

the solution was heated on the steam-cone for 15–30 min. During this time the color faded to yellow. Most of the excess acid was removed under reduced pressure, the residue poured into cracked ice, basified with aqueous sodium carbonate solution and extracted with chloroform. The extract was washed, dried and evaporated.

Oxidation of N-(2-Benzamidophenyl)-piperidine with Peroxytrifluoroacetic Acid.—To a stirred solution of 650 mg. of N-(2-benzamidophenyl)-piperidine in 4 ml. of trifluoroacetic acid was added 2 ml. of 30% hydrogen peroxide.

No reaction was apparent. On heating on the steam-cone with stirring, the color of the solution turned brown and on continued heating for 1 hour a yellow solution resulted. This was worked up as usual to give the crystalline piperidinebenzimidazole, m.p. 96–98°, yield 140 mg. (35%).

Oxidation of N-(2-N',N'-Diacetylamino-phenyl)-piperidine.—Oxidation of N-(diacetylamino-phenyl)-piperidine with peroxytrifluoroacetic acid as described above yielded 120 mg. (28%) of crystalline piperidinebenzimidazole, m.p. 98–99°.

CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIFORNIA]

Adrenocorticotropin (ACTH). XXIII. A Sedimentation Study of the State of Aggregation of Ovine Pituitary ACTH in Acidic and Basic Solutions

BY PHIL G. SQUIRE AND CHOH HAO LI

RECEIVED MARCH 29, 1961

The state of aggregation of ACTH in solution has been the subject of very few quantitative investigations, although suggestions have been advanced that the hormone forms soluble association products near the region of insolubility (pH 6–8) and in basic media. We have studied the sedimentation behavior of ACTH by the sedimentation velocity and sedimentation equilibrium methods in three buffers: (I) HCl-KCl, pH 1.3, ionic strength 0.200; (II) formic acid-formate, NaCl, pH 3.5, ionic strength 0.200; and (III) carbonate-bicarbonate, pH 10.1, ionic strength 0.100. Correction for charge effects was made when necessary and duplicate sedimentation equilibrium experiments at different initial concentration served to distinguish between reversible association and simple heterogeneity. Diffusion coefficients were calculated from data obtained during the approach to equilibrium and from boundary spreading in sedimentation velocity experiments. In buffer I, the dependence of sedimentation and diffusion coefficients and molecular weight (M) upon concentration is slight, and all 3 parameters correspond to the monomer ($M = 4540$). Molecular frictional ratios calculated from s and D indicate that ACTH assumes a highly extended configuration at pH 1.3. In buffer II, evidence of reversible association yielding tetramers (or higher) was found. The first association constant (dimer formation) was found to be 0.33 on the g./100 ml. concentration scale. Evidence is presented that the successive association constants increase in magnitude. In buffer III, soluble aggregates (tetramers) not in equilibrium with monomer were detected. This and evidence from other experiments suggests that ACTH undergoes irreversible association in a basic medium.

Extensive chemical studies^{1,2} have established the complete amino acid sequence of ovine pituitary adrenocorticotropic hormone (α_s -ACTH); consequently the molecular weight of the unit linked by covalent bonds is known with a high degree of certainty to be 4540 at its isoionic point. Little is known, however, of the state of aggregation of the molecular kinetic unit in solution. Brown, *et al.*,³ determined the molecular weight of porcine pituitary ACTH by means of the Archibald procedure in a solution containing 0.05 M KCl and 0.05 M HCl per liter and reported that except for a trace of an impurity of high molecular weight, which was detectable in both the Archibald and synthetic boundary cell experiments, the preparations appeared homogeneous, and the molecular weight calculated from these experiments was in good agreement with that calculated from the amino acid content (4567).

Except for a brief summary² of the results of some preliminary sedimentation and diffusion studies, the molecular kinetic behavior of highly purified ovine ACTH has not been reported. The molecular weight calculated from these studies which were carried out in a 0.100 M HCl, 0.200 M KCl solvent, also was in good agreement with the value calculated from the amino acid composition.

Both studies were performed in the very low pH range because of the insolubility of the hormone in the neighborhood of neutrality and also because of

a rather widely held but experimentally unconfirmed notion that ACTH tends to aggregate when the limiting solubility is approached. It may be that the basis for this contention is largely reasoning by analogy from the rather extensive studies of the association of insulin which has a similar solubility behavior.

Another suggestion that ACTH forms soluble association products was derived from the observation⁴ that α_s -ACTH, which dialyzes quite readily in acidic media, is non-dialyzable when dialyzed against dilute ammonia. This suggested that the non-dialyzability of the hormone in alkaline solution was due to the formation of soluble aggregates.

The object of the investigation to be reported⁵ here was to provide a physicochemical characterization of the hormone by means of sedimentation velocity and sedimentation equilibrium experiments in strongly acidic media where intermolecular association was not expected and to compare these results with those obtained in buffers where association has been postulated, and to provide, if possible, a quantitative description of the association reactions involved. To a very large degree, the course and extent of this investigation has been determined by the scarcity of ACTH polypeptide. The amount required for this study was 25 mg.

Theoretical

Analysis of the sedimentation equilibrium experiments was based largely on the equation of

(1) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 686 (1955).

(2) C. H. Li, *Advances in Protein Chem.*, **11**, 101 (1956).

(3) R. A. Brown, M. Davies, M. Englert and H. R. Cox, *J. Am. Chem. Soc.*, **78**, 5077 (1956).

(4) C. H. Li, *Bull. Soc. Chem. Biol.*, **40**, 1757 (1958).

(5) A brief report of this work was presented at the 138th meeting of the American Chemical Society, New York, September 1960.

