The Preferential Hydration of Proteins in Concentrated Salt Solutions. II. Sedimentation Equilibrium of Proteins in Salt Density Gradients[1]

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The salt density gradient technique of Meselson, *et al.*, has been applied to the measurement of the densities of proteins. The precision of the technique is greatly improved by using a precipitating salt solution (ammonium sulfate–cesium chloride). The density of a protein is the same whether it is dissolved or precipitated. Calculation of the degree of preferential hydration of proteins from their densities as measured by this method gives results comparable with those derived from other techniques. The method appears to be capable of detecting the presence of proteins of different densities in a mixture, since a mixture of proteins yields multiple bands rather than a single band of co-precipitate.

Introduction

In the first paper[3] of this series, the preferential hydration of proteins in salt solutions was studied by the dependence of the sedimentation velocity of the proteins on the density of the salt solutions. The quantity of interest in that work was the hydrodynamic density of the protein—the density of the protein molecule plus any modified solvent region it may carry with it through the solution. The preferential hydration of bovine serum albumin and of ribonuclease were calculated from their hydrodynamic densities, once those densities had been obtained from the sedimentation velocity data, according to

$$\rho_h = \frac{1 + A}{v_p + Av_w} \qquad (1)$$

where ρ_h is the hydrodynamic density, A is the preferential hydration in grams of preferentially bound water per gram of protein and v_p and v_w are the partial specific volumes of the protein and of water. The present paper undertakes to examine another method for obtaining the hydrodynamic densities of proteins and thus for measuring the preferential hydration of these macromolecules.

The sedimentation equilibrium of macromolecules in concentrated salt solutions has been developed and mathematically described by Meselson, Stahl and Vinograd.[4] The technique makes use of the density gradient developed at sedimentation equilibrium when a salt solution is subjected to ultracentrifugation. Let a collection of macromolecules be present in the ultracentrifuge cell, initially distributed throughout the solution. Further, let the salt concentration and the speed of the ultracentrifuge be so selected that the density gradient at equilibrium will "bracket" the hydrodynamic density of the macromolecules. As the density gradient forms during the ultracentrifuge run, the macromolecules near the top of the cell

will begin to sediment, and those near the bottom will begin to float. At sedimentation equilibrium, the macromolecules will have collected in a band at a position in the density gradient corresponding to their hydrodynamic density. Meselson, *et al.*, have shown that, if the macromolecules are homogeneous with respect to molecular weight and hydrodynamic density and if the density gradient is linear in the region of the band, then the band will have a Gaussian shape. The standard deviation of the distribution of the macromolecules will be inversely proportional to the square root of their molecular weight.

The salt gradient method is particularly useful in the study of very high molecular weight materials such as DNA.[5,6] For example, in 7.7 molal cesium chloride, at 30,000 r.p.m. in the AN-D rotor of the Spinco analytical ultracentrifuge, the entire equilibrium distribution of T4 bacteriophage DNA was only one millimeter wide.[4] Since the density gradient was of the order of 0.05 g./cm.,[4] it is evident that the hydrodynamic density of such a substance, with a molecular weight of ten or twenty million, may be determined with very high precision.

Sedimentation equilibrium experiments in concentrated salt solutions may be performed as readily with proteins as with DNA. Vinograd reported one such experiment with bovine serum albumin in cesium chloride.[7] There seem, however, to have been few reports of such studies.[8] The most probable reason for the technique not having been widely applied to proteins is the excessive width of the bands formed by these substances. Since proteins have, in general, molecular weights only a hundreth as large as those of DNA's, protein bands will be ten times as broad as those formed by DNA in the same density gradient. The precision with which the hydrodynamic density of proteins may be measured is, thus, an order of magnitude less than that possible with DNA. Moreover, any density heterogeneity that might be present would be far less readily detectable in protein samples than in DNA.

In the present work, the Meselson technique has been modified to overcome the difficulties caused

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(3) D. J. Cox and V. N. Schumaker, *J. Am. Chem. Soc.*, **83**, 2433 (1961).

(4) M. Meselson, F. W. Stahl and J. Vinograd, *Proc. Natl. Acad. Sci., U. S.*, **43**, 581 (1957).

(5) M. Meselson and F. W. Stahl, *ibid.*, **44**, 671 (1958).

(6) N. Sueoka, J. Marmur and P. Doty, *Nature*, **183**, 1429 (1959).

(7) J. Vinograd, M. Meselson and F. W. Stahl, *Fed. Proc.*, **17**, 603 (1958), presented verbally but not published.

