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The Preferential Hydration of Proteins in Concentrated Salt Solutions. I. Sedimentation Studies[1]

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The sedimentation coefficients of ribonuclease and bovine serum albumin have been measured over a large range of salt concentrations in KCl, NaCl, $(NH_4)_2SO_4$ and LiCl. Sizable variations from the predicted values are observed which may be consistently interpreted in terms of preferential hydration of RNase (0.34 g./g.). Also, for this protein, the preferential hydration is constant from 0 to 40° and from *p*H 4.7 to 9.2. For BSA, the preferential hydration values show differences depending upon the type and concentration of salt, temperature and *p*H. In NaCl and KCl at 20°, *p*H 5.5, the values for BSA are 0.19 and 0.26 g./g., respectively.

Introduction

The problem of the binding of water by proteins has drawn the attention of many workers over a long period of time.[3] Several different experimental techniques have been used to examine protein hydration; as a result, several separate bodies of data have been obtained, each relevant to the definition of protein hydration implicit in the particular experimental approach employed.

A simple, pragmatic definition of protein hydration is the measured decrease in the weight of a protein sample, which has been in equilibrium with an atmosphere having a definite, controlled humidity, when the sample is subsequently thoroughly dried.[4] Although this definition suffers from the experimental difficulty of obtaining the true dry state of the protein,[5] consistent data for water adsorption by a number of proteins have been obtained as a function of relative humidity.[3,6-8] Although the absolute level of water binding varies from one protein to another, the shape of the plot of water bound *versus* relative humidity is the same in all cases. The curves are sigmoid, with a sharp burst of water adsorption at low humidity, a linear portion at humidities around 50% and a further sharp increase at humidities approaching saturation. Pauling[9] has pointed out a correlation between the number of polar groups in particular proteins and the affinity of the proteins for water. The work of Mellon, Korn and Hoover[6-8] has implicated the peptide bonds as well as the polar side chains in the binding of water by proteins.

Sorensen and Hoyrup[10] examined the composition of wet filter cakes of proteins precipitated from ammonium sulfate solutions. They noted that the amount of water in the precipitates was always greater than would have been expected from the amount of salt in the precipitates and the concentration of salt in the mother liquor. The amount of water in excess of what would have been present if the protein had precipitated carrying only adhering mother liquor will be termed preferential hydration. Large, single protein crystals also exhibit a marked preferential hydration.[11] Thus, solid proteins in general not only bind water, but they also bind water in preference to other available components of the solvent.

It has become apparent that both properties of proteins, the binding of solvent in general and the binding of water in preference to other components of the solvent are characteristic of individual protein molecules and not only for large, solid collections of them.[12] It has been especially necessary to take both effects into account in interpreting sedimentation data in terms of the size and shape of macromolecules. The binding of solvent by the sedimenting particle will affect the frictional coefficient of the macromolecule and will render ambiguous the interpretation of the frictional coefficient in terms of the shape of the molecule.[13,14]

While it is difficult to determine the absolute hydration of dissolved proteins, it appears less difficult to determine the preferential hydration of the individual protein molecules. The preferential binding of water will alter the bouyant mass of the particle. The amount of water *preferentially* bound to a protein may be determined from sedimentation data alone; this is done, as will be seen, by measuring the dependence of the sedimentation coefficient of the molecule on the density of the medium and, thus, the effective density of the hydrodynamic particle.

Klotz[15] has suggested that bound water may appreciably modify the chemical behavior of protein molecules; he has pictured the protein molecule as being surrounded by a relatively impenetrable layer of ordered water. The presence of such a carapace of "ice" would help to explain the anomalous titration curves of proteins, the co-operative effects found in "multiheaded" proteins and

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the large initial volume decrement associated with protein denaturation. It would, then, be of considerable interest to examine the hydration of proteins as a function of various physical parameters. The dependence of the hydration on temperature would be of particular interest, since the structuring of water by non-polar gases,[16] which Klotz has chosen as a precedent for his "iceberg" hypothesis, is strongly temperature dependent. In the present work, the preferential hydration of two proteins, bovine serum albumin (BSA) and bovine pancreatic ribonuclease (RNase), has been examined in various salts and as a function of salt concentration, *p*H and temperature.