Goldberg⁶ which gives the weight average molecular weight, $\bar{M}_{w(r)}$, at a distance r from the axis of rotation as

$$\bar{M}_{w(r)} = \frac{RT}{(1 - \bar{v}_p)\omega^2} \times \frac{dc/dr}{cr} \times \left(1 + \frac{c}{dc} \frac{d \ln y}{dc}\right) \quad (1)$$

provided that the partial specific volume is the same for all macromolecular solutes. Here c and dc/dr are the polypeptide concentration and its gradient, and y is the thermodynamic activity coefficient on the g./100 ml. concentration scale. In an associating system we can assume that the partial specific volume is independent of the degree of association. Furthermore, if association occurs to a considerable extent, we might assume that all other contributions to the variation in the thermodynamic activity coefficient with concentration are negligible by comparison and proceed to calculate the association constants from the relationship between the weight average molecular weight and the concentration.

The weight average molecular weight \bar{M}_w of the entire sample used in a sedimentation equilibrium experiment was calculated by equation 2

$$\bar{M}_w = \frac{RT}{(1 - \bar{v}_p)\omega^2} \times \frac{2\Delta c}{c_0(b^2 - a^2)} \left(1 + \frac{c}{dc} \frac{d \ln y}{dc}\right) c = \frac{c_b + c_a}{2} \quad (2)$$

which is Method I of Van Holde and Baldwin⁷ except that we have assumed that, in the concentration range we are investigating, the refractive index increment is a linear function of concentration. The z -average molecular weight, \bar{M}_z , was calculated by means of equation 3 which repre-

$$\bar{M}_z = \frac{RT}{(1 - \bar{v}_p)\omega^2} \times \frac{\left(\frac{1}{r} \frac{dc}{dr}\right)_b - \left(\frac{1}{r} \frac{dc}{dr}\right)_a}{\Delta c} \times \left(1 + \frac{c}{dc} \frac{d \ln y}{dc}\right) c = c_b + c_a \quad (3)$$

sents Method II of Van Holde and Baldwin.

Many of the data to be presented are given in terms of apparent molecular weights, which may be conveniently calculated from the results of a single experiment. The apparent weight average molecular weight, as a function of r , $\bar{M}_{w(r)}(\text{app.})$, the apparent weight average molecular weight, $\bar{M}_w(\text{app.})$, and the apparent z -average molecular weight, $\bar{M}_z(\text{app.})$ are given by equations identical to (1), (2) and (3), respectively, except for the omission of the thermodynamic term in parentheses.

The thermodynamic term can then be evaluated from the variation with concentration of the apparent molecular weight calculated from experiments performed at different concentrations and from the variation of $\bar{M}_{w(r)}(\text{app.})$ with concentration, provided that heterogeneity has been ruled out.

Before interpreting sedimentation equilibrium data in terms of association equilibria, one should demonstrate the presence of association products in equilibrium with monomer. Three possible aggregation phenomena should be considered.

I. A Homogeneous Non-associating Macromolecular Solute.—We would expect the thermo-

dynamic term to be rather small, and values of $\bar{M}_{w(r)}(\text{app.})$ to show little dependence, either on r , on the initial protein concentration. Furthermore these values would be approximately equal to $\bar{M}_w(\text{app.})$ and $\bar{M}_z(\text{app.})$.

II. A Heterogeneous Macromolecular Solute.—Calculated values of $\bar{M}_{w(r)}(\text{app.})$ would increase with r . If duplicate experiments are performed at different initial macromolecular concentrations, the dependence of $\bar{M}_{w(r)}(\text{app.})$ on r would be approximately the same for the two experiments, and $\bar{M}_w(\text{app.})$ and $\bar{M}_z(\text{app.})$ would be the same for the two experiments, neglecting variations in activity coefficients. In both experiments, however, $\bar{M}_z(\text{app.})$ would be greater than $\bar{M}_w(\text{app.})$.

III. A Homogeneous Macromolecular Solute Undergoing Concentration-dependent Association.

—The values of $\bar{M}_{w(r)}(\text{app.})$ would increase with r and the results of a single experiment would be indistinguishable from example II. If, however, a second experiment were performed at a different initial macromolecular concentration, a single plot of $\bar{M}_{w(r)}(\text{app.})$ against $c(r)$ would give points, from the two experiments, which would fall on the same line. Furthermore, $\bar{M}_w(\text{app.})$ and $\bar{M}_z(\text{app.})$ for the entire sample would have higher values when calculated from the experiment having the higher initial protein concentration.

It is customarily assumed that charge effects arising from the sedimentation of charged macromolecules can be effectively suppressed by addition of supporting electrolyte. Lamm has shown, however,⁸ that this assumption becomes inadequate as the charge to weight ratio increases. The known structure of α_s -ACTH^{1,2} and the electrophoretic^{9a} and titration^{9b} data suggest that the molecule might carry a net charge of as high as 6 protons at pH values below 2. Consequently it becomes necessary to take charge effects into account in the analysis of experiments carried out in the very low pH range.

The sedimentation equilibrium of charged macromolecules in the presence of supporting electrolyte has been discussed in detail by Johnson, Kraus and Scatchard¹⁰ and more recently, a similar treatment of the problem has been proposed by Williams, *et al.*¹¹ Since the final equations derived by the latter investigators¹¹ are based on the treatment of data we have chosen, these equations have proven more convenient for our purpose. According to this treatment, the corrected molecular weight is calculated from the apparent molecular weight by equations 4a and 4b

$$\frac{1}{M_{\text{app}}} = \frac{1}{M^*} \left(1 + \frac{Z^2 M_b}{2 \bar{M}_p} \times \frac{c_p}{c_b}\right) \quad (4a)$$

where

$$M^* = M_p \left(1 - \frac{Z M_b (1 - \bar{v}_p \rho)}{2 \bar{M}_p (1 - \bar{v}_p \rho)}\right) \left(1 - \frac{Z M_b \theta_p}{2 \bar{M}_p \theta_p}\right) \quad (4b)$$

where Z is the net charge; \bar{M}_p , v_p and θ_p are the molecular weight, partial specific volume and

(8) O. Lamm, *Arkiv Kemi Mineral. Geol.*, **17A**, No. 25 (1944).

