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The Behavior of a Bacterial Polypeptide as a Polyelectrolyte

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The principal purpose of this study was to compare the properties of a naturally occurring polyelectrolyte containing the peptide group as part of its backbone structure with other polyelectrolytes. Light scattering and viscosity studies have been carried out on the charged and uncharged forms of γ-D-glutamyl polypeptide (M = 89,000) as a function of polymer concentration. The molecule shows fairly typical polyelectrolyte behavior. The principal effect of the peptide group is to enlarge the molecular domain of the charged form. This is shown mainly in the greater resistance to collapse offered by Na glutamyl polypeptide in the presence of NaCl when compared with other polyions. The configuration of the un-ionized form of the molecule shows unperturbed random-coil behavior which would appear to preclude appreciable intramolecular hydrogen bonding. In addition, the properties of glutamyl polypeptide, as a function of its degree of neutralization, have been measured by viscosity, potentiometry and electrophoresis. The viscosity results show the effect of the rather large separation of charge (every sixth atom along chain) since the molecule does not commence to expand until it is about 10% ionized and then stretches rather uniformly with ionization until completely neutralized. The electrical potential of the molecule, as determined from titration and electrophoresis, increases linearly with degree of ionization, essentially over the whole range of ionization.

The capsular material of *Bacillus anthracis* has been obtained by chemical procedures following autoclaving of the bacteria, in the form of a substance of high molecular weight which upon hydrolysis yields only *d*(−)-glutamic acid[2] and which is generally agreed to be a *d*-glutamyl polypeptide. It seems also likely, on chemical,[3,4] physical[4] and enzymological[5] grounds, that the glutamyl peptide units are exclusively of the γ-type. The absence of α-linkages also would eliminate any obvious possibility of a branched chain. Thus, we probably are dealing with a linear polyelectrolyte, the special interest of which lies in the fact that it possesses both the simple repeating structure characteristic of such polyelectrolytes as polyacrylic acid and also, in its simplest form, the peptide residue. This appeared to warrant the survey of physicochemical behavior reported in this paper.

Few comparable studies have appeared in the literature. Hanby and Rydon[2] deduced the thread-like character of the capsular polypeptide molecule from the high viscosity of its solutions; Waley[4] reported on the basis of titration data, in apparent disagreement with similar data given by Hanby and Rydon,[2] that some interaction between the charged groups persists even in presence of N KCl.

(1) National Institutes of Health, Bethesda, Md. Work aided in part by an Institutional Grant from the American Cancer Society.

(2) W. E. Hanby and H. N. Rydon, *Biochem. J.*, **40**, 297 (1946).

(3) V. Bruckner, J. Kovacs and G. Denes, *Nature (London)*, **172**, 508 (1953).

(4) S. G. Waley, *J. Chem. Soc.*, 517 (1955).

(5) W. J. Williams and C. B. Thorne, *J. Biol. Chem.*, **210**, 203 (1954); **211**, 631 (1954); **212**, 427 (1955).

Experimental

Capsular Polypeptide Preparations.—All data refer to preparation 50 unless otherwise noted. Preparation 50 was produced by agar cultures of *B. anthracis* strain M-36, a Vollum strain subjected to 36 serial passages in monkeys. The Hanby and Rydon procedure[2] was used up to the alcohol–NaOH precipitation at *p*H 7–8. The precipitate was dissolved in dilute H_2SO_4, *p*H about 5, centrifuged and clarified by filtering through Celite 545. It was dialyzed for 66 hours at 6° against running distilled water, passed through a Seitz filter and lyophilized.

Solutions were prepared by weighing samples of polypeptide and bringing to volume with standard volumetric equipment. The amount of water in polypeptide stock samples was determined by drying to constant weight at 100° under vacuum. From the titration results the stock sample of preparation 50 is composed of 90% of the sodium salt of glutamyl polypeptide (NaGP) and 10% of the acid form (HGP). Unless stated otherwise all measurements made with ionized glutamyl polypeptide (NaGP) will refer to this 90% neutralized preparation. The concentrations used in the light scattering and viscosity of HGP have been reduced appropriately to account for the conversion of GP from the sodium salt to the acid form. Measurements with the 90% ionized stock refer to the weighed amount of the sample. In the data where the degree of ionization varies,

i.e., viscosity, titration and surface tension, the concentration refers to the weight of stock sample only.

Light Scattering Measurements.—The assembly was based on that of Brice, Halwer and Speiser[6,7] with the following modifications: (a) the galvanometer used to measure the photomultiplier tube output was replaced by a Leeds & Northrop No. 9836 stabilized DC microampere amplifier; (b) additional masks and knife edge slits were inserted in the incident light reducing the width of the incident beam to 0.11 cm. The instrument was calibrated with the opal glass standard recommended by Brice, *et al.*,[6] as well as by two organic solvents, *i.e.*, benzene and toluene.

The turbidity cells (for 90° scattering) were 1 cm. square cuvettes, optically clear on all four sides. For measurement of angular dependence a rectangular cell provided with a cylindrical pocket for solvent was used[8]; the back face and all edges were coated with a dull black lacquer. Small dissymmetries referable to imperfections in the cell or the optical system were corrected for by calibration with a fluorescein solution. The values of $i_\theta \sin\theta$ were constant to within 3% over the range 37 to 135° (i_θ = intensity measured at angle θ to forward direction).

All solvents were prepared with thrice distilled water; solutions were passed through an ultra-fine sintered glass filter, followed by centrifuging at high speed for removal of traces of dust.