Theoretical

Schachman and Lauffer[17] and Katz and Schachman[18] have examined the effect of preferential hydration on the sedimentation behavior of macromolecules. Katz and Schachman dealt with the general case of a sedimenting unit containing one macromolecule, which binds salt and water in some proportion to their availability in the solvent. In addition, an excess of water or salt is bound, over and above the "proportional" binding. They have shown that the partial molar volume of the hydrodynamic unit is the weighted sum of the partial molar volumes of the components since the final volume is the same whether the components are added collectively or separately. Therefore

$$M_h\bar{v}_h = M_p\bar{v}_p + (\alpha + kn_w)M_w\bar{v}_w + kn_sM_s\bar{v}_s \quad (1)$$

In equation 1, the subscripts h, p, w, and s refer to the hydrodynamic unit, protein, water and salt, n_w and n_s are the total number of moles of water and salt in the solution, the $\bar{v}'s$ are partial specific volumes and the M's are molecular weights. The k is a proportionality constant, such that kn_s is equal to the number of moles of salt bound to the protein, and α is the preferentially bound water in terms of moles of water per mole of protein. By introducing the molecular weight of the hydrodynamic unit, $M_h = M_p + (kn_w + \alpha)M_w\rho + kn_sM_s$ and taking the limit as the protein concentration becomes small, equation 1 becomes

$$M_h(1 - \bar{v}_h\rho) = M_p(1 - \bar{v}_p\rho) + \alpha M_w(1 - v_w\rho) \quad (2)$$

where ρ is the density of the medium.[18]

It should be noted that, although equation 2 formally ascribes all effects of the solvent on the buoyant mass of the protein to the preferential binding of water, the preferential binding of salt is equally well described by giving negative values to α. The general expression for the sedimentation coefficient of the hydrodynamic unit S, is given by

$$(S\eta)Nf/\eta = M_p + M_w\alpha - (M_p\bar{v}_p + M_w\alpha\bar{v}_w)\rho \quad (3)$$

where f/η is the intrinsic frictional coefficient, N is Avogadro's number, and η is the viscosity of the medium. The sedimentation coefficient may be determined in a series of solutions of increasing density. If the intrinsic frictional coefficient of the macromolecule (f/η) and other parameters are independent of ρ a plot of $S\eta$ *versus* ρ will be linear; the plot will intercept the density axis at

$$\rho_h = \frac{M_p + M_w\alpha}{M_p\bar{v}_p + M_w\alpha\bar{v}_w} \quad (4)$$

If the molecular weight of the protein is not known the numerator and denominator of the right hand side of equation 4 may be divided by M_p, giving

$$\rho_h = \frac{1 + A}{\bar{v}_p + A\bar{v}_w}; \text{ where } A \equiv \frac{M_w\alpha}{M_p} \quad (4\text{-A})$$

The A is the preferential hydration in terms of grams of excess water per gram of protein. If the experimental plot of $S\eta$ *versus* ρ is not linear, the ultracentrifuge data alone are insufficient to show which of the parameters of equation 3 is varying. Variation in the shape, molecular weight or partial specific volume, as well as in the preferential hydration of the macromolecule, would cause curvature of the $S\eta$ *vs.* ρ plots. It is important to note that the partial specific volumes in equations 3 and 4 are the thermodynamically defined partial specific volumes of the components in the solution as a whole and do not refer to the densities of the components as they occur in the hydrodynamic unit. In determining the *amount* of the preferentially bound water, it is not necessary to make any damaging assumptions about its *state* in the sedimenting unit.

Materials and Methods

Salts.—Ammonium sulfate, sodium chloride and potassium chloride were Merck reagent grade materials and were used without purification. Lithium chloride was recrystallized from hot 0.01 *M* Versene (disodium ethylenediaminetetraacetate) and once more from hot water.