(9) (a) I. D. Raacke and C. H. Li, *J. Biol. Chem.*, **215**, 277 (1955); (b) J. Léonis and C. H. Li, *J. Am. Chem. Soc.*, **81**, 415 (1959).

(10) James S. Johnson, Kurt A. Kraus and George Scatchard, *J. Phys. Chem.*, **58**, 1034 (1954).

(11) J. W. Williams, K. E. Van Holde, R. L. Baldwin and H. Fujita, *Chem. Revs.*, **58**, 715 (1958).

(6) R. J. Goldberg, *J. Phys. Chem.*, **57**, 194 (1953).

(7) K. E. Van Holde and R. L. Baldwin, *ibid.*, **62**, 734 (1958).

specific refractive-index increment of the protein, and M_b , v_b and θ_b refer to the supporting electrolyte. After substitution of known quantities into equations 4a and 4b, they reduce to

$$M_p = \frac{1 + 0.1495c_p}{0.890} M_{app} \quad (5)$$

in the case of the pH 1.3 HCl-KCl buffer (buffer I), and to

$$M_p = \frac{1 + 0.1338c_p}{0.893} M_{app} \quad (6)$$

in the case of the pH 3.28 formate buffer (buffer II). In making these calculations a net charge of +5 was assumed.

Experimental

Ultracentrifugation.—The Spinco Model E ultracentrifuge used in these studies was equipped with schlieren optics including a phaseplate as the schlieren diaphragm. Temperatures were read and regulated to within 0.02° by means of an RTIC unit which had been calibrated at rest. A double sector pure epoxy cell was used for the sedimentation equilibrium experiments and a fluorochemical FC-43¹² was used to provide a visible lower meniscus. A double sector synthetic boundary cell of aluminum-epoxy was used for the determination of the initial concentration of the sedimentation equilibrium experiments and was also used for the sedimentation velocity experiments. All calculations were based upon measurements of the photographic plate which were made by means of the two dimensional Gaertner micro-comparator. Following the nomenclature suggested by Trautman,¹³ radial distances in the cell from the axis of rotation are indicated by the letter r . The corresponding abscissa obtained as a microcomparator reading of the plate is given the symbol X , and the plate is positioned on the microcomparator so that $X = Fr$ where F is the radial magnification factor. The displacement of the diaphragm image from the baseline, which is proportional to the refractive index gradient dc/dr , is designated dc'/dr .

The protein concentrations of the various experiments were determined in either, or both, of two ways; from the optical density measured at 278 m μ in the Beckman DU spectrophotometer and an extinction coefficient, $E_{278}^{1\%} = 17.77$, which was calculated from the amino acid composition² or from a refractometric measurement using the double sector synthetic boundary cell. Here the initial concentration c_0 is related to the peak area at a given time, A_t , by the equation

$$c_0 = \frac{\tan \theta}{Gab \, dn/dc} \times \left(\frac{r_t}{r_0}\right)^2 \times A_t \quad (7)$$

where r_t and r_0 are the peak positions at the time t and the zero time of the experiment, respectively, and the specific refractive increment of the solute is given by the symbol dn/dc . The product of the three instrument constants (the magnification factor of the cylindrical lens system G , the optical lever arm b and the cell thickness a) was determined experimentally with sucrose solutions of known concentration in the same synthetic boundary cell. In these calibration experiments, reagent grade sucrose, dried to constant weight over P₂O₅ was used in preparing the solutions, and the value $1.430 \times 10^{-3} 14$ was taken as the specific refractive increment. As a result of these measurements, we calculated $Gab = 269$ and c (g./100 ml.) = 3.44 c' for a schlieren angle of 60°.

In the experiments with ACTH where concentration c was determined spectrophotometrically and A_t was measured, these data were used to calculate the specific refractive increment. The mean and standard deviation results from six experiments are $dn/dc = (1.882 \pm 0.061) \times 10^{-3}$.

The concentration at any radial distance during the sedimentation equilibrium experiments was calculated by means of the equations

(12) We wish to acknowledge the suggestion of Dr. David Yphantis for the use of fluorochemical FC-43 which was obtained from Minnesota Mining and Manufacturing Co.

(13) R. Trautman, *J. Phys. Chem.*, **60**, 1211 (1956).

(14) L. J. Gosting and M. S. Morris, *J. Am. Chem. Soc.*, **71**, 1998 (1949).

$$c_m' = c_0' + \frac{1}{b^2 - a^2} \left[\int_a^b r^2 \frac{dc'}{dr} dr - b^2 \int_a^b \frac{dc'}{dr} dr \right] \quad (8a)$$

and

$$c_r' = c_a' + \int_a^r \frac{dc'}{dr} dr \quad (8b)$$

where c_0' is the initial concentration, in optical units, determined from an experiment in the synthetic boundary cell.

The partial specific volume of ACTH was calculated from its known amino acid composition² with the values tabulated by Cohn and Edsall¹⁵ used for the partial specific volumes of the amino acid residues. Contrary to customary practice for proteins we have corrected for electrostriction due to ionization of the side chains of the polypeptide, assuming a volume contraction of 9 ml. per charged group. These calculations lead us to a value of $\bar{v} = 0.717$ at pH 1.3, 0.718 at pH 3.5 and 0.704 at pH 10.1.

Diffusion coefficients were calculated from the sedimentation equilibrium experiments as outlined by Van Holde and Baldwin.⁶ Two methods were used in calculating diffusion coefficients from the sedimentation velocity experiments. In the one method, which takes into account the dependence of the sedimentation coefficient on concentration, the data were tabulated as described by Baldwin¹⁸ and the diffusion coefficient was calculated by the method of Fujita.¹⁷ In the other method, the heights and areas of the schlieren peaks were measured and the diffusion coefficient was calculated from a plot of the square of the area to height ratio $(A/H)^2$ as a function of time using the equation

$$D = \frac{1}{4\pi t} \left(\frac{A}{H}\right)^2 \quad (9)$$

Diffusion coefficients calculated by the two methods agreed to within 1%, as might be expected from the slight dependence of S on c .