(8) D. J. Cox and V. N. Schumaker, *Abstracts of the Biophysical Society*, paper F-3 (1959).
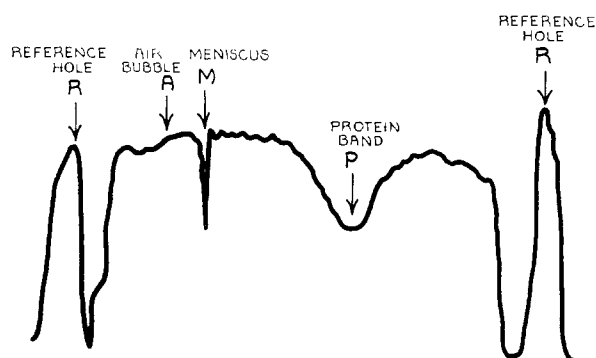
Fig. 1.—Sedimentation equilibrium distribution of dissolved bovine serum albumin in 2.8 molal cesium chloride. The ultracentrifuge run was made at 59,780 r.p.m. in the AN-D rotor of the Spinco analytical ultracentrifuge (Model E). The figure is a photodensitometer tracing of an ultraviolet photograph of the centrifuge cell.

by the width of the protein bands. Salt solutions have been selected which are dense enough to float the proteins and are, at the same time, of a composition which will cause the proteins to precipitate. When such a procedure is followed, the precipitate collects in an exceedingly narrow band at equilibrium; such a band may then be located very precisely in the density gradient. The present experiments have all been done in various mixtures of cesium chloride and ammonium sulfate. Appropriate mixtures of these salts have given the expected sharp bands with all of the seven proteins examined.

Materials and Methods

Salts.—The ammonium sulfate was Merck reagent grade material. It had no ultraviolet absorption and was used without further purification. Cesium chloride was the Fisher "purified" product. It contained a small amount of heavy, ultraviolet-absorbing contaminant which distorted the baseline in the ultraviolet tracings (see Fig. 1). The density of the solutions of the salt conformed to established values in the International Critical Tables.

Proteins.—Bovine serum albumin (BSA) was taken from two lots of the Armour crystallized product. In addition, one experiment was carried out with a preparation, supplied by Dr. George Braun, from which fatty acid had been removed. No differences were observed in the behavior of the three preparations. A crude sample of horse cytochrome c was obtained from Dr. Julian Marsh. Chicken myosin and the H- and L- meromyosins produced by tryptic splitting of myosin were provided by Dr. John Marshall, as was a commercial preparation (Worthington) of crystalline lysozyme. Partially purified samples of lobster hemocyanin and of human hemoglobin were prepared by standard methods in our laboratory.

Instrumentation.—A Spinco analytical ultracentrifuge (Model E) equipped with schlieren and ultraviolet optics was used with the AN-D rotor for all experiments. Several standard and synthetic boundary cells were used, all of which had an optical path of 12 millimeters. The Spinco Analytrol with a photodensitometer attachment was used to scan the photographs taken with the ultraviolet optical system. It was found that the very high refractive index gradients produced in these experiments were sufficient to deflect the light in the ultraviolet system against the side of the optical track. It was, therefore, essential to move the ultraviolet light source off center to compensate for the deflection of the light in order to obtain ultraviolet photographs of the runs. The off-center position of the light source was the same to within one millimeter in every run.

In the DNA experiments of Meselson, et al.,[4] the duration of the runs was 30 hr. or more. With soluble proteins,
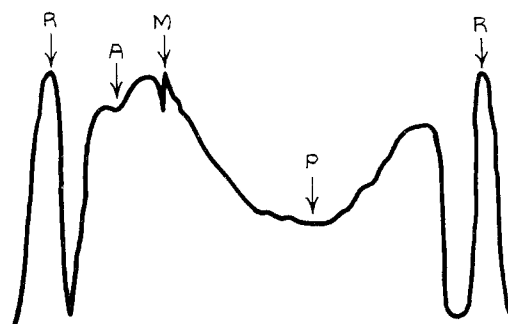

Fig. 2.—Sedimentation equilibrium of dissolved horse cytochrome c in 2.8 molal cesium chloride at 59,780 r.p.m. in the AN-D rotor. The protein band extends over the entire length of the liquid column.

equilibrium is established in 5 or 6 hr. In the case of protein precipitates, which, as a consequence of their enormous size, migrate immediately to their appropriate places in the density gradient, the limiting factor in reducing the time required for the experiments is the establishment of the equilibrium of the salt. It was found that the runs could be considerably shortened if the salt solutions were introduced into the centrifuge cell in two or three layers of differing concentration; in this way, the equilibrium distribution was approximated as closely as possible at the beginning of the run. The system could then be brought to equilibrium in 2 or 3 hr.