Proteins.—Bovine serum albumin was the Armour crystallized product. Analysis of the sedimentation patterns revealed the presence of a second component with a sedimentation rate around $1^1/_2$ times that of the major peak; the faster component was presumed to be albumin dimer and was present to the extent of 8–10%. It was, however, observed that the movement of the maximum of the entire boundary gave straight log x *vs.* t plots and was equal to the sedimentation velocity of the albumin monomer only within experimental error. Bovine pancreatic ribonuclease was the Worthington salt-free product. The material was analyzed chromatographically on an Amberlite XE-64 column in phosphate buffer at *p*H 6.51, according to the procedure of Hirs, Moore and Stein.[19,20] Ninety-five per cent. of the ninhydrin-positive material was found in a single symmetrical peak corresponding in chromatographic behavior to ribonuclease A. This material was used for measurements of sedimentation coefficients and partial specific volumes without further purification.

Sedimentation Velocity Measurements.—Ultracentrifugation was performed in a Spinco analytical ultracentrifuge (Model E), using an An-D rotor and a synthetic boundary cell with a 12 millimeter optical path. The runs were of twenty to thirty minutes duration. The relative amounts of protein solution and solvent placed in the centerpiece and the cup, respectively, were adjusted so that the peaks were formed at or just above the middle of the cell. This procedure facilitated reading the plates and also insured that the density of the medium through which the protein in the neighborhood of the peak was sedimenting did not change appreciably during the runs.

Density and Viscosity Determinations.—Densities were determined in triplicate in one milliliter vented-cap pycnometers and are considered accurate to within 0.001 g./cm^3. Salt concentrations were calculated from the densities and these were routinely used to calculate the viscosity increment due to the salt from the values given in the

(16) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).

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International Critical Tables. The validity of this procedure was spot-checked, using a one-bulb capillary viscometer with a shear gradient of 300 sec.$^{-1}$; these checks revealed no discrepancy between the measured values and those calculated from the observed solution densities and the density and viscosity values in the International Critical Tables. Temperature corrections of the viscosity of water were taken from the Chemical Rubber Handbook.

Plate Measurements.—All measurements were made with a Bausch and Lomb bench comparator capable of measurements to 0.001 mm. The position of the top of the schlieren peak was taken as the location of the central interference fringes under the peak.[21] This feature could be located reproducibly within 0.002–0.005 millimeters, depending on the width of the peak and the general condition of the photograph. It is estimated that the sedimentation coefficients calculated from plates measured in this way are accurate to within 0.05 Svedbergs or better.

Measurements of Partial Specific Volumes.—Since the standard vented-cap pycnometer can not be used to obtain very precise values for $\bar{v}$, special flasks were designed.[22] These were approximately 25 ml. in volume and were fused at the top with one centimeter of one-millimeter precision-bore capillary. Above the capillary was an overflow well, and the top of the entire bottle was closed by a ground-glass stopper. The bottles were filled through the capillary with a syringe to within one milliliter of the capillary and were then allowed to equilibrate with a bath whose temperature was constant to within 0.01°. After equilibration, the bottles were filled to the bottom of the capillary tubes and allowed to equilibrate again with the bath. The final addition of water to a mark in the capillary was carried out in the bath; the filling could be done to within 0.1 millimeters of the mark, that is, to within 0.1 mg. in 25 g. The bottles were removed from the bath, capped, wiped clean and allowed to equilibrate with the atmosphere in the balance room for 1 to 2 hr. The loss by evaporation in this time was negligible; it amounted to less than 0.5 mg. in 24 hr. After equilibration, the filled flasks were weighed to 0.1 mg. All weights were corrected to vacuum. In the calibration of the flasks with distilled water, it was found that the entire operation could be accomplished reproducibly to within 0.1 mg. Distilled water was used for all operations, and the water had been freshly boiled to prevent the formation of bubbles in the bottles.