Materials.—Two samples of α_s -ACTH were used in these studies. Both were prepared by the method reported by Li, *et al.*,¹⁸ except for a final process⁴ designed to remove trichloroacetate remaining as a contaminant following the preceding countercurrent step. In Sample I, the preparation had been dissolved in 0.1 M Na₂CO₃ and dialyzed against dilute ammonia. In Sample II, the trichloroacetate had been removed from the ACTH solution by passage through an Amberlite 1R-4B ion exchange column.

The compositions of the three buffers were as follows: The pH 1.30 buffer of ionic strength 0.200 contained 0.070 mole of HCl and 0.130 mole of KCl per liter; the pH 3.25 formate buffer of ionic strength 0.200 contained 0.150 mole of NaCl, 0.050 mole of sodium formate and 0.100 mole of formic acid per liter; the pH 10.1 carbonate-bicarbonate buffer of ionic strength 0.100 contained 0.025 mole of NaHCO₃ and 0.025 mole of Na₂CO₃ per liter. Density and viscosity data were taken from the tables compiled by Svedberg and Pedersen¹⁹ and from the International Critical Tables.

Results

The ACTH used in the early experiments of this study was sample I, which had been prepared by dialysis in basic solution prior to the final lyophilization. Two sedimentation equilibrium experiments were performed at pH 1.3 at initial concentrations of 0.248 and 0.869 g./100 ml. in order to test the homogeneity of this sample. The conditions of these experiments are recorded in Table I as experiments 1 and 2 along with the weight average and z average molecular weights. The calculated values of $\bar{M}_{w(r)}$ (app.) are plotted against concentration in Fig. 1. The points were calculated from measurements made at 0.2 mm. intervals of r , starting 0.2 mm. from the air-solu-

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Pub. Corp., New York, N. Y., 1943, p. 375.

(16) R. L. Baldwin, *Biochem. J.*, **65**, 503 (1957).

(17) H. Fujita, *J. Phys. Chem.*, **63**, 1092 (1959).

(18) C. H. Li, I. I. Geschwind, J. S. Dixon, A. L. Levy and J. I. Harris, *J. Biol. Chem.*, **213**, 171 (1955).

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

TABLE I
ANALYSIS OF α_s -ACTH BY SEDIMENTATION EQUILIBRIUM

Expt. no.	pH of soln.	R.p.ni.	c_0 , g./100 ml.	$\frac{d \ln y}{dc}$	$\bar{M}_w(\text{app})$	\bar{M}_w	$\bar{M}_z(\text{app})$	\bar{M}_z
1 ^a	1.30	20410	0.869	0.17	6540	7000	11900	10700
2 ^a	1.30	31410	.248		6310		10200	
3	1.30	24630	.773	.20	4880	5100	5580	5400
4	1.30	24630	.286		4540		5220	
5	10.1	24630	.235		5230		6600	
6	10.1	24630	.970	.22	630	5100	9000-15000	8000-12000
7	10.1	24630	.487		5910		8000-12000	
8	3.53	29500	.435		5270		6780 ^b	
9	3.46	23150	.901		10800		19500 ^b	

^a The ACTH used in experiments 1-2 was sample I and that used in the remaining experiments was sample II. See text for definition of terms. ^b Calculated by equation 3. The values calculated from an associating system have a different significance than \bar{M}_z , however.

tion meniscus but are plotted *vs.* the concentration at that radial distance. Applying the interpretation presented earlier to the data recorded for these two experiments in Fig. 1 and Table I,

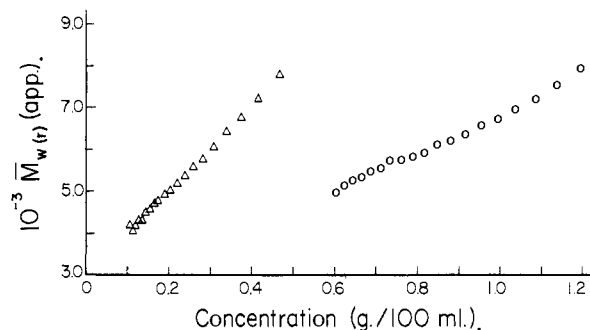


Fig. 1.—Sedimentation equilibrium of α_s -ACTH (sample I) at pH 1.3 (HCl-KCl) and ionic strength 0.200. The points indicated by the symbol O were calculated from expt. 1 and those indicated by Δ from expt. 2 (see Table I).

we observe that sample I behaves as a heterogeneous macromolecular solute. If we take the molecular weight of the monomer, $M_1 = 4690$ (ACTH·Cl₄) and let $x =$ weight fraction polymer and $1 - x =$ weight fraction monomer and N the degree of polymerization, we obtain the simultaneous equations

$$\bar{M}_w = \frac{w_1 M_1}{w_i} = \frac{(1-x)M_1 + xN M_1}{1} = 7000$$

$$\bar{M}_z = \frac{w_1 M_1^2}{w_i M_1} = \frac{(1-x)M_1^2 + xN^2 M_1^2}{(1-x)M_1 + xN M_1} = 10,700$$

Solving these equations²⁰ for N and x , we obtain $N = 3.90$ and $x = 0.126$. Thus we can account for our data with the postulate that the sample contains 13% tetramers not in equilibrium with monomer.

This evidence of heterogeneity in sample I demonstrated that it was unsuitable for the contemplated experiments; consequently, we turned our attention to sample II which had been prepared by the use of ion-exchange chromatography rather than dialysis prior to the final lyophiliza-

(20) Solution of these simultaneous equations is simplified by making the substitutions $\alpha = \bar{M}_w/M_1$ and $\beta = \bar{M}_z \bar{M}_w / M_1^2$. Then the equation

$$n^2(\alpha - 1) + n(1 - \beta) + (\beta - \alpha) = 0$$

may be solved for n as a quadratic, and x may be calculated from $x = (\alpha - 1)/(n - 1)$.

tion. Experiments 3 and 4 were performed using conditions similar to 1 and 2 except that the new sample was used.