Density Measurements.—The initial densities of the salt solutions were measured in triplicate in one-milliliter vented-cap Gay-Lussac pycnometers. The measurements were reproducible to 0.001 g./ml. The salt concentration gradients were calculated by comparing the areas under the schlieren curves with the area corresponding to a known salt concentration increment as measured in the synthetic boundary cell. The schlieren patterns were converted to concentration gradients by numerical integration of the schlieren curves. The final interpretation of the schlieren curves in the terms of concentration or density gradients for the salt mixtures was done by trial and error, to give the best agreement with both the measured concentration gradient and the known amount of salt present in the entire solution.

Results

Figures 1 and 2 show the type of distribution obtained when the unmodified salt gradient technique is applied to proteins. These figures are photodensitometer tracings of ultraviolet photographs of the ultracentrifuge cell. They show the equilibrium distribution of bovine serum albumin and cytochrome c, respectively, in 2.8 molal cesium chloride. The centrifuge was operated at 59,780 r.p.m. The width of the serum albumin band was around four millimeters; since the density gradient, under these conditions, is around 0.09 g./cm.,[4] it was very difficult to locate the center of the peak to within 0.002 density units. Such precision in the density measurement is necessary if the preferential hydration of the protein is to be determined to within 0.01 g. of preferentially bound water per gram of protein. In the case of BSA, the maximum of the peak is at 1.295 ± 0.005 g./cm.[3] The partial specific volume[9] of BSA is 0.734, so that, according to equation 1, the preferential hydration of BSA in cesium chloride is 0.17 ± 0.02 gram per gram.

The application of the density gradient technique to dissolved proteins does, however, offer

(9) J. T. Edsall, in H. Neurath and K. Bailey, ed., "The Proteins," Vol. I, Academic Press, Inc., New York, N. Y., 1953, Chapter 7.

TABLE I

HYDRODYNAMIC DENSITIES AND APPARENT PREFERENTIAL HYDRATION VALUES FOR PROTEIN PRECIPITATES IN CONCEN-
TRATED SALT SOLUTIONS

Protein	Salts, m		Initial density	ρ_h	$v_p{}^a$	A
Human hemoglobin	CsCl	2.8	1.306	1.295 ± 0.002	0.749	0.10
Chicken myosin	CsCl	2.4	1.300	$1.280 \pm .002$	.74	.19
	$(NH_4)_2SO_4$	0.3				
L-Meromyosin	CsCl	2.2	1.304	$1.280 \pm .002$	(.74)	.19
	$(NH_4)_2SO_4$	0.6				
H-Meromyosin	CsCl	2.1	1.301	$1.285 \pm .002$	(.74)	.18
	$(NH_4)_2SO_4$	0.8				
BSA	CsCl	0.7	1.253	$1.255 \pm .002$	.734	.31
	$(NH_4)_2SO_4$	3.6				
Lysozyme	CsCl	0.8	1.267	$1.290 \pm .002$	.722	.24
	$(NH_4)_2SO_4$	3.6				
Lobster hemocyanin	CsCl	0.8	1.267	$1.270 \pm .002$	.740	.22
	$(NH_4)_2SO_4$	3.6				

^{a} Values for the preferential hydration A are calculated using values of the partial specific volume of the proteins cited by Edsall.[9]

one advantage not available with the precipitated material. Meselson, et al.,[4] showed that the standard deviation σ of the Gaussian equilibrium distribution is related to the preferentially hydrated molecular weight M_h of the macromolecule by

$$M_h = \frac{RT}{\sigma^2\,\bar{v}_h\,(d\rho/dr)\omega^2 r_0} \qquad (2)$$

where R is the gas constant, T is the absolute temperature, $d\rho/dr$ is the density gradient, ω is the angular velocity of the centrifuge, and r_0 is the distance of the center of the band from the axis of rotation. $\bar{v}_h$ is the partial specific volume of the preferentially hydrated protein and is equal to the reciprocal of the density of the solution at the center of the band, $1/\rho_h$. M_h is the preferentially hydrated molecular weight of the macromolecule

$$M_h = M_p + \alpha M_w \qquad (3)$$

where α is the number of moles of water preferentially bound to a mole of protein, and M_p and M_w are the molecular weights of the protein and water. If σ can be measured with any precision, then the preferential hydration of the protein can be measured by this method, provided M_p is known. Unfortunately, in the experiment of Fig. 1, the curve is not quite Gaussian, probably because the density gradient is not linear across the entire band. The standard deviation could be measured only approximately as 0.130 ± 0.005 cm. Under the conditions of the experiment, this value for σ yields a value of $83,000 \pm 5,000$ for M_h. Since the molecular weight of BSA is 67,000, A is 0.24 ± 0.07 gram per gram. In this experiment, the error is too large to allow a really meaningful measurement of preferential hydration. Nonetheless, the potential advantage of the unmodified technique, in providing two nearly independent approaches to the measurement of preferential hydration, deserves mention. Alternatively, if the preferential hydration is measured from the location of the band in the density gradient, the preferentially hydrated molecular weight found from the width of the band can be corrected to yield the true molecular weight. Thus, the preferential hydration and the molecular weight of a protein can be both measured in one

experiment, provided only that the partial specific volume of the protein is known.