The procedure was checked by measurements with solutions of crystalline bovine serum albumin (Armour); the dissymmetry in 0.1 *M* acetate buffer, *p*H 5.3 from 25.8 to 126.8° was 1.00 ± 0.02. The molecular weight compared favorably with recorded values determined by similar methods.[9] The turbidity data were corrected for internal interference. The 90° scattering function Kc/R_{90} was corrected by the factor $P(90)$ derived from the dissymmetry, $z(=i_{45}/i_{135})$ by use of tables given in reference 10.

In the above expressions $K = 2\pi^2 n_0^2 (dn/dc)^2/N\lambda^4$ where n_0 is the refractive index of solvent, c, the concentration in g./ml., N, the Avogadro constant, 6.02×10^{23}, λ, the wave length of light *in vacuo* 4358 Å., and R_{90}, the reduced intensity, $i_{90}r^2/I_0$, calculated from the 90° scattered intensity and various calibration data.[6]

Refractive Index Increments.—The refractive index increments of aqueous solutions of polypeptide were determined at 4358 Å. with the Brice differential refractometer[11] using the apparatus constant calculated by the manufacturer.

Viscosity.—Solution viscosity was measured in Ostwald viscosimeters held in a fixed position in a water-bath kept at 25.00 ± 0.02°. The flow times for water were ~2 minutes at 25°. Kinetic energy corrections were determined but were unimportant. Rates of shear were varied by applying pressure with a column of dibutyl phthalate. No effect of shear rate was observed with NaGP at very low salt concentrations and average velocity gradients of 3000 to 650 sec.$^{-1}$

Potentiometric Titrations.—A water-jacketed beaker served as the titration vessel. Constant temperature was maintained by circulating water from a constant temperature bath through the water jacket. All runs were performed at 25.0 ± 0.1°. A Beckman *p*H meter, model GS, was used to measure *p*H. Standard buffers[12] were used to calibrate the instrument; from *p*H 2.11 to 9.18 the *p*H values were within 0.02 unit of those of the standards. No corrections were applied to the observed values. Solutions of GP in NaCl (4 ml.) were brought to *p*H 7.5 with NaOH and titrated from a microburet with 0.39 *M* HCl. Solutions were mixed by magnetic stirrers. Bound H^+ was determined as the difference between the amount of acid added to the solution and to the solvent; compensation was made for the small difference of volume between solution and solvent.

The potentiometric properties of GP and other polyelectrolytes depend rather markedly on concentration. Measurements should be made therefore in very dilute solutions. The titration curve of GP extends to *p*H ~ 2, where solvent corrections constitute an appreciable fraction of the added acid. Precise values of the degree of dissociation, in the very early stages of ionization, were therefore difficult to obtain.

Moving Boundary Electrophoresis.—The Klett apparatus was used. Solutions were prepared by dissolving GP in buffer and dialyzing for at least 24 hours at 5° against 4 liters of buffer. Boundaries were photographed with the Longsworth scanning procedure. A single sharp boundary was observed in all cases. The maximum ordinates of the boundaries were found to represent the displaced boundaries quite adequately. In all runs the areas were constant and the peaks moved with constant velocity. Conductivities were measured in the electrophoresis bath at 1.3°. Mobilities were calculated by routine methods.[13] All solutions were adjusted to ionic strength 0.125, excluding the contribution of the polyion, 0.10 contributed by NaCl (except at *p*H 1.8) and the remainder by the buffer.

Electrophoresis of Polypeptide Coated Pyrex Particles.—The microscope method of electrophoresis was used with the flat rectangular electrophoresis chamber in the "lateral" orientation.[14] Agglomeration of particles caused some difficulty.

Sedimentation.—Sedimentation analyses were done with the Spinco analytical ultracentrifuge Model E at room temperature; data were not corrected for changes in rotor temperature during accleration[15] but were adjusted to 20° by correcting for solvent viscosity. At *p*H 4.8 in 0.20 *M* NaCl the patterns showed a single sharp boundary the position of which, distance x from center of rotation, was sufficiently defined by the position of the maximum ordinate. Sedimentation constants, s, were calculated by plotting ln x against time, t, since $s = \Delta \ln x/\Delta t\omega^2$ where ω is angular acceleration.

Surface Tension.—Surface tensions were measured by the ring method, a commercial du Noüy tensiometer being modified in order to allow the solution to be enclosed in a glass jacket at constant temperature.[16] Instrument readings, P, were converted into surface tensions, σ, using calibration data plotted according to the empirical equation[17] $\sigma/\rho = a(P/\rho) - b$, where ρ is solution density and a and b are constants.

Results

Light Scattering and Refractive Index Increment.—The results of light scattering measurements on un-ionized and 90% ionized polypeptide are given in Figs. 1 and 2. The values of the scattering function $KcP(90)/R_{90}$ at *p*H 4.9 in 0.1 *M* NaCl are based on a refractive index increment, dn/dc, for sodium polyglutamate of 0.20 ml./g.

The plot of $KcP(90)/R_{90}$ against c permits the molecular weight to be estimated. At *p*H 4.9 in presence of 0.1 *M* NaCl, the second virial coefficient B (defined by $KcP(90)/R_{90} = 1/M + 2Bc$) has a small positive value equal to 2.5×10^{-4} mole cc./g.^{2}, while the intercept, 1.12×10^{-5}, gives M = 89000. The dissymmetries under these conditions are considerably greater than those observed at *p*H 2.5, with an intrinsic dissymmetry, [z], of 1.27.

In the case of the solutions at *p*H 2.5, the relationship is linear, with zero or very slightly negative slope while the intercept for solutions with or without added salt is 0.90×10^{-5}. Since the second

(6) B. A. Brice, M. Halwer and B. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

(7) Brice-Phoenix Light Scattering Photometer: Phoenix Precision Instrument Co. Bulletin B-P1000.