The calculated values of $\bar{M}_{w(r)}(\text{app.})$ obtained from experiments 3 and 4 are plotted against concentration in Fig. 2. These data are typical of those one might expect for a homogeneous non-associating macromolecular solute. The slight

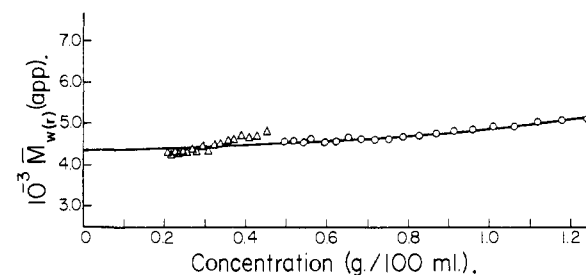


Fig. 2.—Sedimentation equilibrium of α_s -ACTH (sample II) at pH 1.3 (HCl-KCl) and ionic strength 0.200. The points indicated by the symbol O were calculated from expt. 3, and those indicated by Δ from expt. 4 (see Table I).

departure of the points from expt. 4 from the extension of the line drawn through the points from expt. 3 can easily be attributed to experimental error in reading the plates recording sedimentation equilibrium at this low concentration. These errors, amounting to about a 2% error in dc'/dr are unfortunately not random; bias regarding the center of the schlieren diagram can be retained from point to point. The agreement between $\bar{M}_w = 5100$ and $\bar{M}_r = 5400$ is also good evidence for a high degree of homogeneity. These values are also in reasonable agreement with the known molecular weight of the monomer which we presume to be $M(\text{ACTH}\cdot\text{Cl}_4) = 4690$. The limiting value of $\bar{M}_{w(r)}(\text{app.})$ at infinite dilution is 4350. With correction for charge effects according to equation 5, this becomes $\bar{M}_{w(r)} = 4890$. The most obvious sources of error would include (1) improper choice of net charge Z , (2) incorrect partial specific volume or (3) presence of a trace of polymer—a 1/2% octamer would be sufficient to account for these discrepancies.

Figure 3 shows a plot of $\bar{M}_{w(r)}(\text{app.})$ as a function of concentration for three experiments, 5, 6 and 7 performed at pH 10.0 in the carbonate-bicarbonate buffer at 0.1 ionic strength. If we

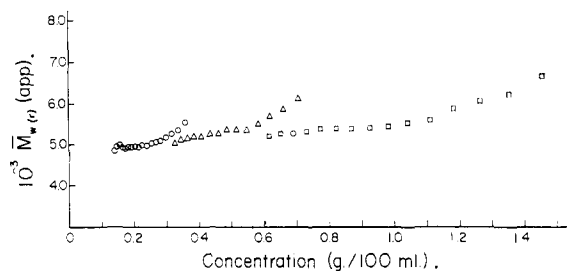


Fig. 3.—Sedimentation equilibrium of α_8 -ACTH in pH 10.1 carbonate-bicarbonate buffer of ionic strength 0.100. The points indicated by the symbol O were calculated from expt. 5 and those by Δ from expt. 7 and those by \square from expt. 6 (see Table I).

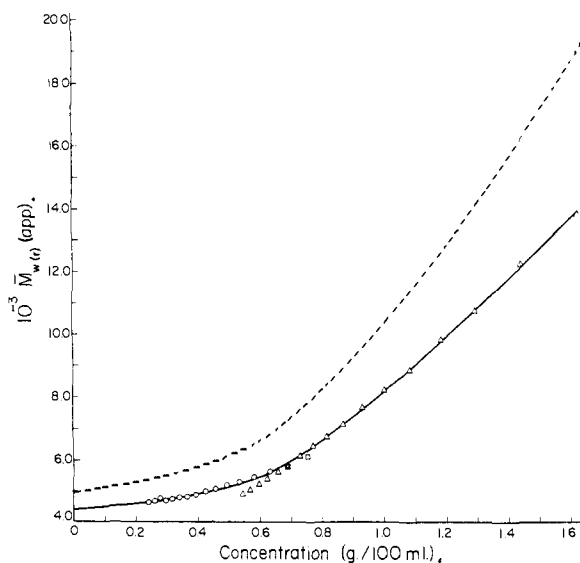


Fig. 4.—Sedimentation equilibrium of α_8 -ACTH in a pH 3.5 formate buffer of 0.200 ionic strength. The points indicated by the symbol O were calculated from expt. 8 and those by Δ from expt. 9 (see Table I). A smooth line drawn through all the points is presented as a solid line, and correction for charge effects by equation 5b, assuming $z = 5$, gives M_p as a function of c , represented by the dashed line ---.

apply the interpretation presented above, we conclude that the sedimentation equilibrium diagram displays appreciable heterogeneity, but judging from the variation in the activity coefficient $d \ln y/dc$, recorded in Table I, there is no evidence of reversible association.

The results of two experiments in which we studied the sedimentation equilibrium of ACTH in a formate buffer of pH 3.28 and 0.200 ionic strength are recorded in Fig. 4. (The pH of the solution was 3.52 in expt. 8 and 3.46 in expt. 9.) A smooth curve can be drawn through most of the experimental points; the slight lack of congruence of the data from these two experiments can be attributed possibly to microcomparator error or more likely to a trace of irreversibly aggregated ACTH in the sample, but it is apparent that the bulk of the polypeptide is in a state of reversible equilibrium with monomer.

