The value of 6.5, which is obtained for the pK of this group when the correction for the effect of urea is applied, falls into the range of normal values for imidazole side chains in proteins, 6.4 to 7.0,[10] and is far enough out of the range of normal values for α-amino groups, 7.4 to 7.9,[10] that tentative identification of G_4 as an imidazolium group is reasonable.

Since salts reduce the rates of denaturation without increasing the activation energy, the influence of electrostatic forces, as discussed by Kauzmann,[24] must be considered in any explanation of the pH dependence of the rates of denaturation. It is obvious that the increases in rate on both sides of pH 5.7 are not due to the electrostatic repulsion of charges on the molecule as a whole; if this were the case the rate would be minimal at the isoelectric pH, which is above 9.[18] Also, the bonds holding the molecule together are probably not electrostatic, or the rate would increase at pH 5.7 upon the addition of salt. However, the possibility that electrostatic forces contribute to the weakening of critical bonds in a small portion of the molecule at pH values on either side of 5.7 is not eliminated. Such a portion of the molecule would correspond to the "critical seam" in the theory of London, *et al.*[25]

It is interesting to note that Eisenberg and Schwert found that the extent of reversible heat denaturation of chymotrypsinogen is related to the ionization of three acid groups.[26] It would seem reasonable that these are the same groups found to be critical in the urea denaturation. Since the mechanism of the two types of denaturation are not necessarily the same and since the values for heat denaturation were determined under very different conditions and from equilibrium constants rather than rate constants, the difference in apparent pK values found (pK 2.5 found for heat denaturation) is not surprising.

ADDED IN PROOF.—After this work was accepted for publication a private communication was received from Dr. P. E. Wilcox describing the results of prior experiments on the titration of denatured chymotrypsinogen. These data show that after denaturation in 4 M guanidinium chloride three additional protons are taken up by the protein in the pH region of carboxylic acid ionization.

(24) W. Kauzmann, in W. D. McElroy and B. Glass, "The Mechanism of Enzyme Action," The Johns Hopkins Press, Baltimore, Md., 1954, p. 70.

(25) M. London, R. McHugh and P. B. Hudson, *Arch. Biochem. Biophys.*, **73**, 72 (1958).

(26) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).

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

The Sedimentation Behavior of Human Pituitary Growth Hormone

BY PHIL G. SQUIRE AND KAI O. PEDERSEN[1]

RECEIVED JULY 15, 1960

An attempt to determine the molecular weight of human pituitary growth hormone was complicated by the fact that molecular association of the protein occurred between pH 2 and 10. Sedimentation velocity experiments, performed at protein concentrations ranging from 0.53 to 36.0 g./l. in a phosphate buffer of pH 2.38 revealed in addition to the major component the presence of material with a higher sedimentation coefficient. The amount of rapidly sedimenting material increased with total protein concentration, and the weight average sedimentation coefficient calculated from the rate of movement of the second moment of the entire schlieren diagram also had a strong positive slope, both observations indicating an association that is dependent upon concentration. Values for the molecular weight of the hormone were calculated from studies of the approach to equilibrium (Archibald procedure). The values calculated from the top and bottom menisci did not follow the same smooth curve when plotted as functions of concentration, suggesting that the reaction rate was slow compared with the time of the experiment (1–2 hr.). The best values for the molecular weight of the monomer were obtained by extrapolating the upper miniscus values to infinite time and by extrapolating the zero time values to infinite dilution. Both methods give a value of about 29,000 as the molecular weight of the hormone.

Introduction

Marked biological and physicochemical differences have been demonstrated between growth hormone preparations obtained from bovine and human pituitaries.[2,3] Preliminary investigations of the molecular weight of human growth hormone have been reported from three different laboratories. The results of Ehrenberg and Heijkenskjöld[4] obtained by sedimentation velocity, and Ehrenberg's application[5] of the Archibald procedure, showed that the sample was quite heterogeneous. These investigators drew the conclusion that the molecular weight was 15,000–20,000, but they pointed out that the sample also contained material of higher molecular weight. Li and Papkoff[6] reported sedimentation velocity experiments carried out in a phosphate buffer of pH 2.3, in which a sedimentation coefficient of 2.47 S was obtained. Using this value and a diffusion coefficient determined from an independent experiment in the same buffer, they calculated a molecular weight of 27,100. The sedimentation velocity studies of Lewis and Brink[7] yielded results which were substantially in agreement with those reported by Li and Papkoff.[6]

The availability of additional material has permitted a more extensive study of the molecular weight than had been possible previously. These studies have demonstrated that the hormone undergoes a reversible association reaction, as evidenced by sedimentation behavior. The molecular weight of the hormone has been determined by the Archibald procedure.