In Table I are listed the results of the sedimentation equilibrium experiments with protein precipitates in concentrated solutions of ammonium sulfate and cesium chloride. All of the centrifuge runs were done at 59,780 r.p.m. The pH in all experiments was 4.8–5.5. The values of the partial specific volumes of the proteins are those cited by Edsall.[9] It has been assumed that the partial specific volumes of both meromyosins are the same as that of myosin.

In a number of cases, the solid protein formed more than one band, indicating density heterogeneity among the particles of precipitate The experiments with myosin, H-meromyosin and lysozyme (Fig. 3) gave very sharp single bands.

Fig. 3.—Sedimentation equilibrium of precipitated lysozyme in 0.8 molal cesium chloride and 3.6 molal ammonium sulfate at 59,780 r.p.m. in the AN-D rotor. The protein appears as a single prominent spike near the bottom of the centrifuge cell.

Hemoglobin (Fig. 4) showed a pair of sharp bands about 0.3 millimeters apart. L-meromyosin gave two somewhat broader bands, about 0.5 millimeters apart. In both cases, the average density of the two bands is given in Table I. BSA gave two, possibly three rather diffuse bands about one millimeter apart; the densest of these was the most prominent, and it has been listed in Table I. Hemocyanin gave a single continuous band about
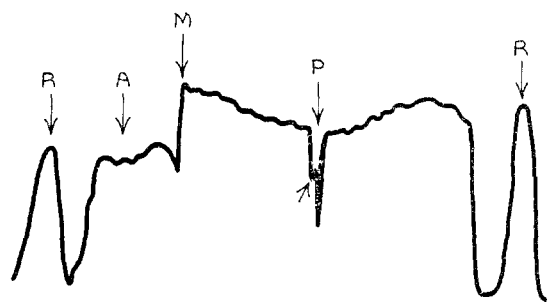
Fig. 4.—Sedimentation equilibrium of precipitated human hemoglobin in 2.8 molal cesium chloride at 59,780 r.p.m. in the AN-D rotor. The protein is seen as a double band near the center of the centrifuge cell. The smaller band (arrow) appeared late in the run, about 3 hr. after the major band had formed.

1.5 mm. wide. The density at the center of this rather broad band is given in Table I. The fact that more than one discrete band of protein can appear in a single experiment was something of a surprise, since it was to be expected that any protein mixture would precipitate either as a single band of intermediate density or as a continuous distribution of materials between the densities of the component proteins. A mixture of lysozyme and BSA in 3.6 molal ammonium sulfate and 0.8 molal cesium chloride gave two bands at sedimentation equilibrium. The bands were both somewhat diffuse, with maxima at 1.265 and 1.270 g./cm.^{3}, intermediate between the density of BSA (1.255 g./cm.^{3}) and that of lysozyme (1.290 g./cm.^{3}). Both bands were probably mixtures of the two proteins. It is not at all clear why the precipitates should be found at two separate densities rather than in a single band containing all the precipitated protein.

Where a broad band of precipitated material was found, it was sometimes difficult to decide whether it was composed of a large quantity of a material of uniform density or of a series of narrow bands of slightly varying density. The distinction can be made by decelerating the centrifuge and allowing the salt to diffuse toward a less steep density gradient. When this is done, a wide band of uniform density will migrate as a unit along with its proper location in the density gradient. Neighboring bands of different density may either break up into separate bands or be detected by the spreading of an already diffuse band.