The sedimentation coefficients calculated from two series of sedimentation velocity experiments

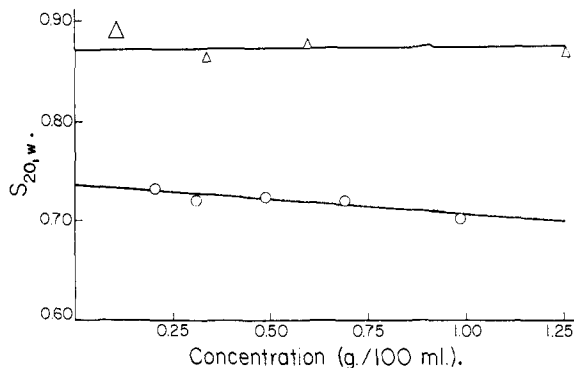


Fig. 5.—Dependence of the converted sedimentation coefficient of ACTH, in Svedberg units ($s_{20,w}$), upon concentration at pH 1.3 (O), and pH 10.1 (Δ). An enlarged Δ symbol representing one of the points reflects a greater error in the slope of $d \log r/dt$ in the calculation of this value.

are plotted against protein concentration in Fig. 5. The sedimentation coefficients have been converted to $s_{20,w}$ and expressed in terms of Svedberg units. In the one series, the buffer was HCl-KCl of pH 1.3, and in the other, carbonate-bicarbonate of pH 10.1. The sedimentation coefficient in the HCl-KCl buffer can be represented, as a function of concentration, by the equation: $s = 0.736(1 - 0.0393c)S$, with a standard deviation of 0.0042. The peaks were symmetrical throughout all the experiments at pH 1.3, and the sedimentation coefficient was calculated from the rate of movement of the peak bisector.²¹

The sedimentation velocity studies at pH 10.1, done on the same sample of ACTH, yielded schlieren peaks which were slightly skewed in the sense that the trailing edge was steeper than the leading edge. Again the sedimentation coefficients were calculated from the rate of movement of the peak bisector. The sedimentation coefficients calculated from these studies, also plotted in Fig. 5, are significantly higher than those calculated from the pH 1.3 experiments, but again, little concentration dependence is observed.

Diffusion coefficients obtained from both the sedimentation velocity and sedimentation equilibrium experiments at pH 1.3 are plotted as a function of the initial polypeptide concentration in Fig. 6, and a representative plot of the data used in the calculation of the diffusion coefficient by the method of heights and areas is presented in Fig. 7.

The values calculated by the two methods are in very good agreement, although there seems to be somewhat more scatter in the data calculated from the sedimentation velocity experiments. The regression line for the dependence of the diffusion coefficient on concentration (Fig. 6) is given by the equation

$$D_{20,w} = [13.19 (1 - 0.0788c) \pm 0.27] \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$$

(21) The sedimentation coefficient was also calculated from the rate of movement of the first moment of the schlieren peak photographed during the experiment in which the protein concentration was 0.99%. The two values differed by only 1%. The values based on the peak bisector were those used, however, in order to minimize errors arising from diffraction fringes which distort the extremities of the schlieren peak.

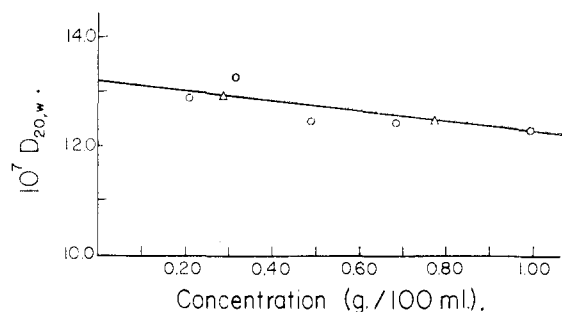


Fig. 6.—Diffusion coefficients of ACTH, corrected to 20°, in water, are presented here as a function of the initial polypeptide concentration of the experiment. Values indicated by the symbol Δ were calculated from sedimentation equilibrium experiments and those by O from sedimentation velocity experiments. The buffer used in all experiments was the pH 1.3 HCl-KCl buffer of 0.200 ionic strength.

where the precision of the data is expressed in terms of standard deviation.

Discussion

Several sensitive criteria of purity have been applied to the ACTH sample II in the course of the sedimentation equilibrium and sedimentation velocity studies at pH 1.3. The calculated values of $\bar{M}_{w(r)}$ (app.), for a homogeneous protein sample, may be a function of concentration but should not change with r , except to the extent that c changes with r . That this condition is met, or very nearly so, is demonstrated in Fig. 2, and the sensitivity of this criterion may be judged by comparing Fig. 2 with Fig. 1, which corresponds to the plot one would expect if the ACTH sample were contaminated with 13% of another substance having a four times greater molecular weight. According to this criterion, sample II may be judged pure since any discrepancy falls within the range of experimental error.

According to the second criterion, the weight average molecular weight and the z average molecular weight should equal the molecular weight of the monomer. This condition is not completely fulfilled since the three averages show some discrepancy, *i.e.*, $M(\text{ACTH}\cdot\text{Cl}_4) = 4690$, $\bar{M}_w = 5100$, $\bar{M}_z = 5400$. These data could be taken as an indication of the possible presence of a trace of a contaminant of a higher molecular weight, possibly an ACTH association product, but it is also important to point out that all three of these values are somewhat dependent upon the choice of the net charge Z , which was somewhat arbitrary. The titration data of Léonis and Li⁹ as well as the known amino acid composition of α_s -ACTH,² indicate a net charge of 9 protons, at pH 1.3, in the absence of ion binding. A comparison of the computed isoionic point, pH 8.5, with the determined isoelectric point, pH 6.6⁸ suggests that two anions are bound at pH 6.6. Studies of anion binding as a function of pH (*cf.*, for example ref. 22) show an increase in anion binding as the pH is decreased. We have assumed that an additional 2 anions are bound at pH 1.3, giving a

(22) G. Scatchard, Y. V. Wu and A. L. Shen, *J. Am. Chem. Soc.*, **81**, 6101 (1959).