(1) University of Uppsala, Sweden.

(2) C. H. Li, *Federation Proc.*, **16**, 775 (1957).

(3) C. H. Li, H. Papkoff and C. W. Jordan, Jr., *Proc. Soc. Exptl. Biol. Med.*, **100**, 44 (1959).

(4) A. Ehrenberg and F. Heijkenskjöld, *Acta Chim. Scand.*, **10**, 1675 (1956).

(5) A. Ehrenberg, *ibid.*, **11**, 1257 (1957).

(6) C. H. Li and H. Papkoff, *Science*, **124**, 1293 (1956).

(7) U. J. Lewis and N. G. Brink, THIS JOURNAL, **80**, 4429 (1958).

Experimental

Material and Methods.—The growth hormone used in these studies had been prepared from human pituitary glands by the method previously described.[2,6] The tests of purity performed by means of electrophoresis and chromatography and the physicochemical characterization indicate that the hormone possesses a high degree of molecular homogeneity.[2,8] Ribonuclease and α-chymotrypsinogen were employed as reference proteins for sedimentation experiments. The ribonuclease was a commercial product (Armour, lot no. 381–059); the crystalline α-chymotrypsinogen was kindly supplied by Dr. H. Neurath.

In most of the experiments, the weighed sample was dissolved in a known weight of solvent and the concentration was calculated directly. In a few of the earlier experiments, however, the sample was dissolved in water, one drop of 0.2 *M* HCl was added, and the sample was dialyzed overnight against the buffer. In these experiments, the concentration was calculated from the optical density measured in a Beckman Spectrophotometer, model DU, at 278 mμ (extinction coefficient, $E^{1\%}_{278m\mu} = 8.3$).

Sedimentation analysis of human growth hormone was performed in a phosphate buffer of *p*H 2.3 and ionic strength 0.11 (0.100 *M* in NaH_2PO_4) and in a borate buffer of *p*H 9.94 and ionic strength 0.24 (0.100 *M* H_3BO_3, 0.150 *M* NaCl and 0.09 *M* NaOH). Sedimentation of α-chymotrypsinogen was performed in a *p*H 3.20 glycine buffer consisting of 0.05 *M* glycine, 0.15 *M* NaCl and 0.0125 *M* HCl. A solution composed of 0.020 *M* Na_2HPO_4 and 0.100 *M* NaCl was used in the sedimentation analysis of the ribonuclease sample.

Sedimentation was performed in the Spinco model E ultracentrifuge equipped with schlieren optics, with a Wolter phase plate as the schlieren diaphragm. The temperature was regulated and measured by means of the "RTIC" unit which had been calibrated at rest. In each experiment, odometer readings were taken at the beginning and end of the time at which the rotor is at full speed. In all cases, the speed calculated from the odometer readings agreed with the set speed to within 0.1%. The radial magnification factor, determined by photographing the ruled disc supplied by the manufacturers, was found to be 2.020.

Most of the sedimentation velocity experiments were carried out in a capillary type synthetic boundary cell. The capillary was placed 9 mm. from the bottom of the cell, a position which was found very nearly optimal for these studies. Within this cell, it was possible to determine sedimentation constants at concentrations as low as 0.53 g./l. When samples having a very low protein concentration were run, the precaution was taken of accelerating very slowly (3 amps. drive) during boundary formation inasmuch as we had only a slight density gradient to stabilize the boundary.

The cells used in most of the experiments contained double-sector aluminum-filled epoxy centerpieces. To rule out possible involvement of Al^{+++} ions in the association phenomenon, an Archibald experiment was performed with a Kel F centerpiece. No significant difference in results was observed.

The schlieren image was photographed on metallographic plates with an exposure of 3 seconds and was developed in D-19 photographic developer for six minutes. A Gaertner toolmakers microcomparator, with a two-way movement and a projection attachment, was used to read the photographic plates. Following the nomenclature suggested by Trautman,[9] radial distances in the cell, from the axis of rotation, are indicated by the letter r. The corresponding abscissa obtained by microcomparator readings on the plate is given the symbol X, and the plate is positioned on the microcomparator so that $X = mr$ where m is the radial magnification factor. The displacement of the diaphragm image from the baseline, which is proportional to the refractive index gradient dc/dr and is obtained as a microcomparator reading, is designated ΔY.