In the measurements of partial specific volumes, the material under study, protein or salt or both, was weighed in a beaker to 0.1 mg. and dissolved in the minimum amount of water. It was found that lyophilizing the protein and putting the salt through a fine sieve was helpful in dissolving the materials. The concentrated solution was carefully transferred to the weighing bottle with a syringe. The beaker, the syringe and the neck of the bottle were carefully washed out with the water remaining to be introduced into the bottle. The final filling of the bottles was carried out as described above. In all cases, blank flasks filled with water were carried through the procedure with the protein and salt solutions, so that it was not necessary to rely on the absolute accuracy of the thermometer in the constant temperature bath.

Where it was desired to find the variation of $\bar{v}$ with temperature, the filling and weighing were carried out at the highest temperature first. Then the bottles were equilibrated at the next lower temperature, "topped off," and weighed again.

The proteins used in the experiments were lyophilized and equilibrated for several days with the air in the balance room; the samples thus contained a certain amount of water. The amount of this water was determined by heating a weighed sample for two days at 105° in the air. When the samples were removed from the oven, they were placed under vacuum over calcium chloride until they cooled. They were then weighed, and the loss in weight was taken as the amount of water in the samples. No further loss in weight was observed after two days at 105°.

Determination of Partial Specific Volumes by Sedimentation in Deuterium Oxide.—Triplicate determinations of the sedimentation coefficients of BSA and RNase were made in 99.5% D_2O–0.1 *M* KCl. The D_2O was the product of Volk Radiochemical Co. The viscosity data used were those of Hardy and Cottington.[23] The sedimentation coefficients were corrected for deuterium exchange, so that the partial specific volumes calculated by the method of Svedberg and Erikssen-Quensel[24] were those of the undeuterated protein. For RNase, the deuterium-exchange data of Hvidt[25] were used directly. For BSA, the extent of deuterium exchange was calculated from the amino acid composition reported by Tristram.[26] The sedimentation coefficients both in water and in deuterium oxide were determined at 20°.

Results

Pycnometric Determination of Partial Specific Volumes.—In calculating the preferential hydration of a protein from its hydrodynamic density, it is essential to know the partial specific volume of the protein. For the purposes of these experiments, it was, in particular, necessary to know whether $\bar{v}$ varies with salt concentration and temperature. The partial specific volumes of BSA and RNase were, therefore, determined as a function of temperature in distilled water and in 1.4 molal potassium chloride. The results appear in Table I. Edsall quotes values of 0.730 and 0.734 for the partial specific volume of BSA at 20° and 0.709 for RNase at 25° C.[27] Considering the uncertainties that may arise in measuring the moisture content of protein samples,[5] the agreement of the measured values with the literature is very good. The agreement between the measured partial specific volumes in KCl and in water is excellent. The partial specific volumes of both proteins are unaffected by the presence of very considerable amounts of salt. On the other hand, the partial specific volume is a rather strong function of temperature.

TABLE I

PARTIAL SPECIFIC VOLUMES OF RNase AND BSA AS A FUNCTION OF TEMPERATURE AND SALT CONCENTRATION

Protein	Salt	Temp., °C.	$\bar{v}$	Protein	Salt	Temp., °C.	$\bar{v}$
RNase	..	34.1	0.712	BSA	..	29.9	0.738
RNase	..	34.1	.709	BSA	..	29.9	.741
RNase	..	20.0	.703	BSA	..	10.0	.726
RNase	..	20.0	.703	BSA	..	10.0	.725
RNase	..	8.9	.699	BSA	KCl	29.6	.742
RNase	..	8.9	.699	BSA	KCl	10.1	.729
RNase	KCl	29.6	.708				
RNase	KCl	10.1	.699				

Preferential Hydration in Various Salts.—Figure 1 shows the dependence of the "viscosity-corrected" sedimentation coefficient, $S\eta$, of BSA on the density of the medium for four salts: potassium chloride, sodium chloride, lithium chloride and ammonium sulfate. The temperature in all runs was 20–25°, and in any one run the change was less than 0.5°. The *p*H was 5.5. A number of features of this series of experimental results are of interest. First of all, the plots for BSA in sodium and potassium

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(22) G. P. Baxter and C. C. Wallace, *ibid.*, **38**, 259 (1916).