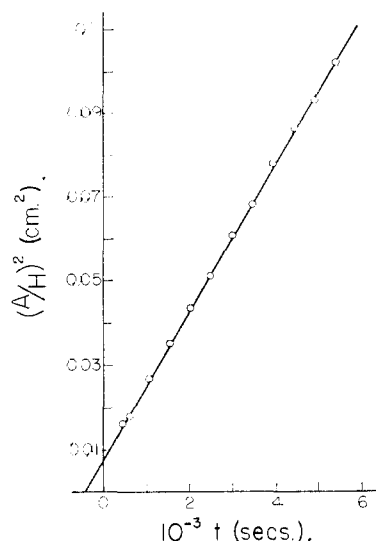


Fig. 7.—Calculation of the diffusion coefficient from a sedimentation velocity experiment by the method of heights and areas. The protein concentration was 0.99 g./100 ml. in HCl-KCl pH 1.3. The straight line relationship observed here was common to all the experiments.

net charge of 5 and resulting in a monomer having the composition $\text{ACTH}\cdot\text{Cl}_4$. If anion binding is greater than we have assumed, the discrepancy in the above figures would be decreased.

If the sample were homogeneous, the limiting value of $\bar{M}_{w(r)}$ (app.) at infinite dilution should be that of the pure monomer. This value after correction for charge effects is 4890, in somewhat better agreement with the known molecular weight of the monomer than \bar{M}_w and \bar{M}_z .

Finally, the plot used in the calculation of the diffusion coefficient from sedimentation velocity data should give a straight line for a homogeneous macromolecular solute. An examination of Fig. 7 shows that this criterion is also fulfilled.

If the sample were homogeneous, the molecular weight calculated from the sedimentation and diffusion coefficients would agree with the known molecular weight of the monomer. The molecular weight calculated by the Svedberg equation

$$M = \frac{Rts}{D(1 - \bar{v}\rho)}$$

with $s_{20,w}^0 = 0.736S$ and $D_{20,w}^0 = 13.19 \times 10^{-7}$ cm.²/sec. calculated from the sedimentation equilibrium studies, is $M = 4790$. In this instance, the agreement is quite satisfactory.

Having demonstrated the high degree of homogeneity of the sample, we would like to draw some conclusions from its hydrodynamic behavior. In calculating the frictional ratio, we can calculate the molecular frictional coefficient of the equivalent anhydrous sphere, f_0 from the known molecular weight, using the equation

$$f_0 = 6\pi\eta \left(\frac{3M\bar{v}}{4\pi N} \right)^{1/3}$$

where η is the viscosity of water at 20° (0.01005 poise). Taking $M = 4690$ and $\bar{v} = 0.717$, we obtain $f_0 = 2.085 \times 10^{-8}$.

The experimentally determined molecular frictional coefficient f can be calculated from the sedimentation coefficient, s , using the equation, $f = M(1 - \bar{v}\rho)/Ns$, and from the diffusion coefficient, with the equation $f = Rt/ND$; thus, the frictional coefficient calculated from the sedimentation coefficient ($s_{20,w}^0 = 0.736S$) is 3.01×10^{-8} and that calculated from the diffusion coefficient ($D_{20,w}^0 = 13.19 \times 10^{-7}$), 3.07×10^{-8} . The two values of f are in excellent agreement. From the mean value, 3.04×10^{-8} , a frictional ratio, f/f_0 for ACTH of 1.46 is obtained.

The interpretation of the frictional coefficient in terms of shape parameters usually requires an assumed model. The model usually employed for globular proteins is that of a hydrated ellipsoid of revolution.²³ If we assume that the molecule is hydrated to the extent of 20% and calculate the axial ratio of the "equivalent prolate ellipsoid of revolution," we find that this frictional ratio corresponds to an axial ratio of 7:1. It should be pointed out that the pronounced asymmetry of the polypeptide at this pH is very likely due to intramolecular electrostatic repulsions. Of the 39 amino acids, residues number 1, 6, 8, 11, 15, 16, 17, 18 and 21 from the NH₂-terminal end² could carry a positive charge at pH 1.3. Perhaps the true configuration would be more like a semi-rigid polypeptide of 21 amino acids attached to a random coil of 18 amino acids. Important also in this regard is the observation by Léonis and Li^{9b} that the shape factor w , calculated from the titration data, differs considerably in the acidic and basic portions of the titration curve, indicating an appreciable deformability of the molecule.

In an attempt to determine the association equilibria which are involved at pH 3.5, as well as the corresponding equilibrium constants, we encounter a serious theoretical limitation. Our basic data are in terms of the concentration dependence of the weight average molecular weight, but a small part of this concentration dependence may be due to interactions other than association of the macromolecular solute. Since we have not been able to find a way to subtract the other effects, we have neglected this correction and treated the entire variation in the apparent weight average molecular weight in terms of association, in order to estimate the association constants.

The method used in determining the association equilibria and calculating the corresponding equilibrium constants is similar to the approach used by Steiner²⁴ and Rao and Kegeles²⁵ in their studies of protein associations by other experimental methods. We first attempted to interpret the association reaction in terms of a single association reaction



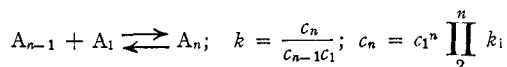
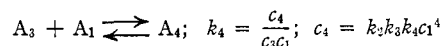
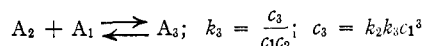
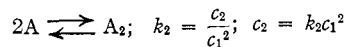
where n , the degree of association, is to be determined experimentally, and the corresponding equilibrium constant, k_n , is defined by the equation

$$k_n = \frac{c_n}{c_1^n} \quad (11)$$

where c_1 and c_n are the concentrations of monomer and n -mer on the g./100 ml. concentration scale.

This approach was completely unsuccessful inasmuch as it was impossible to fit the experimental data with a single association constant regardless of the choice of n .