Treatment of Sedimentation Velocity Data.—Analysis by sedimentation velocity of samples with a protein concentration greater than a few tenths of a per cent. revealed the presence of a small amount of material which sediments much more rapidly than the bulk of the protein (see Fig. 1). Since the entire schlieren diagram can be resolved into a large symmetrical peak preceded by an additional increment of faster-sedimenting material, the sedimentation coefficient of the "slow component" (s_s) was calculated from the rate of movement of the maximum ordinate. A mean sedimentation coefficient of the fast material (s_f) was computed from the rate of movement of the center of the area of the remaining portion of the schlieren diagram. In addition, the weight average sedimentation coefficient (s_w) was calculated from the rate of movement of the square root of the second moment of the entire schlieren diagram.[10] Inasmuch as calculation of the second moment involves calculation of the term $X^2 \Delta Y$ for intervals of X, it was convenient to plot these values as a function of X; hence, the diagrams in Fig. 1 are corrected for dilution.

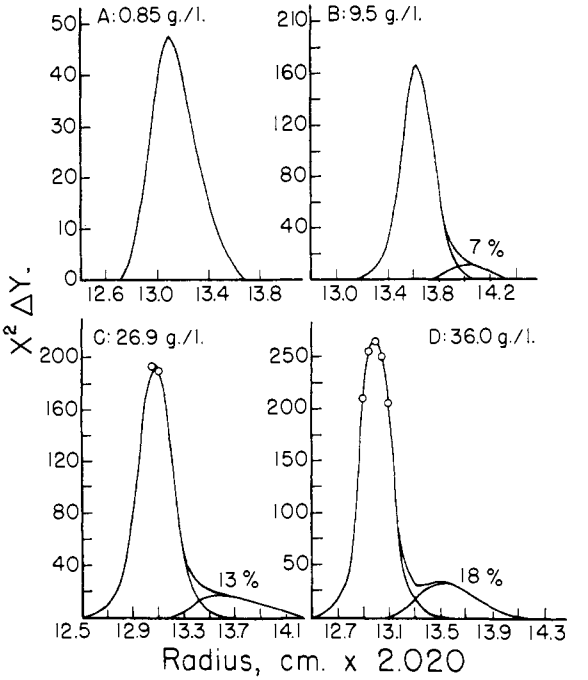

Fig. 1.—Sedimentation diagrams of human growth hormone obtained during sedimentation velocity experiments over a wide range of protein concentration. The abscissa is the radius of rotation in cm. × 2.020 and the ordinate the product of the displacement of the schlieren diagram from the baseline (ΔY) and the radial distance squared (x^2). The total protein concentrations and the percentage of fast material are given in the diagrams. Circled values were introduced by interpolation (see text).

Calculations of $s_{\bar{w}}$ and s_f were limited to data from the first 30 minutes of sedimentation, owing to the disappearance of a "plateau region" after this time. Furthermore, calculation of moments from the early exposures at high concentrations presented a problem since the tops of the peaks were not in view. Since, however, these experiments at higher concentration (3.60, 2.69 and 1.79%) were performed on samples diluted quantitatively from the same stock solution, the value for $X^2 \Delta Y$ as a function of time could be calculated from the experiment at 1.79%.[11] The peak tops of early pictures were thus constructed by applying the requirement that $\Sigma X^2 \Delta Y$ for that entire diagram should equal the calculated value. The interpolated values are denoted by circles in Fig. 1.

(8) C. H. Li, "Symposium on Protein Structure," A. Neuberger ed., Methuen and Co., Ltd., London, Eng., 1958, p. 302.

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

(10) R. J. Goldberg, *ibid.*, **57**, 194 (1953).

(11) The function $\Sigma x^2 \Delta Y$ was plotted as a function of time for all experiments. This plot was not constant but decreased slightly, due presumably to the presence of a small amount of protein in a very high state of aggregation and for a given time of sedimentation was proportional to the initial concentration. Therefore, the expected value for $\Sigma x^2 \Delta Y$ for the early times before the entire peak was visible was obtained by extrapolation.