An extreme case of density heterogeneity was examined quite by accident when a sample of myosin was run in a cell which had been contaminated with chymotrypsin. A series of thirty or more bands appeared, varying in density over a range of 0.05 density units. Five of these bands are numbered in Fig. 5-A. Dr. John Marshall had found a small amount of nucleotide material attached to this sample of myosin; the presence of variable proportions of this very dense material in the various protein fragments would account for the very great variation among the densities of the bands. When the centrifuge was slowed down, the bands migrated in echelon toward the ends of the cell as the salt density gradient de-
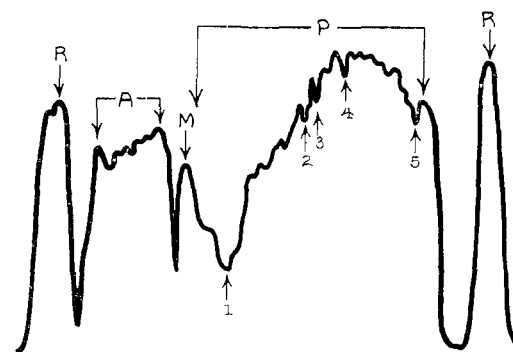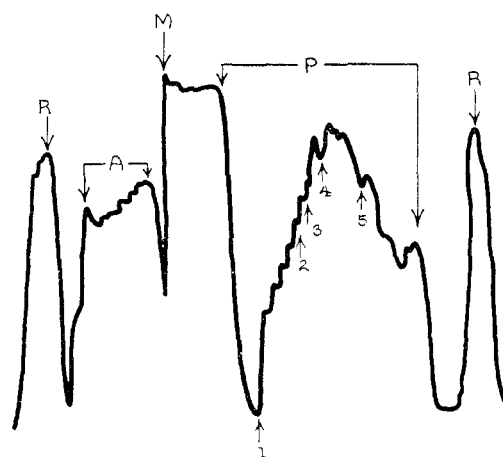

Fig. 5.—A. Chymotrypsin-degraded myosin in 2.2 molal cesium chloride and 0.6 molal ammonium sulfate at sedimentation equilibrium after 384 minutes at 59,780 r.p.m. B, run of Fig. 5-A, after an additional 124 minutes at 29,500 r.p.m. Bands labelled 1–5 correspond to those similarly labelled in Fig. 5-A.

creased. A few new bands were revealed, but none disappeared or combined with any other (Fig. 5-B). The resolving power of the method for the detection of density heterogeneity seems to be very considerable.

The apparent preferential hydration of bovine serum albumin listed in Table I is very much higher than that of the other proteins examined. Moreover, BSA produced multiple bands over a considerable range of densities when there was no reason to suspect gross heterogeneity in the protein samples. It was decided, therefore, to investigate in some detail the behavior of BSA in ammonium sulfate–cesium chloride mixtures. The density of dissolved BSA in various salt mixtures was measured with the help of the layering technique described earlier. Concentrated ammonium sulfate–cesium chloride was introduced into the center-piece of a synthetic boundary cell, while the protein was added from the cup in a less concentrated salt solution. By this procedure, it was possible to introduce dissolved protein into a solution whose average ammonium sulfate concentration was sufficient to precipitate it. If the protein had then sedimented into the lower part of the cell, it would have precipitated. Under the conditions of this series of experiments, however, the protein remained high in the cell, where the salt concentra-

tion was not sufficient to cause it to precipitate. The results are listed in Table II. The preferential hydration of BSA in the presence of a constant concentration of ammonium sulfate decreases as the concentration of cesium chloride is increased. No explanation will be attempted here for this surprising but consistent set of data. The preferential hydration of bovine serum albumin, at least, is radically dependent on the composition of the salt solution.

TABLE II

SEDIMENTATION EQUILIBRIUM STUDIES ON SOLUBLE BSA IN CONCENTRATED SALT SOLUTIONS

Salts, m		Initial density	ρ_b	A^a
$(NH_4)_2SO_4$	3.6	1.267	1.250 ± 0.005	0.35 ± 0.003
CsCl	0.82			
$(NH_4)_2SO_4$	3.6	1.238	$1.225 \pm .005$	$.45 \pm .03$
CsCl	0.52			
$(NH_4)_3SO_4$	3.6	1.224	$1.210 \pm .005$	$.54 \pm .03$
CsCl	0.30			
$(NH_4)_2SO_4$	3.6	1.210	$1.200 \pm .005$	$.60 \pm .04$
CsCl	0.25			
$(NH_4)_2SO_4$	3.6	1.196	$1.190 \pm .005$	$.67 \pm .04$
CsCl	0.13			
$(NH_4)_2SO_4$	3.6	1.182	$1.175 \pm .005$	$.79 \pm .05$
CsCl	0			

^{a} Values for the preferential hydration A are calculated on the assumption that the partial specific volume of BSA is 0.734 cm.^{3}/g. in all of the solutions.