The remaining alternative is to treat the data in terms of stepwise association equilibria of the form



The weight average molecular weight is given by the relation

$$\bar{M}_w = \frac{\sum c_i M_i}{\sum c_i} = \frac{c_1 M_1 + 2k_2 c_1^2 M_1 + 3k_2 k_3 c_1^3 M_1 + \dots}{c} \quad (12)$$

where c is the over-all macromolecular concentration on the g./100 ml. concentration scale and c_1 is the monomer concentration on the same scale. If $\bar{\alpha}_w$ is introduced as the weight average degree of association, we have

$$c\bar{\alpha}_w = \frac{c\bar{M}_w}{M_1} = c_1 + 2k_2 c_1^2 + 3k_2 k_3 c_1^3 + \dots + c_1^n \prod_2^n k_i \quad (13)$$

Following the approach of Steiner²⁴ we define x as the weight fraction of monomer. Then $c_w = xc + 2k_2(xc)^2 + 3k_2 k_3(xc)^3 + \dots +$

$$n(xc)^n \prod_2^n k_i \quad (14)$$

and we evaluate x by graphical integration of the function (see Table II)

$$x = \int_0^c \frac{(\alpha^{-1} - 1)}{c} d\alpha \quad (15)$$

The first association constants, k_2 , may then be evaluated from the power series

$$F_1 = \frac{\bar{\alpha}_w - 1}{xc} = 2k_2 + 3k_2 k_3(xc) + 4k_2 k_3 k_4(xc) + \dots + \quad (16)$$

by plotting the function F_1 as a function of (xc) and taking the intercept as $(xc) \rightarrow 0$ as equal to $2k_2$. The first association constant calculated in this manner is 0.30. The second constant may be evaluated from the function

$$F_2 = \frac{F_1 - 2k_2}{xc} = 3k_2 k_3 + 4k_2 k_3 k_4(xc) + \dots \quad (17)$$

in a similar manner. The value obtained for k_3 is 0.33.

Because of the accumulation of errors in this type of analysis as higher terms are evaluated,

(23) J. T. Edsall in "The Proteins," Vol. IB, Neurath and Bailey, Eds., Academic Press, Inc., New York, N. Y., 1953, pp. 549-726.

(24) R. F. Steiner, *Arch. Biochem. Biophys.*, **39**, 333 (1952).

(25) M. S. N. Rao and G. Kegeles, *J. Am. Chem. Soc.*, **80**, 5724 (1958).

TABLE II

ESTIMATION OF THE ASSOCIATION CONSTANTS OF ACTH IN pH 3.5 FORMATE BUFFER OF IONIC STRENGTH 0.200

Concn., g./100 ml.	$\bar{\alpha}_w$	$\frac{\bar{\alpha}_w^{-1} - 1}{c}$	x	xc
0.0	1.000		1.000	
.2	1.069	0.32	0.937	0.187
.4	1.172	.37	.873	.349
.6	1.343	.43	.805	.483
.8	1.665	.50	.729	.583
1.0	2.099	.52	.657	.657
1.2	2.604	.51	.593	.712
1.4	3.162	.49	.537	.752
1.6	3.779	.46	.489	.782

we have not attempted to calculate the higher association constants. Instead, we have attempted to treat the data in terms of intrinsic association constants, assuming that all the constants have the same value, in order to discover whether a single intrinsic association constant, \bar{K} , could be found which would fit the data over the entire concentration range. In this analysis equation 14 assumes the form

$$\frac{\bar{\alpha}_w}{x} - 1 = 2Kxc - 3(Kxc)^2 + 4(Kxc)^3 + \dots + (n-1)(Kxc)^n \quad (18)$$

The left-hand side of the equation may be solved for a given value of the product xc , and the corresponding value of the product Kxc may be determined graphically from a plot of the right-hand side of the equation as a function of Kxc . The mean equilibrium constant for a given value of xc may then be calculated from the relation

$$\bar{K}(c) = \frac{Kxc}{xc} \quad (19)$$

The results of this analysis appear in Table III. Here we may see that no single value for an intrinsic association constant fits over the entire concentration range. Instead, the mean association constant increases with concentration starting in the low concentration range with 0.34, in good agreement with the first association constant, k_2 , calculated previously. From the increase in the calculated value of the mean association constant, with increase in concentration, we may infer that the successive association constants have increasingly larger values.

We should point out that the choice of concentration scale used in these calculations, the g./100 ml. scale, while not conventional, is in conformance with the suggestions of Rao and Kegeles²⁴ who

TABLE III

VARIATION OF THE AVERAGE ASSOCIATION CONSTANT WITH CONCENTRATION

xc	c , g./100 ml.	No. of terms ^a	\bar{K}
0.10	0.11	2	0.34
.35	.40	4	.40
.52	.66	6	.50
.55	.72	7	.55
.63	.91	8	.63
.70	1.17	15	.65

^a Here are recorded the number of terms in the power series which make a significant contribution to the sum.

pointed out the desirability of using standard states that are experimentally realizable. Taking 0.33 as the first association constant (dimerization), we calculate a standard free energy change

$$\Delta F^0 = -Rt \ln K = +64 \text{ cal.}$$

This may be compared with a value of +70 cal. calculated by Rao and Kegeles²⁴ for the dimerization of α -chymotrypsin but converted to the concentration scale and direction of reaction used here. It is interesting to note that k_3 for chymotrypsin is less than k_2 while the successive association constants of ACTH appear to increase.

The results of the sedimentation experiments at pH 10.1 demonstrate a small amount of irreversible polymer formation at this pH. The sample which appeared to be homogeneous at pH 1.3 revealed only a very small amount of polymer when the sample had been in contact with the pH 10.1 buffer only for the duration of the experiments, but the sample which had been prepared by dialysis in basic medium contained about 13% tetramers. We are not sure whether this higher content of polymerized material is due to longer contact with a basic buffer or whether the polymerization occurred during lyophilization from basic solution. In any case, the extent of polymer formation is not sufficient to explain the nondialyzability of ACTH in basic solution; some other explanation must be found, perhaps in terms of repulsive interactions between the negatively charged polypeptide and the negatively charged membrane.

Acknowledgments.—Appreciation is expressed to Mrs. Elizabeth Russell for her able technical assistance in the microcomparator work and for some of the calculations and to Dr. Robert R. Baldwin for several fruitful discussions during the course of the investigation. This work was supported in part by a grant from the National Institutes of Health of the United States Public Health Service (RG-2907).