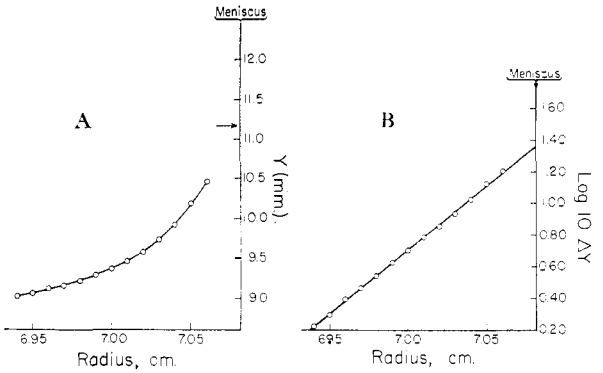

Fig. 2.—(A) Microcomparator readings of the schlieren diagram near the bottom meniscus as a function of radial distance in order to illustrate the difficulty of extrapolating these values to the bottom meniscus. The picture was taken with a diaphragm angle of 70°, after 102 minutes of sedimentation at 7447 r.p.m. The initial protein concentration was 10.4 g./l. The arrow indicates the extrapolated value obtained from the logarithmic plot as shown in B. (B) The logarithm of the displacement of the schlieren diagram from the baseline as a function of the corresponding radial distance (data, same as A).

Determination of Molecular Weights by the Archibald Procedure.—The experiments which were designed to give the molecular weight from observations taken during the approach to equilibrium (the Archibald procedure) were carried out essentially as described by Schachman.[12] From any exposure, a value for the molecular weight of the protein can be calculated from measurements of the refractive index gradient near the meniscus and near the bottom of the solution. The two values for the molecular weight are designated M_m and M_b, respectively, and are calculated from the equations

$$M_m = \frac{RT}{(1 - \bar{v}_p)w^2} \frac{(dc/dr)_m}{C_m r_m}$$

$$M_b = \frac{RT}{(1 - \bar{v}_p)w^2} \frac{(dc/dr)_b}{C_b r_b}$$

The refractive index gradients at the two limits of the cell must be obtained by extrapolation of ΔY to the meniscus (for $(dc/dr)_m$) and to the outer limit of the protein solution (for $(dc/dr)_b$).

In experiments with ribonuclease and α-chymotrypsinogen, the plotted values of ΔY as function of r fell on a curve with a gentle slope and were easily extrapolated to the top meniscus, although the extrapolation to the bottom meniscus was somewhat more uncertain. In the case of the human growth hormone, the curve to the upper meniscus also had only a slight slope, but the plot of ΔY against r near the bottom meniscus had a pronounced curvature (Fig. 2A) and extrapolation could not be done with certainty. If the logarithm of ΔY is plotted as a function of r, then the points usually fall on a nearly straight line (Fig. 2B) and can easily be extrapolated to the bottom. Consequently, the logarithmic plot was used to calculate the molecular weights of the human growth hormone on the basis of data derived from the bottom of the cell. Otherwise, our treatment of data was essentially according to Schachman[12] except that we made use of a "radius cubed scale," as recommended by Trautman[9] as an aid in performing the integration necessary for the calculation of C_m and C_b.

Results

Studies with Ribonuclease and α-Chymotrypsinogen.—Before human growth hormone was investigated by the Archibald method, it seemed prudent to apply this method to the study of one or two well-characterized proteins. For these studies we used ribonuclease and α-chymotrypsinogen.

The ribonuclease was studied at a 1.0% concentration in a solution containing 0.100 M NaCl and 0.020 M Na_2HPO_4. The rotor temperature was 22.8° and the speed was 11,573 r.p.m. Carbon tetrachloride was layered under the protein solution as recommended by Klainer and Kegeles.[13] The exposures used in the calculation of the experimental results were taken at intervals between 45 and 193 minutes after the rotor had reached full speed. Results are summarized in Table I; two different methods were employed to analyze the plates; namely, pencil drawings of the enlarged exposures (giving a 10.00-fold enlargement of the cell coördinates) or reading of the plates with the microcomparator. The first method gave, from 3 exposures, values at the meniscus and bottom of M_m = 14,640 and M_b = 13,890, respectively, whereas the second method from 5 exposures gave M_m = 13,760 and M_b = 13,830. A Trautman plot[9] in which all the measurements were utilized gave M = 13,970. The molecular weight calculated from the amino acid analysis is 13,683.[14]

The α-chymotrypsinogen was studied as a 1.0% solution in a glycine buffer of pH 3.20 and ionic strength 0.16. The rotor temperature throughout the experiment was 26.10°, and the speed was 8766 r.p.m. The eight values for the molecular weight, calculated from the meniscus and bottom of the cell as recorded in four exposures during the run are shown in Table II. For purposes of comparison, the molecular weights calculated from the bottom of the cell were calculated with values of $(\Delta Y)_b$ obtained by plotting ΔY *vs.* r (direct plot) and by plotting log ΔY *vs.* r (log plot). There is no significant difference between the mean values for the experiment obtained by the two methods, nor is there any convincing evidence of a trend with time, or of a difference between M_m or M_b. This mean (± standard deviation) of the eight values for M_m and M_b (log plot) is 25,500 ± 1000. The value obtained by a Trautman plot[9] is 24,800. These values agree within 2% with the value 25,100 obtained by Wilcox, *et al.*,[15] from amino acid analysis.