If the banding method is to be used to obtain information about preferential hydration, then the question must be asked whether the density of a protein precipitate is the same as that of the dissolved protein. A comparison of Tables I and II shows that, in 3.6 molal ammonium sulfate–0.7–0.8 molal cesium chloride, the density of precipitated BSA is a little higher than that of the dissolved material. Since the precipitated band (Table I) originally formed in the lower part of the cell, where the composition of the salt–water solvent was not the same as that near the top of the cell, these two experiments were not identical. Therefore, an experiment of the layering type was done, in which the protein (BSA) was introduced as crystals taken straight from the bottle and carefully floated on the concentrated salt solution in the centerpiece of the synthetic boundary cell. When the less concentrated salt solution was added from the cup, the protein crystals at first stayed in the middle of the cell and then migrated slowly toward the meniscus. At equilibrium, they had reached a point corresponding to a density of 1.250 g./cm.^{3} The average salt concentrations were 3.6 molal ammonium sulfate and 0.82 molal cesium chloride, as they were in the first experiment in Table II. The agreement between the densities of dissolved and precipitated serum albumin was exact.

Another experiment relevant to the question of dissolved and precipitated densities is shown in Fig. 6. Figure 6-A shows the equilibrium distribution of H-meromyosin in 2.1 molal cesium chloride and 0.8 molal ammonium sulfate at 59,780 r.p.m. The schlieren photograph shows a single band of precipitated protein flanked by the characteristic diphasic pattern of a band of dissolved
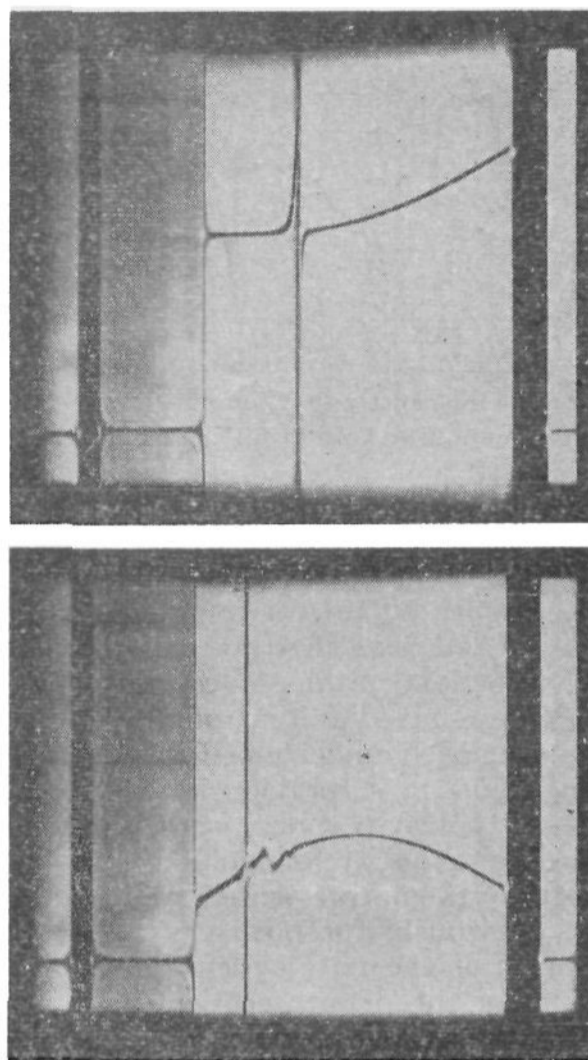

Fig. 6.—A, schlieren photograph of H-meromyosin in 2.1 molal cesium chloride and 0.8 molal ammonium sulfate after 256 minutes at 59,780 r.p.m. The heavy band of precipitate is flanked by a sharp diphasic pattern, indicating a band of dissolved protein. B, run of Fig. 6-A, after an additional 112 minutes at 29,500 r.p.m. The precipitated and dissolved protein have separated as a result of the different speeds with which they respond to the changing density gradient.

protein. Evidently, the salt concentration was such that H-meromyosin had a slight but appreciable solubility. Figure 6-B shows the situation when the salt gradient had begun to decrease after 112 minutes at 29,500 r.p.m. The heavy precipitate, responding instantaneously to the changing density, has moved toward the meniscus. The dissolved material has also moved up the cell, but much more slowly, since it is composed of much smaller particles. The band of dissolved protein is now clearly revealed. In this case too, there is an exact correspondence between the densities of the dissolved and precipitated protein.