(8) Pyrocell Manufacturing Co., 207–211 E. 84th St., New York 28, New York.

(9) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(10) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **18**, 1211 (1950).

(11) B. A. Brice and M. Halwer, *J. Opt. Soc. Amer.*, **41**, 1033 (1951).

(12) R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).

(13) R. A. Alberty, *J. Chem. Ed.*, **25**, 426, 619 (1948).

(14) R. S. Hartman, J. B. Bateman and M. A. Lauffer, *Arch. Biochem. Biophys.*, **39**, 56 (1952).

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

(16) W. D. Harkins and H. F. Jordan, *ibid.*, **52**, 1751 (1930).

(17) D. G. Dervichian and C. Clark, *Compt. rend. Acad. Sci. Paris*, **207**, 277 (1938).

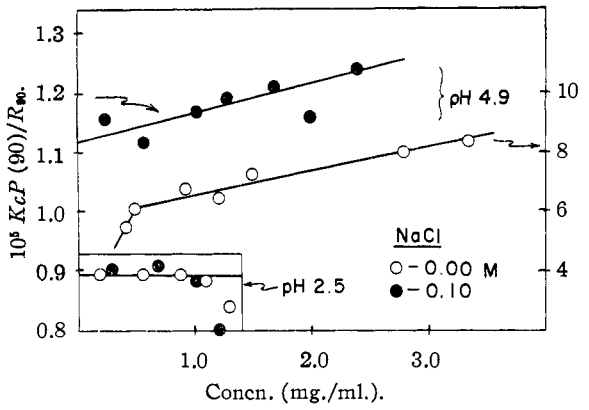

Fig. 1.—Light scattering by solutions of glutamyl polypeptide at *p*H 2.5 (small insert) and 4.9 without added salt (open circles) and in 0.10 *M* NaCl (closed circles). Right ordinate applies only to *p*H 4.9 data in the absence of NaCl.

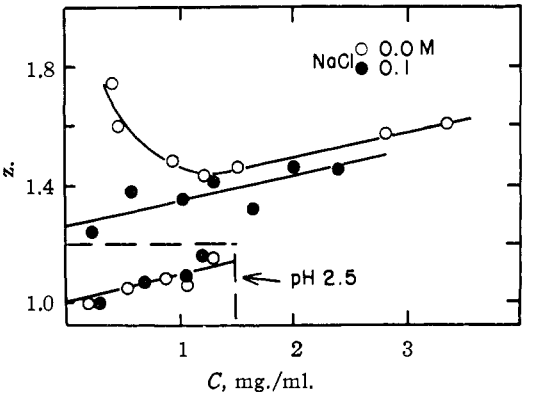

Fig. 2.—The dissymmetry of light scattering ($z = R_{45}/R_{135}$) corresponding to the 90° scattering data given in Fig. 1.

virial coefficient is close to zero the solutions can be considered thermodynamically ideal. The corresponding dissymmetry data (Fig. 2) also are unchanged by addition of neutral salt. The slope is positive and constant, while the intercept is not significantly different from unity.

In absence of small ions other than the counterions, the transparency of the solutions at *p*H 4.9 becomes 5 to 10 times greater than in presence of 0.1 *M* NaCl (Fig. 1). The corresponding dissymmetries are only slightly greater, at concentrations above 1.2 mg./ml., than in the presence of 0.1 *M* NaCl, but below 1.2 mg./ml. the dissymmetry increases sharply with dilution, with $[z]$ equal to about 2.0 when estimated by plotting $1/(z - 1)$ against c.

Viscosity.—The reduced specific viscosity, η_{sp}/c, of HGP appears to be independent of concentration from 0.10 to 0.35 g./dl. and is not affected by 0.10 *M* NaCl (Fig. 3). The intrinsic viscosity is close to 0.25 dl./g. under these conditions.

When GP is highly ionized and only counterions are present, pronounced upward curvature is shown at concentrations below 0.15 g./dl. The data conform closely to the equation of Fuoss.[18] If D is

$$\eta_{sp}/c = D + A/(1 + Bc^{1/2})$$

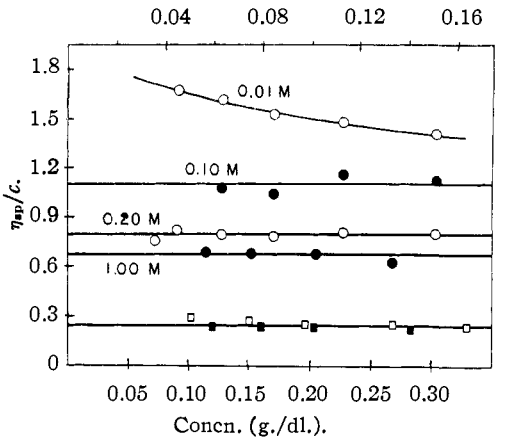

Fig. 3.—Circles: variation of reduced specific viscosity with NaGP ($\alpha = 0.90$) concentration at four levels of NaCl concentration; $T = 28.00°$. Squares: reduced specific viscosity of HGP: closed, without NaCl; open, with 0.10 *M* NaCl. Upper abscissae refers to NaGP; lower to HGP. $T = 25.00°$.

considered to be the value of the reduced specific viscosity of HGP (0.25), then the constants of this equation, determined from the plot of the data shown in Fig. 4, are $A = 125$ dl./g. and $B = 40$. Addition of simple electrolyte tends to reduce the concave portion of the curve and to displace it to