Analysis of Human Growth Hormone by Sedimentation Velocity.—A series of twelve sedimentation velocity experiments were performed with phosphate buffer of pH 2.38 used as the solvent. The initial protein concentration ranged from 0.53 to 3.60 g./l. Sedimentation diagrams, after buffer baseline correction and correction for radial dilution, for four representative experiments, selected to represent the concentration range studied are given in Fig. 1. As indicated, the total diagram has been resolved into a "slow component," symmetrical around the maximum ordinate, and "fast material" containing the excess portion of the curve. The notable feature is the fact that the amount of fast material increases with total protein concentration, indicating that association of

(12) H. K. Schachman, in "Methods in Enzymology," Vol. IV, S. P. Colowick, ed., Academic Press, Inc., New York, N. Y., 1957, pp. 32–104.

(13) S. M. Klainer and G. Kegeles, *J. Phys. Chem.*, **59**, 952 (1955).

(14) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **221**, 151 (1956).

(15) P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. Biophys. Acta*, **24**, 72 (1957).

TABLE I

MOLECULAR WEIGHT OF RIBONUCLEASE[a]

Time (min.)	$Y_{(m)}$	Data from the meniscus C_m	$q' \times 10^6$	$-P$	M_m[b]	Y_b	Data from the bottom C_b	$q \times 10^6$	P	M_b[b]
45[c]	28.4	175.8	3.26	17.8	15,090	39.1	216.5	3.77	22.9	14,200
61	25.46	174.2	2.92	19.4	13,660	38.08	217.5	3.68	23.9	13,770
109[c]	26.4	166.5	3.03	27.1	14,820	39.3	227.9	3.79	34.3	13,560
109	24.55	167.1	2.82	26.5	13,730	39.59	226.6	3.82	33.0	13,740
125	24.85	165.2	2.85	28.4	14,050	39.19	228.6	3.78	35.0	13,830
173	23.14	161.7	2.65	31.9	13,370	40.85	234.6	3.94	41.0	13,700
189[c]	24.0	160.2	2.75	33.4	14,000	42.3	239.2	4.08	45.6	13,910
193	23.9	159.9	2.74	33.7	13,970	42.0	239	4.09	45.4	14,120

[a] Protein conc., 10 g./l.: solvent, 0.02 M Na_2HPO_4 in 0.100 M NaCl. The parameters q and q', used in constructing the Trautman plot,[9] are defined by the equations:

$$q' \text{ (meniscus)} = \frac{\Delta Y_m}{w^2 r_m} = -(s/D)_m \left[1.2967 \frac{\Delta z}{r^2_m} \Sigma \Delta Y_i\right] + (s/D)_m \Delta r \Sigma \Delta Y$$

$$q \text{ (bottom)} = \frac{\Delta Y_b}{w^2 r_b} = (s/D)_b \left[1.2967 \frac{\Delta z}{r^2_b} \Sigma \Delta Y_i\right] + (s/D)_b \Delta r \Sigma \Delta Y$$

The equation for calculating the molecular weight, from the Trautman plot, and our result for ribonuclease are

$$M = \frac{RT}{1 - \bar{v}\rho} \frac{q}{D} = \frac{RT}{1 - \bar{v}\rho} \frac{3.26}{193.6} = 13{,}970$$

[b] Molecular weight computed by the method of Klainer and Kegeles.[13] [c] Indicates plates read with a photographic enlarger all others were read with a microcomparator.

TABLE II

MOLECULAR WEIGHT OF α-CHYMOTRYPSINOGEN[a]

Time (min.)	Mol. wt. calcd. from the meniscus M(m)[a]	Mol. wt. calcd. from the bottom of the cell M_b (direct plot)[a]	M_b (log plot)[b]
126	26,000	24,000	25,800
190	25,700	23,500	24,500
254	24,100	25,000	26,200
308	24,700	26,100	26,800
Mean	25,100	24,700	25,800

[a] Protein concn. 10.0 g./l.; solvent, pH 3.20 glycine buffer of ionic strength 0.16; values of molecular weight were calculated by the method of Klainer and Kegeles.[13] [b] The values for ΔY used in the calculation of the molecular weight at the meniscus and those in column 3 were obtained by extrapolation of ΔY *vs.* r. [c] The values recorded in the fourth column were obtained by extrapolating log ΔY as a function of r.

monomers occurs. The percentage of polymers (fast material) is given in these diagrams,

The three sedimentation coefficients calculated from these experiments, s_s, s_f, and $s_{\bar{w}}$, are represented graphically in Svedberg units in Fig. 3. The regression line for s_s is given by the equation $s_s = 2.179 - 0.0522C (\pm 0.027)$, with a standard deviation of points around the line of regression of 1.5%.

