed measurements on urea solutions of fibrinogen by Scheraga,[46] *et al.* These conclusions were reached from considerations of the significance of values of β[44] computed from experimental values of $[\eta]$ and s (or $[\eta]$ and D). β is a measure of the shape of an "effective hydrodynamic particle" which they assumed to have the form of an ellipsoid of revolution. A similar parameter to β, identical in dimensions, has been shown by Mandelkern and Flory[50] to be a universal constant, equal to $2.5 \pm 0.1 \times 10^{-6}$, for flexible coil configurations of linear polymers.[51] β may be determined by equation 1 from combined viscosity and

(41) A. Oth and P. Doty, *J. Phys. Chem.*, **56**, 43 (1952).

(42) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954); C. Tanford, J. G. Buzzell, David G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

(43) G. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1633 (1956); W. F. Harrington, P. Johnson and R. H. Ottewill, *Biochem. J.*, **62**, 569 (1956).

(44) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

(45) P. Doty and S. Katz, cited by P. Doty and J. T. Edsall, "Advances in Protein Chemistry," Vol. VI, Academic Press, Inc., New York, N. Y., 1951.

(46) H. A. Scheraga, W. R. Carroll, L. F. Nims, E. Sutton, J. K. Backus and J. M. Saunders, *J. Polymer Sci.*, **XIV**, 427 (1954).

(47) H. Neurath and A. M. Saum, *J. Biol. Chem.*, **128**, 347 (1939).

(48) J. F. Foster and E. G. Samsa, THIS JOURNAL, **73**, 5388 (1951).

(49) For an excellent review, the chapter by F. Putnam in "The Proteins," by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, should be consulted.

(50) L. Mandelkern and P. J. Flory, *J. Chem. Phys.*, **20**, 212 (1952).

(51) L. Mandelkern, W. R. Krigbaum, H. A. Scheraga and P. J. Flory, *ibid.*, **20**, 1392 (1952).

sedimentation data in the same solvent

$$\beta = Ns[\eta]^{1/3}\eta_0/M^{2/3}(1 - \bar{v}\rho) \quad (1)$$

Values of β and V_e (effective volume of ellipsoid) for native and denatured pepsin are listed in Table III. A molecular weight of 30,700, as determined

TABLE III

MOLECULAR PARAMETERS OF PEPSIN FROM SEDIMENTATION AND VISCOSITY

		Native (*p*H 6.0)		Denatured (*p*H 7.0)	
	Γ/2	0.010	0.100	0.010	0.100
100 $[\eta]$		3.40	3.35	20.8	10.2
$s^0_{20,w} \times 10^{13}$		3.20	3.20	1.84	2.17
a/b[a]			3.9	16.1	9.9
f/f_0[b]			1.17	1.84	1.54
$M \times 10^{3}$[c]			36.7	31.4	30.9
$\beta \times 10^{6}$[d]			2.28	2.66	2.49
a/b[e]			6.0	19	13
$V_e \times 10^{15}$[e]			2.80	2.94	2.60
$M \times 10^{3}$[f]				33.8	30.5

[a] Simha equation, neglecting hydration effects. [b] Obtained from Perrin equation using viscosity data—neglecting hydration effects. [c] Obtained from eq. 2 in text. [d] Obtained from eq. 1 in text. [e] Assuming an "effective hydrodynamic ellipsoid." [f] Obtained from eq. 3 in text.

from s and D (Table I), was used in the computation of β for denatured pepsin. It should be noted that the precision of β is much less dependent on errors in viscosity than on sedimentation values because the cube root of $[\eta]$ is taken. This places the values of β on a relatively firmer basis since the s values are for the principal component only while the $[\eta]$ values are average values for all the components present. The results, insofar as they measure the physical properties of the major constituent, are quite conclusive in attributing the total change in the hydrodynamic behavior to that of the *shape* (a/b) of the effective ellipsoid with the volume remaining essentially unchanged. This type of behavior is exactly opposite from that undergone by horse albumin in urea solutions mentioned above. The values of the axial ratio a/b of the equivalent ellipsoid increase from 6.0 in native pepsin to 13 in 0.100 Γ/2 and 19 in 0.010 Γ/2 denatured pepsin.