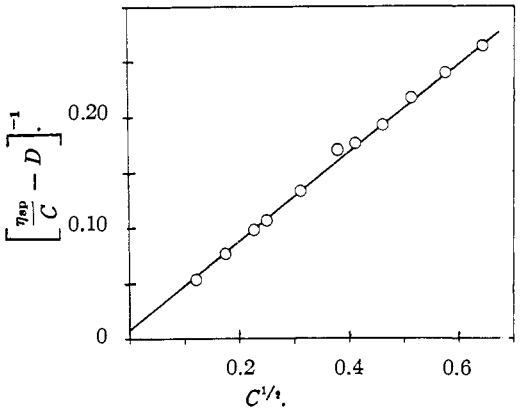

Fig. 4.—Fuoss plot of viscosity data in the absence of added salt; concentration in g./dl.; *p*H 5.2; $\alpha = 0.95$; $T = 25.00°$.

lower concentrations, eventually eliminating it entirely; this phenomenon is well known, and has been reported with many types of polyelectrolytes, *e.g.*, Na pectinate,[19] polyvinyl-N-butylpyridinium bromide[18] and polyacrylate.[20] The effect of NaCl on the reduced viscosity of NaGP is shown also in Fig. 3. Increasing concentrations of NaCl serve to depress the intrinsic viscosity, but even 1.00 *M* NaCl fails to reduce the size of NaGP to that of its unperturbed state, as in HGP. These results differ markedly from those of Schneider and Doty[21] on Na carboxymethylcellulose where 1 *M* NaCl sufficed to reduce the viscosity to within 25% of its value when

(18) 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; *cf.* R. M. Fuoss and U. P. Strauss, *Ann. N. Y. Acad. Sci.*, **51**, 836 (1949).

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

(20) W. Kern, *Z. physik. Chem.*, **181**, 283 (1938).

(21) N. S. Schneider and P. Doty, *J. Phys. Chem.*, **58**, 762 (1954).

un-ionized. Flory and Osterheld[22] reported similar behavior with Na polyacrylate. Unpublished experiments[23] show that whereas NaCl is relatively ineffective, the viscosity of NaGP is reduced to that of HGP by 0.1 M $BaCl_2$.

The variation of viscosity with degree of ionization has been measured over a 100-fold range in NaCl[24] (Fig. 5). The curves are similar in shape and

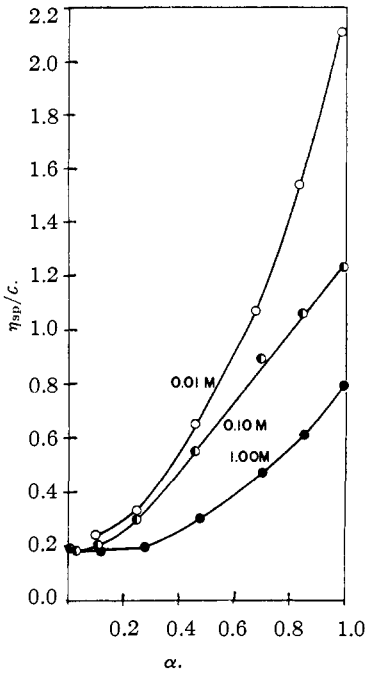

Fig. 5.—Plot of reduced specific viscosity as a function of the degree of ionization at three concentrations of NaCl. Concentration of NaGP = 0.164% when α = 1.00 and 0.156% when $\alpha \cong 0.10$; T = 25.00.°

slightly concave to the α-axis. The strong dependence of viscosity on ionization is apparent at all stages of neutralization above α = 0.1 at the two lower levels of NaCl and α = 0.3 in 1.00 M NaCl.

Electrophoresis.—The results from the moving boundary and microscope methods are given in Table I. The data for dissolved polypeptide are in agreement with similar data obtained by Watson, *et al.*,[25] with 1% solutions of capsular polypeptide of *B. subtilis* and with preparations of "inflammatory factor" from anthrax lesions.[26] The small maximum at *p*H 6.6 in the *p*H–mobility curve probably reflects minor differences in cation binding (Na^+ or buffer ions).

Electrokinetic potentials (ζ) have been calculated by the equation $\zeta = 300\ \eta\mu C/D$ (Table I) where η is the viscosity of the solvent, D, the dielectric constant of water and C, a proportionality constant. We have set C equal to 6 π for dissolved GP and 4 π when adsorbed on Pyrex particles.[27] If we plot $0.43\ \epsilon\zeta/kT$ *versus* α for dissolved GP, the electrical potential varies linearly with α from $\alpha \cong$ 0.03 to 1.00 and has a slope of 0.55. Below $\alpha \cong$ 0.03 the curve must fall off steeply in order to pass through the origin when $\alpha \cong 0$ (Fig. 6).

TABLE I

MOBILITIES AND ELECTROKINETIC POTENTIALS OF DISSOLVED AND ADSORBED GP

Moving boundary electrophoresis: ionic strength — 0.125, of which 0.100 M contributed by NaCl and 0.025 by buffer. Experiments at *p*H 1.80 contained only indicated buffer ions. GP concentration varied between 1.3 and 1.6 mg./ml. Microelectrophoresis: buffer is composed of 0.10 M NaCl and 0.10 M glycine; Pyrex particles migrating in a 0.10 mg./ml. solution of GP. Titration values of GP in solution were used for evaluating α of GP, when adsorbed on Pyrex particles.

$\Gamma/2$	*p*H	α	Buffer	$-10^5\ \mu$ Asc.	$-10^5\ \mu$ Des.	$\frac{0.43\ \zeta^a}{kT}$
			Dissolved GP, 1.3°			
0.125	1.80		Glycine	1.00	0.80	0.049
	2.40	0.03	Glycine	5.77	5.58	.34
	3.50	0.33	Acetate	8.55	8.20	.49
	4.70	0.83	Acetate	13.92	12.75	.76
	6.58	1.00	Phosphate	17.09	15.42	.93
	8.02	1.00	Veronal	15.69	14.21	.85
	9.70	1.00	Glycine	15.63	14.55	.87
			Adsorbed GP, 25°			
0.10 NaCl	1.85		Glycine	>0		
	2.29	0.02	Glycine	1.8		0.044
	3.00	0.18	Glycine	10.3		0.25
	3.52	0.33	Glycine	16.0		0.39

[a] Calculated with text equation, using D = 87 for moving boundary data (descending boundary) and 78 for microscope method. T = 298.1° K.