The Archibald experiments provide evidence (*vide infra*) that the association–dissociation reactions are slow compared with the time of sedimentation (1–3 hr.); consequently it is essential to provide evidence that the sedimentation velocity experiments were done on systems which were at equilibrium at the start of the experiment. All the experiments in this series had been allowed to equilibrate in the sedimentation buffer for at least 18 hr. A portion of one of these samples at a protein concentration of 9.5 g./l. was studied by sedimentation velocity after 18 hr., and another portion of the same sample was submitted to sedimentation velocity studies six days later. There was no significant difference in the sedimentation diagrams, nor in the sedimentation coefficients. The two values for s_s are recorded in Fig. 3.

Calculation of the Molecular Weight of the Monomer by the Archibald Procedure.—The results obtained from an Archibald experiment at an initial protein concentration of 10.4 g./l. and at 2.6

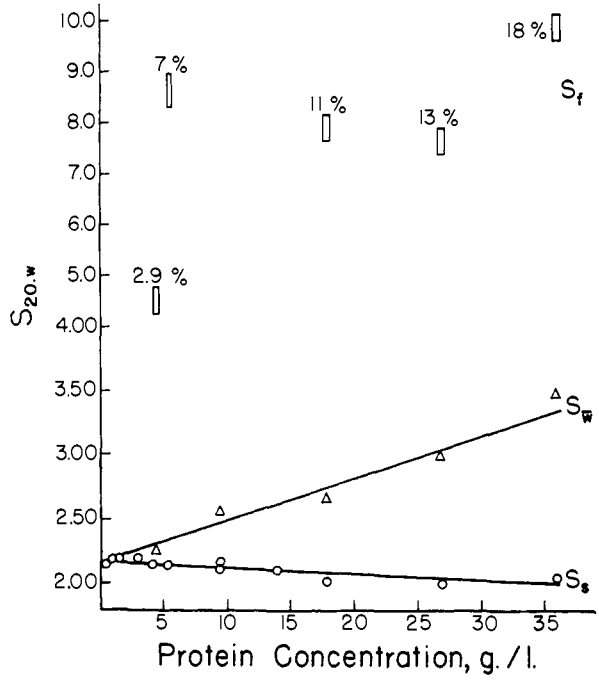

Fig. 3.—Concentration dependence of sedimentation coefficients. The sedimentation coefficient of the major component is designated s_s, and the mean sedimentation coefficient of the rapidly sedimenting material, s_f. The weight average sedimentation coefficient is designated $s_{\bar{w}}$.

g./l. in the buffer of pH 2.38 are presented in Figs. 4 and 5, respectively. Molecular heterogeneity is indicated by the lack of agreement of the molecular weight values obtained from the top M_m and bottom M_b. The downward trend of M_m values, especially in Fig. 4, indicates that fractionation and

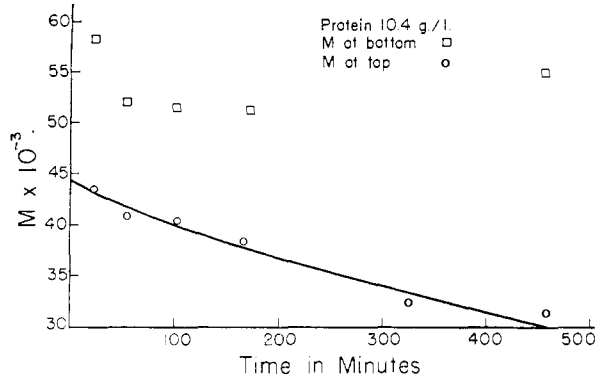

Fig. 4.—Analysis of human growth hormone by the Archibald procedure at an initial protein concentration of 10.4 g./l. Average molecular weights calculated from data obtained from the bottom (□) and from the top (O) are given as function of time.