It is perhaps of some interest to note that fairly similar values of a/b, *i.e.*, 9.9 and 16.1, respectively, are obtained from the viscosity data by application of the Simha equation.[41] Since denatured pepsin is in solution only by virtue of its being ionized and appreciably solvated at *p*H 7.0, these axial ratios should be reduced accordingly. If frictional ratios are obtained from the Perrin equation[38] for the above axial ratios, then it is possible to calculate a molecular weight from eq. 2 by combining this information with the known sedimentation constants.

$$M = ((f/f_0)s)^{3/2}\frac{\bar{v}^{1/2}}{(1 - \bar{v}\rho)^{3/2}}\frac{1}{(1.19 \times 10^{-15})^{3/2}} \quad (2)$$

In this manner values of 31400 and 30900 were obtained for the molecular weight in solutions of 0.010 Γ/2 and 0.100 Γ/2. These values agree with that obtained from s and D.

Since denatured pepsin has been shown to exhibit random coil-like behavior, the β values listed in Table III should be considered also in terms of the Mandelkern–Flory theory. The latter assumes that the molecular chain may be described as an effective hydrodynamic sphere rather than an ellipsoid as was done by Scheraga and Mandelkern for proteins. The β values of denatured pepsin of 2.66 (0.01 Γ/2) and 2.49 (0.10 Γ/2) $\times 10^{-6}$ are well within the range found for a number of linear polymers of quite different composition in a variety of solvents.[51] The molecular weight may also be calculated by eq. 3 from $[\eta]$ and s, if we accept $\Phi^{1/3}P^{-1}$ as a universal constant equal to 2.5×10^{6}

$$M = [\eta]^{1/2}s^{3/2}\eta_0 N/\Phi^{1/3}P^{-1}(1 - \bar{v}\rho) \quad (3)$$

By this means values of M of 33800 (0.010 Γ/2) and 30500 (0.100 Γ/2) are obtained. The latter is quite reasonable and in accord with the value obtained from s and D while the former may indicate a departure from random-coil behavior as a result of molecular expansion induced by electrostatic repulsions between charged chain segments.

Conclusions

The viscometric behavior of denatured pepsin parallels that observed with many types of linear polyelectrolytes. This observation automatically precludes the concept of an ellipsoid of revolution, of either a real or an equivalent hydrodynamic particle, as representing the molecular configuration of denatured pepsin. However, the equations based on these two models do provide what appear to be realistic molecular parameters of denatured pepsin.

It is also very likely that permanent cross-links occur between chain elements through disulfide or phosphorus bridges known to be present in native pepsin. However, it is not known in which fraction of denatured pepsin they occur. In addition, intramolecular "hydrophobic bonding" between non-polar residues probably occurs, as suggested by the viscometric data in alcohol–water mixtures. Both factors would limit the rotational flexibility of the molecule and tend to preserve various features of the ellipsoidal model. In 0.100 Γ/2 where intramolecular repulsions are small, the possibility of forming "hydrophobic bonds" is substantially increased and the shape of the molecule may well resemble that of an ellipsoid either real or effective, though undoubtedly not a very rigid one. Since all three hypotheses that have been tested lead to results that are not unreasonable, the conclusion appears inescapable that either the equations are not very discriminating between different models in the molecular weight range under investigation or to put it in another way, the molecular configuration of denatured pepsin bears certain resemblances to all the theoretical models.

It is also quite possible that the viscosity measurements (and diffusion coefficient) on denatured pepsin are unduly influenced by the low molecular weight polypeptides present. It is hoped that the major residue present in denatured pepsin may be isolated by suitable fractionation methods and studied separately. Perhaps with better values of $[\eta]$ and M derived with this component a more significant choice may be made between the various models that have been considered.

KANSAS CITY, KANSAS