These changes in electrical potential may be interpreted qualitatively in terms of the configurational changes that take place in the structure of GP as it is neutralized. In the initial stages of ionization, GP remains compact so that the electrokinetic potential rises extremely rapidly. Thereafter, the molecular volume increases on account of intramolecular electrostatic repulsions and consequently ζ increases much less rapidly (Fig. 6). When GP is adsorbed on Pyrex particles the size is stabilized and ζ increases linearly with the degree of ionization (Fig. 6).

Potentiometric Titrations.—The general form of the potentiometric equation, as applied to polymeric electrolytes, may be expressed as[28]

$$pH + \log \frac{1-\alpha}{\alpha} = pK_0 + \frac{0.43\ \epsilon\psi}{kT} \qquad (1)$$

where ψ is the electrical potential of the polyion, pK_0, the dissociation constant and ϵ the electronic charge.

(22) P. J. Flory and J. E. Osterheld, *J. Phys. Chem.*, **58**, 653 (1954).

(23) Unpublished experiments of H. Edelhoch.

(24) Since the dimensions of the un-ionized (h_0 = 220 Å.) and ionized (h = 720 Å.) forms of GP are available (see Table IV), the viscosity $-h$ relationship of the theory of H. Kuhn (*J. Colloid Sci.*, **5**, 331 (1950)) may be tested. Using values of b = 3.09 Å., d_h = 2.37 Å. and Z = 1400, the calculated intrinsic viscosity is 0.36 and 8.7 for HGP and NaGP, respectively. The experimental values are 0.25 and 1.14 dl./g. (see Fig. 3).

(25) D. W. Watson, W. J. Cromartie, W. L. Bloom, R. J. Heckley, W. J. McGhee and N. Weissman, *J. Infectious Dis.*, **80**, 121 (1947).

(26) W. L. Bloom and F. G. Blake, *J. Infectious Dis.*, **83**, 116 (1948); W. L. Bloom, *et al.*, *ibid.*, **80**, 41 (1947).

(27) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publishing Corp., New York, N. Y., 1942.

(28) G. S. Hartley and J. W. Roe, *Trans. Faraday Soc.*, **36**, 101 (1940).

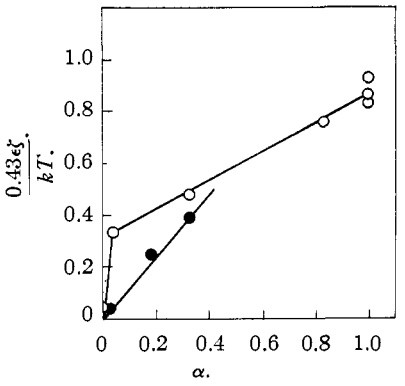

Fig. 6.—Variation in electrokinetic potentials with degree of neutralization. Open circles, ζ-potentials derived from descending electrophoretic mobility data of dissolved GP at 1.3°. Closed circles, potentials of GP coated Pyrex particles, measured at 25° and adjusted for solvent viscosity to 1.3°.

Potentiometric data may be analyzed by use of eq. 1 provided ion binding effects[29] are negligible and solutions are sufficiently dilute to approach ideal behavior. Neither of these conditions can be realized completely nor are the necessary correction factors known in the present case. The above factors are minimal in the first stages of neutralization. Unfortunately this happens to occur in the pH range least accessible to precise potentiometric evaluation with GP (*i.e.*, pH 2.0–2.5).

The electrical potential is related to the electrostatic free energy (F_5, *cf.* ref. 29) by $Ze\psi = \partial F/\partial\alpha$[30,31] where Z is the charge of the polymer when completely ionized. If the term dependent on the ionic strength is abstracted from F, eq. 1 may be rewritten as[32]

$$pK' = p\mathrm{H} + \log\frac{1-\alpha}{\alpha} = pK_0 - \frac{0.43e^2\kappa}{3DkT} + \frac{0.43}{kT}\left(\frac{\partial F}{\partial\nu}\right) \quad (2)$$

where $\nu = \alpha Z$ and κ is the reciprocal Debye radius.

The potentiometric behavior of aqueous solutions of GP, at 0.010 M monomer concentration, over a 100-fold variation in NaCl,[33] is illustrated graphically in Fig. 7 and the pertinent parameters collected in Table II. All three curves vary linearly

TABLE II

POTENTIOMETRIC PARAMETERS OF GP IN NaCl[a]

NaCl, M	$\frac{\Delta pK'}{\Delta\alpha}$	$\frac{0.43e^2\kappa}{3DkT}$	$pK'_{\alpha=0}$	pK_0
0.01	0.53	0.03	3.73	3.76
0.10	.31	.10	3.69	3.79
1.00	.20	.33	3.36	3.69
			Av.	3.75 ± 0.05

[a] Monomer concentration of $GP = 0.010$ M.

(29) F. E. Harris and S. A. Rice, *J. Phys. Chem.*, **58**, 725 (1954); S. A. Rice and F. E. Harris, *ibid.*, **58**, 733 (1954).

(30) J. Th. G. Overbeek, *Bull. soc. chim. Belg.*, **57**, 252 (1948).