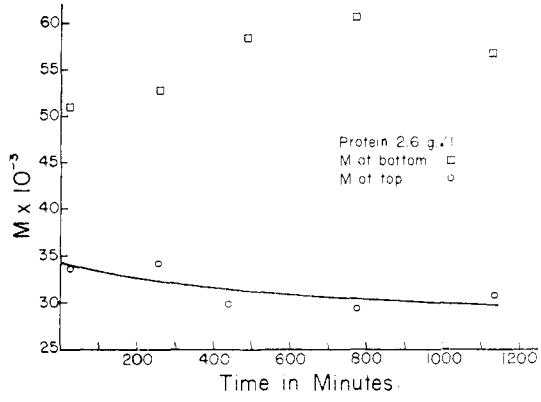

Fig. 5.—Analysis of human growth hormone by the Archibald procedure at an initial protein concentration of 2.6 g./l. Average molecular weight calculated from data obtained from the bottom (□) and from the top (O) are given as function of time.

dissociation is taking place during sedimentation. At a protein concentration of 2.6 g./l., however, the decrease in M_m with time is very slight.

As Rao and Kegeles have demonstrated,[16] one can ascertain whether the rate of association–dissociation reaction is slow or fast, compared with the time of sedimentation, by plotting the molecular weight obtained at the top and bottom against concentration. If the equilibrium is rapid, the molecular weights plotted against concentration should fall on the same curve, regardless of whether they were calculated from the top or from the bottom. The data these investigators report for α-chymotrypsinogen do this, or nearly so. The data obtained with human growth hormone, when plotted in the same manner, do not, as may be seen in Fig. 6. Apparently the association-dissociation reactions do not attain equilibrium during the sedimentation experiment.

If we can assume that the system is at equilibrium at the start of the two Archibald experiments, then the molecular weight can be calculated by extrapolating these values to infinite dilution. Due to errors inherent in this extrapolation, the extrapolated

(16) M. Rao and G. Kegeles, THIS JOURNAL, **80**, 5724 (1958).

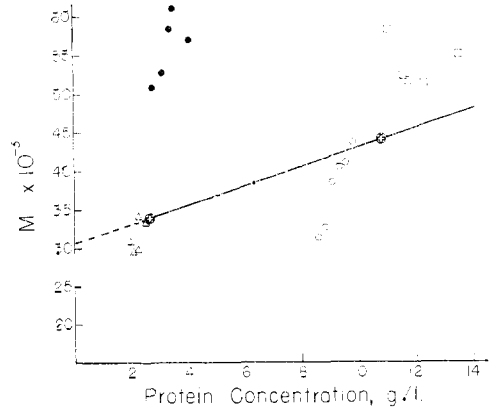

Fig. 6.—Molecular weights obtained from the bottom (□) and top (O) in the experiments reported in Fig. 4 and those from the bottom (●) and top (△) in Fig. 5, as a function of concentration. The average molecular weight values calculated from data at the top of the cell, and extrapolated to zero time of sedimentation, are denoted by ⊕.

value, 30,000, could be in error in either direction. From the shape of the theoretical curve for M_w *vs.* C,[16] this value, obtained by the two-point extrapolation, is more likely to be too high than too low.

A second estimate of the molecular weight of the monomer can be made from the top meniscus after sedimentation is carried out for a long period of time at a low concentration. Since the material of high molecular weight has not been completely removed from the top of the cell, this value would be in the direction of being too high. Thus, 30,000 can probably be set as the upper limit for the molecular weight.

A lower limit can be calculated by making a generous estimate of the amount of aggregated material remaining at the meniscus after 15 hr. of centrifugation. The sedimentation velocity data suggests that at this protein concentration there should not be more than 2% of aggregated material remaining at the meniscus and thus that the average extent of aggregation is probably not greater than tetramers at this low concentration. If this is correct, then the upper limit of 30,000 should not be more than 6% too high, and the best value for the molecular weight would be between 28,000 and 30,000.

Sedimentation analysis experiments were also performed by the sedimentation velocity and Archibald methods with a borate buffer of *p*H 9.94 and at a protein concentration of 10.4 g./l. The results of the experiments in this buffer were very similar to those obtained at *p*H 2.38; 8% of the protein sedimented as fast material possessing an $s_{20,w}$ of 3.5×10^{-13}, and the remaining 92% has an $s_{20,w}$ of 2.5×10^{-13}.

Discussion

The sedimentation velocity experiments demonstrate that human pituitary growth hormone undergoes association in *p*H 2.38 phosphate buffer. This is shown conclusively by the definite positive slope of the weight-average sedimentation coefficient and by the increase in the amount of rapidly sedimenting material as protein concentration increases.

Since the values for the weight average sedimentation coefficient and the sedimentation coefficient of the major component (presumably the monomer) extrapolate to the same point at infinite dilution, one may conclude that all the protein in the sample is in equilibrium with the monomer, within the limits of error incurred by calculating the weight average sedimentation coefficient in the short interval during which the entire peak is in view.