In Table I, it may be noted that the apparent preferential hydration of human hemoglobin is significantly lower than that of the other proteins. It seems likely that the hemoglobin sample was denatured; native hemoglobin should not precipitate from 2.8 molal cesium chloride. Further, when the sample was removed from the cell and diluted after the run, little, if any, of the precipitated material went back into solution. It was decided, therefore, to see whether a protein known to be denatured was more dense than its native analog in this system; if so, the low apparent hydration of hemoglobin would be adequately explained by presuming that the protein was de-
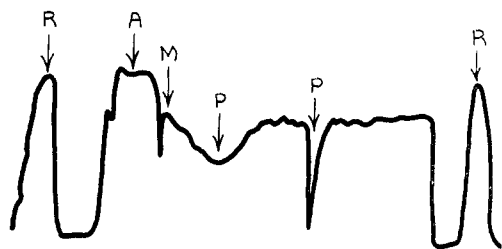
Fig. 7.—Sedimentation equilibrium of bovine serum albumin, native and denatured, in 0.8 molal cesium chloride and 3.6 molal ammonium sulfate at 59,780 r.p.m. in the AN-D rotor. The native protein appears as a broad band near the top of the cell, the denatured material as a spike near the middle of the cell.

natured. Figure 7 shows a controlled comparison of the native and denatured forms of bovine serum albumin. Portions of dissolved and heat-coagulated BSA were mixed and introduced from the cup of the synthetic boundary cell in an experiment of the layering type previously described. The average salt concentrations were 3.6 molal ammonium sulfate and 0.82 molal cesium chloride. The density of the native protein was 1.250 g./cm.^{3}, as found previously for both crystalline and dissolved forms of the native material. The single, sharp band of the denatured protein appears farther down the cell at a density of 1.265 g./cm.^{3}. The denatured protein is thus 0.015 g./cm.^{3} more dense than its native analog.

Discussion

The salt gradient technique, whose application to proteins has been examined in the present work, offers measurements of three different characteristics of macromolecules: hydrodynamic density, preferential hydration and, possibly, density heterogeneity.

The technique provides a reasonably unequivocal measure of the hydrodynamic density of a protein, whether it be examined in solution or as a precipitate. The method is far more precise for precipitated than for dissolved proteins, and the measured density is the same in both states for the two cases which have been checked.

The high density of denatured BSA as compared with that of the native form deserves some emphasis. Linderstrøm-Lang has noted that denaturation of protein substrates by trypsin is accompanied by a decrease in volume unaccounted for by electrostriction.[10] Waugh has discussed this volume decrease in terms of packing space inside the protein molecule,[11] while Klotz has suggested that it is due to the "melting" of a crystalline "ice layer" around native proteins.[12] The data presented here do not distinguish between these alternatives, but they do corroborate the observation that denatured proteins are more dense than their native analogs. It is also worth noting that Sueoka, Marmur and Doty, using the salt density gradient technique, have observed a similar

increase in the density of DNA upon heat denaturation.[6]

In all cases examined, the measured densities are considerably lower than the reciprocals of the partial specific volume of the proteins. It has been assumed here that all of the depression of the density below $1/\bar{v}_p$ is due to preferential hydration. The technique, then, may provide a measure of a second characteristic of a protein, its preferential hydration. Such an interpretation of the density data will be legitimate only if the partial specific volume of the precipitate is the same as that of the dissolved protein. The data at hand bear on this question in two ways. First, the preferential hydration values obtained by the salt gradient technique are reasonable; they fall in the range 0.2–0.3 g. of preferentially bound water per gram of protein, in agreement with data obtained by other methods.[3,13,14] Secondly, the densities of the dissolved and precipitated forms of the same protein are the same in the two cases examined. Any change in the partial specific volume of the protein upon precipitation would have to be exactly cancelled out by a compensating change in preferential hydration. So exact a compensation seems to us unlikely. The interpretation of the density of a protein precipitate in terms of preferential hydration seems to be justified.

One set of preferential hydration values, that for dissolved bovine serum albumin in concentrated ammonium sulfate–cesium chloride, does not, at first sight, appear reasonable. The values are far higher than any reported for any protein by other workers. Nonetheless, these six experiments were consistent with each other; further, sedimentation velocity versus density plots previously reported[3] indicate an abnormally low density for bovine serum albumin in ammonium sulfate. The density values, then, are probably real. The interpretation of the low density as high preferential hydration requires the measurement of the partial specific volume of BSA in concentrated ammonium sulfate, which has not yet been done.

It may be possible to derive a third kind of information from this type of experiment; the method may be applicable to the detection of density heterogeneity in protein samples. It might be supposed that a mixture of proteins would precipitate as a conglomerate with a density intermediate between those of its components. The experimental results indicate that some co-precipitation does occur, but in no case studied did a known mixture of proteins appear as a single precipitated band. In fact, the difficulties with the method may be of just the opposite sort. Spurious heterogeneity can arise if the density of a protein precipitate is a function of the composition or concentration of the salt mixture. Both the total concentration and the relative proportions of ammonium, sulfate, cesium and chloride change from one end of the centrifuge cell to the other. It might be possible to produce several bands, each of a density appropriate to the particular region of the salt gradient in which it occurs.