(31) A. Katchalsky and J. Gillis, *Rec. trav. chim.*, **68**, 879 (1949).

(32) F. E. Harris and S. A. Rice, *J. Polymer Sci.*, **15**, 151 (1955).

(33) We have not included the contribution of the polymer and its gegenions to the ionic strength of solutions. The gegenion concentration (Na^+) is constant at 0.010 M. The contribution of the fixed charges of the polymeric chain has been shown to be negligible.[35] The effect of this concentration of Na^+ would be to increase the ionic strength by 50% in 0.01 M NaCl and would be negligible in 0.10 and 1.00 M NaCl solutions.

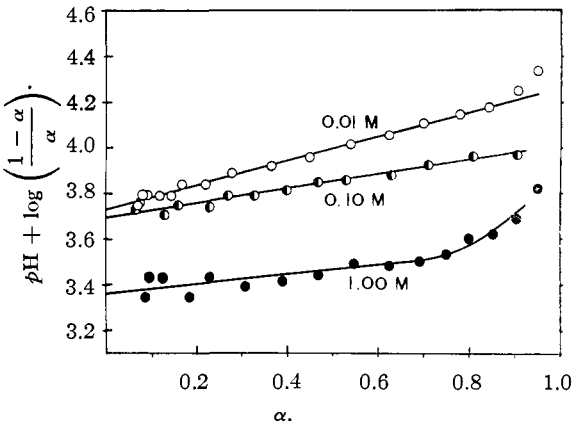

Fig. 7.—Potentiometric titration data plotted against the degree of ionization (α) at three concentrations of NaCl; $T = 25.0°$.

with α from $\alpha \cong 0.06$ to near complete neutralization (except above 0.7 in 1.00 M NaCl). The slopes of the curves decrease with increasing NaCl because of the building up of the Debye–Hückel ion atmosphere about each charged center with a consequent reduction of the electrostatic field. In the limit $\alpha = 0$, $\partial F/\partial\nu$ will vanish and the intrinsic dissociation constant of the ionizable groups may be evaluated from eq. 2. If the curves in Fig. 7 are extrapolated linearly to $\alpha = 0$, and corrected for the ionic strength term in eq. 2, a common value for pK_0 equal to 3.75 (± 0.05) is found. This procedure is questionable since other polyions (*e.g.*, polymethacrylic acid) show a very rapid change in $\partial F/\partial\nu$ in the first few per cent. of ionization.[34]

Katchalsky, *et al.*,[35,36] have shown that the electrostatic potential ψ may be identified with ζ and thus obtained from electrophoretic data. Values of $0.43\ e\psi/kT$ obtained in this way are included in Table I. The slope of this curve is 0.55 (at 0.125 $\Gamma/2$) which is to be compared with a value of 0.31 obtained from the titration data in 0.10 M NaCl. Below $\alpha \cong 0.03$, the electrophoretic potentials must show a sharp decline. Whether the titration data show similar behavior is not known since the high precision required in determining α to give meaningful values of $\log(1-\alpha)/\alpha$ was not obtainable. If this decline in ψ does occur below the limits of α recorded in Fig. 7, then the value of pK_0 will have to be revised accordingly.[37]

The factor $\partial F/\partial\nu$ may also be estimated from theoretical expressions which relate the electrical free energy of the polymer to its dimensions and

(34) A. Katchalsky, *J. Polymer Sci.*, **7**, 393 (1951); R. Arnold and J. Th. G. Overbeek, *Rev. trav. chim.*, **69**, 192 (1950).

(35) A. Katchalsky, N. Shavit and H. Eisenberg, *J. Polymer Sci.*, **13**, 69 (1954).

(36) S. Lifson and A. Katchalsky, *ibid.*, **11**, 409 (1953).

(37) If the identification of the zeta potential with the titrimetric potential (ψ) is correct the undetected decline in ψ at very low values of α must occur in order to bring the values of $\zeta_{\alpha=1}$ and $\psi_{\alpha=1}$ into agreement. This would necessitate a revision of pK_0 to about 3.45. There is some evidence that the slopes in the region reliably measured by titration, $\Delta pK'/\Delta\alpha$, may vary considerably according to the conditions of measurement, an extreme case being that shown in the data of Hanby and Rydon[2] where the slope is close to zero. Such variability may perhaps be attributed to differences in the suddenness with which molecular expansion takes place in the early stages of ionization. In Hanby and Rydon's preparation it would occur completely and "explosively" over a rather narrow range of α.

charge. Reasonable values of $\partial F/\partial \nu$ have been calculated by Schneider and Doty[21] for sodium carboxymethylcellulose and by Katchalsky, *et al.*,[35] for polymethacrylic acid, by employing the equation of Lifson and Katchalsky[36]

$$\frac{0.43}{kT} \times \frac{\partial F}{\partial \nu} = \frac{0.86\nu e^2}{DkTh}\left[\ln\left(1 + \frac{6h}{\kappa h_0^2}\right) - \left\{\frac{\alpha(s_1 - s_0)}{s}\right\} \times \frac{6h/\kappa h_0^2}{1 + 6h/\kappa h_0^2}\right] \quad (3)$$

Values of s, the number of monomers per statistical element in Kuhn's theory,[38] for HGP and NaGP are 3.6 and 43 (see Discussion).

At ionic strength 0.10 the electrical free energy term on the left side of equation 3 is not greatly affected from h = 220 to 720 Å. When $\alpha = 0$, $0.43(\partial F/\partial \nu)/kT$ is equal to 0.0066 ν and only falls by about 40% to 0.0038 ν when $\alpha = 1$. The transition with α should be gradual since the viscosity changes occur rather uniformly with α. It is not surprising therefore that the values of pK' are linear with α. On the other hand not very good agreement is obtained between experiment and theory in the magnitude of the variation of $0.43(\partial F/\partial \nu)/kT$ with ν. In the electrophoretic experiments this variation is about 20% as large as in eq. 3 and even poorer agreement is evident in the potentiometric data. Of course, the theory of Lifson and Katchalsky has not provided for ion-binding effects nor has a realistic value of the dielectric constant been used in their equations. Correction for these effects would serve to reduce the discrepancy.