The scatter of data on the fast material is too great to determine with any accuracy the dependence of this sedimentation coefficient on concentration, but it is likely that s_f increases with C. If the sedimentation coefficient of the rapidly sedimenting material is proportional to the 2/3 power of the molecular weight, then the average degree of association varies from 3 to 10.

The results of the Archibald experiments indicate that the association and dissociation reactions are slow compared with the time of sedimentation; consequently the sedimentation velocity diagrams were interpreted in terms of a slow component present along with rapidly sedimenting material. Since these calculations were made from pictures taken during the first 30 minutes of sedimentation, these values may be assumed to correspond approximately to the equilibrium composition at infinite time of dialysis (at 4°) and zero time of sedimentation. Had the evidence indicated that the association–dissociation equilibria were rapid, it would have been more appropriate to treat the data in the manner recently described by Gilbert.[17] In that case, however, it would still be necessary to conclude that the association reactions involved primarily states of aggregation higher than dimers in order to explain the double peaks observed at the higher concentrations. Unfortunately, neither of these extreme assumptions (*i.e.*, very rapid or very slow equilibria) completely explains the phenomenon observed in these experiments, but either view leads to the same qualitative conclusion, namely, that association equilibria involving highly aggregated species are involved.

The preliminary studies of the Archibald method with the well-characterized proteins, ribonuclease and α-chymotrypsinogen, demonstrated the high accuracy and convenience of the Archibald method when applied to homogeneous non-associating systems, as has also been reported by others.[18,19] This was true whether the photographs were analyzed by the Kegeles–Klainer method[13] or by means of the Trautman plot.[9] Molecular weights obtained by these procedures agreed within 1/2% with those calculated from the known amino acid composition. The greatest apparent source of error arises from the extrapolation of the dc/dr (ΔY) data to the bottom of the cell. If ΔY is plotted as a function of r, the points fall on a line which curves upward, and the curvature increases as r approaches r_b, so that some uncertainty is introduced into the extrapolation; however, with ribonuclease and α-chymotrypsinogen this was not a serious source of error. If, on the other hand, log ΔY is plotted as a function of r, the curve, which in some pictures is initially concave in a downward direction, becomes linear as r approaches r_b, and the extrapolation is more precise. In the studies of human growth hormone by the Archibald procedure, extrapolation by a direct plot was virtually impossible[20]: however, the logarithmic plot was usually linear near the lower meniscus as shown in Fig. 2B.

The use of the logarithmic plot for the extrapolation of data at the bottom of the cell is largely empirical. Archibald's equations give us the relationship between the concentration and its gradient at the meniscus and bottom during the transient period, *i.e.*, the period before equilibrium is reached, but does not tell us the form the refractive index gradient should take, as a function of r, during this period. We know, however, that at equilibrium, log ΔY should be a linear function of r, and it is not surprising that during the transient period this relationship might be approximately linear as r approaches r_b.

It is interesting to compare Figs. 4 and 5 of this work with Fig. 9 reported by Ginsberg, *et al.*[18] The latter investigators calculated molecular weight values from the top (M_m) and bottom (M_b) of a cell containing a mixture of sucrose and ribonuclease. For the first 60 minutes, the values for M_m and M_b were identical, then M_b began to increase with time and M_m began to decrease. In Figs. 4 and 5, it may be seen that M_b has assumed a high value, ~ 50,000 within the first 15 minutes of centrifugation, and that it changes in a rather erratic manner with time. This is probably due to the presence of highly aggregated material ($n \sim 6$–10) which begins to accumulate at the bottom of the cell very soon after the rotor has reached top speed. Changes in M_b (a z average molecular weight according to Erlander and Foster[19]) with time, reflect the accumulation of more aggregated material on the cell bottom, counter-balanced in part by dissociation.

Calculations of the molecular weight of the monomer were based entirely on measurements of M_m. The calculated value, 29,000 ± 1000, is in fair agreement with the value reported previously[6] from sedimentation and diffusion studies and is consistent with the known content of cystine and tyrosine in the hormone. The difference in biological activity between human and bovine growth hormone can thus be related to an appreciable difference in molecular weight, since the latter has a molecular weight[21] of 45,000.

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(20) Difficulties in calculating molecular weights from the bottom of the cell are not uncommon. For example, Erlander and Foster[19] have recently reported that the z average molecular weights calculated from the bottom of the cell in experiments with known mixtures gave results which were much too high.

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