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In the case of bovine serum albumin, this possibility is lively indeed. Sharp single bands were, however, obtained with myosin, H-meromyosin and lysozyme. Multiple bands were observed with all of the other proteins studied, but in each of these latter cases, excepting only that of BSA, there was reason to doubt the homogeneity of the protein sample. The question as to how commonly a single protein species will give more than one band of precipitate requires further study, but the method deserves closer examination as a criterion of the homogeneity of protein samples.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PA.]

The Preferential Hydration of Proteins in Concentrated Salt Solutions. III. Theoretical[1]

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Equations are developed showing the relation between the binding of ions by macromolecules and preferential hydration. It is suggested that the hydration of the salt ions in solution is the primary factor responsible for the preferential hydration of the macromolecule.

Equations governing the interaction between ions and macromolecules have been developed by a number of investigators.[2] These binding equations are based upon the mass law and treat the macromolecule as possessing a number of specific ion binding sites having definite association constants for interaction with the small ions. Equations have been developed by other workers who have studied the phenomenon of preferential hydration of macromolecules.[3] The macromolecule is considered by these workers to interact with a number of water molecules and a smaller number of ions. If the proportion of water molecules to salt ions in the vicinity of the macromolecule is greater than the proportion of water to salt in the solvent, the preferential hydration is positive. If salt is in excess, the preferential hydration is negative.

Interestingly, investigators studying ion binding often find that proteins bind sizable numbers of salt ions in solution. Other investigators studying preferential hydration find that proteins bind large quantities of water to the exclusion of salt.

In this communication we have joined together the ion binding equations and the preferential hydration equations. As might be expected, the resulting expression clearly shows that at low concentrations of salt ions, the macromolecules preferentially bind salt while at high concentrations of salt, the macromolecules preferentially bind water. Indeed, it becomes clear that ion binding and preferential hydration are, experimentally, difficult to separate.

One result of this treatment is to suggest that the interpretation of ion binding results, such as determined in the e.m.f. cell or by equilibrium dialysis,

may contain small but definite errors if a correction for preferential hydration is not made.

In addition, we present in this communication a reasonable physical mechanism for preferential hydration of macromolecules in salt solutions. In this mechanism, attention is directed to the ionized salt which interacts strongly with adjacent water molecules through ion-dipole bonds. As the salt ion is brought to the surface of the macromolecule, electrostatic allegiances shift, new bonds may arise and water molecules may be freed. Should these events be energetically unfavorable, the ion concentration will drop sharply in the surface layer. Effectively, the macromolecule will be preferentially hydrated even though there is little or no positive interaction between it and the water.

Theory

In this section it is shown that preferential hydration may be expressed mathematically in terms of ion binding by considering all the binding energies, positive and negative, over the entire surface of the macromolecule. The surface layers surrounding the macromolecule will be defined as the monolayer of solvent in contact with atoms of the macromolecule together with several adjacent layers of solvent. For ease of mathematical formulation the sizes of the water molecules and the unhydrated salt ions are considered to be equal so that the surface layers will contain λ water molecules and salt ions regardless of the composition of the layers. In general the surface of the large molecule will be inhomogeneous in binding affinities for the salt ions, and it can be divided into groups of sites i, each group containing λ_i members possessing an intrinsic affinity constant k_{ij} for a particular ion, j.

For interaction between small molecules and a macromolecule, the mass law may be conveniently written[2]

$$r_{ij} = \frac{\lambda_i k_{ij} m_j}{1 + k_{ij} m_j} \tag{1}$$

where r_{ij} is equal to the number of moles of salt ions of species j bound at sites of type i, m_j is the molality of the free (unbound) ion, and k_{ij} is the intrinsic association constant for this reaction. Now, if the composition of solvent bound at the λ_i sites is to be the same as the composition of the free solvent, then there would have to be $r_{ij}(55.51/m_j)$ water molecules bound at the sites, λ_i. The difference between this number and the number of water molecules actually bound, $(\lambda_i - r_{ij})$ will be termed the preferential hydration, α_{ij}

$$\alpha_{ij} = (\lambda_i - r_{ij}) - r_{ij} \frac{55.51}{m_j} \tag{2}$$

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(2) Reviewed by I. M. Klotz, in "Proteins," Vol. I, Part B, H. Neurath and K. Bailey, Editors, Academic Press, Inc., New York, N. Y., 1953, p. 763.

(3) S. Katz and H. K. Schachman, Biochem. et. Biophys. Acta, 81, 28 (1955).