Sedimentation.—The sedimentation patterns for NaGP in 0.20 M NaCl (pH 4.8) showed a single sharp peak; the sedimentation coefficient was markedly dependent on concentration and fitted the equation $s = s_0(1 + k_s c)$ where $s_0 = 3.12 \times 10^{-13}$ sec.$^{-1}$ and $k_s = 1.72$ when c is in g./dl. (Fig. 8). At pH 2.0 in 0.1 M NaCl the sedimentation

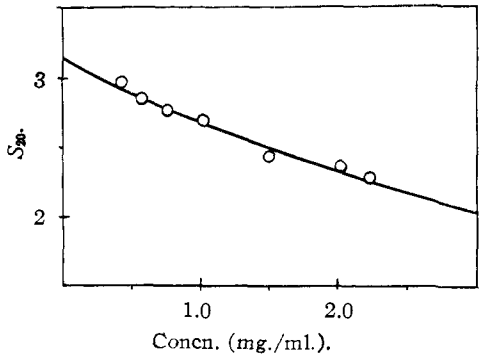

Fig. 8.—The concentration dependence of sedimentation of NaGP in 0.20 M NaCl, pH 4.8. Smooth curve corresponds to $s_0 = 3.12 \times 10^{-13}$ sec.$^{-1}$ and $k_s = 1.72$. See text.

boundaries were fairly broad, although symmetrical. The sharp boundaries observed at pH 4.8 are undoubtedly due to artificial boundary sharpening effects resulting from the strong concentration dependence of sedimentation.

Surface Tension.—Measurements were made at three values of α (8 to 30%) and several concentrations. Complex variations were associated with aging of the surface and with removal of the tensiometer ring. In keeping with the behavior observed for other polyelectrolytes, the surface activity is depressed considerably in the early stages of ionization and is not much affected thereafter (*cf.* Fig. 9). At concentrations above 0.2 mg./ml. the

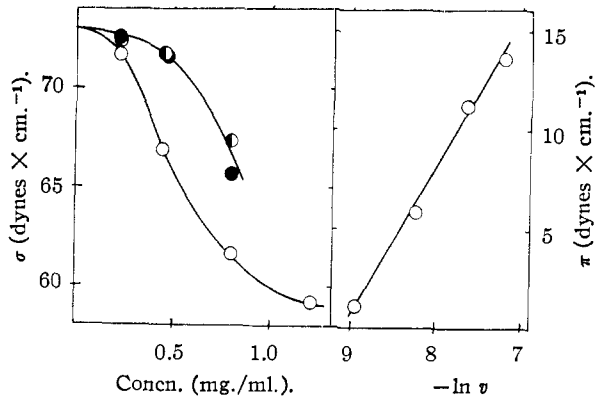

Fig. 9.—Left side: effect of GP concentration on surface tension: open circles, α = 0.08; shaded circles, α = 0.15; closed circles, α = 0.26. Right side: variation of surface pressure with natural logarithm of GP volume fraction, α = 0.08.

surface pressure, π ($= \sigma_0 - \sigma$), of solutions at about 8% ionization follows the equation $\pi = A + B \ln v$ where v is volume fraction GP (taken equal to $0.77c$). The theory developed by Katchalsky and Miller[39] permits calculation of the adsorbed film thickness τ and the free energy of adsorption λ, since $\tau = \varphi B/RT$ and $\lambda \approx RT(A/B - 0.577)$ where φ is molecular volume. The data are summarized in Table III which contains for comparison our results with sodium lauryl sulfate. The value of B for GP is some 9 times greater than that found by Katchalsky and Miller for polymethacrylic acid of comparable degree of polymerization, and the resulting calculated film thickness is unreasonably large. We conclude that the surface activity of GP is dominated by components of lower molecular weight than those which determine the other macromolecular properties, a conclusion illustrated by the alternative values for τ and λ (Table III) calculated on the assumption that the effective volume fraction and molecular weight are both reduced by a factor $2e$. The free energy of adsorption for the latter case is 6210/94 or 66 cal. per glutamyl residue mole. Since λ for a single glutamyl residue mole is about 340 cal.[40,41] it seems that under the above assumption only about one residue in five would contribute to the surface pressure of GP solutions. Wide latitude in the assumptions made can be permitted without changing the qualitative conclusion that this behavior is similar to that observed for PMA,[39] in contrast to that of Na lauryl sulfate, for which the values of τ and λ (Table III) are compatible with the root-mean-square end-to-end distance of the dodecane chain and with the free energy of adsorption esti-

(38) W. Kuhn and H. Kuhn, *Helv. Chim. Acta*, **26**, 1394 (1943).

(39) A. Katchalsky and I. Miller, *J. Phys. Colloid Chem.*, **55**, 1182 (1951); "Atti 1° Congresso internaz. Materie plastiche," Torino, 1949.

(40) J. B. Bateman and J. Godwin, estimated from unpublished surface tension measurements on N-methylsuccinamic acid.

(41) I. Langmuir, THIS JOURNAL, **39**, 1838 (1917).

mated as an additive resultant of the contributions of the component radicals.

TABLE III

SURFACE TENSION DATA

	$-\ln v$	A, dynes/cm.	B, dynes/cm.	M	τ, Å.	λ, cal./mole	λ, cal./residue mole
HGP, *p*H 2.7	7–9	68.8	7.6	90000	2120	5025	7
	9–11	84.0	7.6	12200	290	6210	66
Na lauryl sulfate	6–8	91.7	7.5	288	~9	~6900	~6900

Discussion

The Un-ionized Polypeptide.—Calculation of the root-mean-square end-to-end distance of the HGP molecule from intrinsic viscosity data using the Flory equation[42] based on random-coil behavior, $\Phi = [\eta]M/(\bar{h}^2)^{3/2} = 2.1 \times 10^{21}$ leads to a value of 220 Å. This value is quite compatible with the intrinsic dissymmetry value of 1.01 of the un-ionized polypeptide.

Since the repeating unit of GP is a composite of a fairly stiff peptide and a freely rotating ethylenic unit, we have made the simplifying assumption that there are two monomer units per glutamyl residue with average length 3.09 Å. (3.64 Å. for the peptide residue and 2.54 Å. for $2CH_2$ groups) and the same effective bond angle as the aliphatic chain. Calculation of the $(\bar{h}^2)^{1/2}$ distance of a random-coil configuration by Eyring's formula[43] leads to a value of 165 Å. which is somewhat smaller than that deduced from $[\eta]$ and M. On the basis of the equation of Kuhn,[38] $\bar{h}_0^2 = Zsb^2$, an s value of 3.6 is calculated using $b = 3.09$ and the degree of polymerization (Z) as 1400. The small s value and the agreement in $(\bar{h}_0^2)^{1/2}$ with random-coil behavior indicates that HGP is quite flexible with little steric constraint between monomer units.

The unperturbed random-coil configuration of the uncharged polypeptide is probably due to a tendency to form polymer–polymer contacts in preference to polymer–solvent "bonds," water thus being a relatively poor solvent The increase in dissymmetry with concentration provides unequivocal evidence of polymer–polymer interaction. Thermodynamic support is supplied by the independence of turbidity and concentration. The polydispersity manifested in the diffuse and rapidly spreading sedimentation boundary at *p*H 2.0 could be due in part to a tendency to association, although doubtless also due to a distribution of chain lengths, as the surface tension data suggest. In this respect GP resembles a fractionated synthetic polymer rather than a purified protein.

The Highly Ionized Polypeptide at Infinite Dilution,—The properties of the isolated NaGP molecule in aqueous solution can be deduced qualitatively by extrapolation of viscosity and dissymmetry data. The pertinent values are summarized in Table IV. They conform to the usually accepted picture of a linear polyelectrolyte, with a highly expanded configuration at very low ionic strengths, arising from intramolecular electrostatic repulsions. These are readily "quenched" by addition of simple electrolyte; however, in contrast to other polyelectrolytes, the molecule remains significantly expanded in relation to that of the neutral molecule, even in 1.0 M NaCl.

TABLE IV

DIMENSIONS OF GLUTAMYL POLYPEPTIDE FROM LIGHT SCATTERING AND VISCOSITY

	Added NaCl	$[z]$	$[\eta]$	$(\bar{h}^2)^{1/2}$[a]	$(\bar{h}^2)^{1/2}$[b]
HGP ($\alpha < 0.10$)	0	1.01	0.25	220	
	0.10	1.01	0.25	220	
NaGP ($\alpha = 0.90$)	0	2.0	125		1300
	0.10	1.27	1.1	350	720

[a] Based on Flory–Fox eq. 42. [b] Based on tables of Doty and Steiner for random-coil model (10).

In 0.10 M NaCl a value of 43 is calculated for the Kuhn parameter s which indicates that the ionized polymer chain is appreciably less flexible than when uncharged. In the absence of neutral salt s values become very large (~100) and the "length" of the molecule is increased to about $1/3$ or $1/2$ the contour length (4350 Å.) depending on whether we interpret the light scattering data in terms of a random-coil or rod-shaped model. The polymer chain is now quite stiff with only long range kinking and the shape is undoubtedly quite unsymmetrical.

Interactions at Finite Concentrations,—The viscosities of solutions of NaGP at low ionic strength conform to the Fuoss[18] behavior pattern of linear polyelectrolytes. The light scattering properties, in particular the sharp rise of dissymmetry at high dilutions and the decreased and strongly concentration-dependent turbidity resemble those of other highly charged polymers[44–46] as well as bovine serum albumin.[47] The considerations put forward by Oth and Doty[44] to explain these phenomena are equally applicable to GP and are likewise not amenable to quantitative treatment.

Addition of 0.1 M NaCl depresses the dissymmetry curve and eliminates the minimum; the molecule remains however considerably expanded in spite of the fact that the reduced turbidities show intermolecular repulsions to have been drastically reduced. The value of $[z]$ leads to a root-mean-square end-to-end distance of 720 Å., considerably in excess of the value 350 Å. calculated from the Flory–Fox equation and the freely rotating coil value of 165 Å. The high specific viscosities of NaGP, even in the presence of 1.0 M NaCl (Fig. 3), reinforce the evidence from dissymmetry. It would seem that the molecule of GP shows a tendency to be stabilized in a relatively extended configuration by intramolecular forces (attractive or repulsive) other than the electrostatic repulsions which are usually considered dominant in bringing about the expansion of polyelectrolyte molecules.

Molecular Changes with Ionization.—The shapes of the viscosity curves (Fig. 5) contrast markedly with those of polymethacrylic acid[34] and more

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(43) H. Eyring, *Phys. Revs.*, **39**, 746 (1932).

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(46) H. J. L. Trapp and J. J. Hermans, *J. Phys. Chem.*, **58**, 757 (1954).

(47) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **20**, 85 (1952).

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.

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

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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.

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

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

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