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(25) A. Hvidt, *Biochim. et Biophys. Acta*, **18**, 306 (1955).

(26) G. R. Tristram, in H. Neurath and K. Bailey, Ed., "The Proteins," Vol. I, Academic Press, Inc., New York, N. Y., 1953, p. 215.

(27) J. T. Edsall, in H. Neurath and K. Bailey, Ed., "The Proteins," Vol. I, Academic Press, Inc., New York, N. Y., 1953, pp. 634–639.

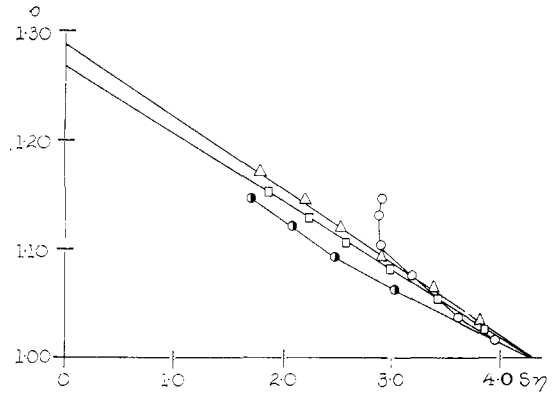

Fig. 1.—Dependence of $S\eta$ on ρ for bovine serum albumin, pH 5.5, 20 to 25°. The salts used to increase the density are: NaCl, △; KCl, □; $(NH_4)_2SO_4$, ◑; LiCl, ○.

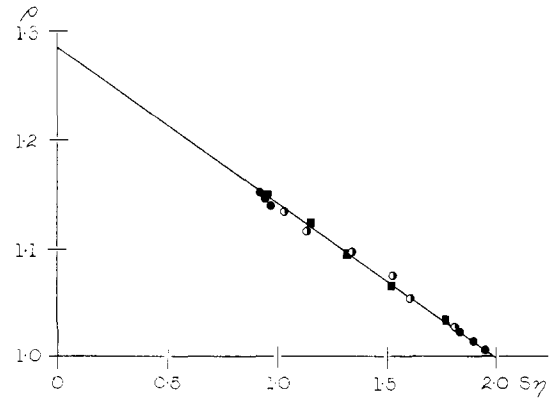

Fig. 2.—Dependence of $S\eta$ on ρ for ribonuclease, pH 7, 20–25°. The salts used to increase the density are: NaCl, ■; $(NH_4)_2SO_4$, ◑; KCl, ●.

chloride are almost linear. There is, in both cases, a slight downward curvature, but straight lines fit the data within experimental error. Extrapolation of the data in NaCl and KCl to the vertical axis gives hydrodynamic densities of 1.288 g./cm.^{3} in NaCl and 1.268 g./cm.^{3} in KCl. Taking the partial specific volume as 0.734, the preferential hydration of BSA in sodium chloride is 0.19 g./g.; in KCl, A is 0.26 g./g. The difference is outside the range of experimental error.

In lithium chloride and ammonium sulfate, the points do not fall on straight lines. Both sets of points define plots that curve upward rather sharply. These two series of experiments have two other features in common. At low concentrations of LiCl and at all concentrations of $(NH_4)_2SO_4$, the points fall well below the points of the KCl and NaCl series. If a hazardous extrapolation is carried out from the S_w^{20} intercept through the lowest point on each of these two plots, it intercepts the vertical axis at the very low density of 1.22 for both salts. The other common feature of the two sets of experiments is that high concentrations of either lithium chloride or ammonium sulfate will precipitate BSA, while the protein is soluble in saturated solutions of either sodium or potassium chloride.

Figure 2 shows $S\eta$ as a function of density for RNase in KCl, NaCl and $(NH_4)_2SO_4$. Here, in

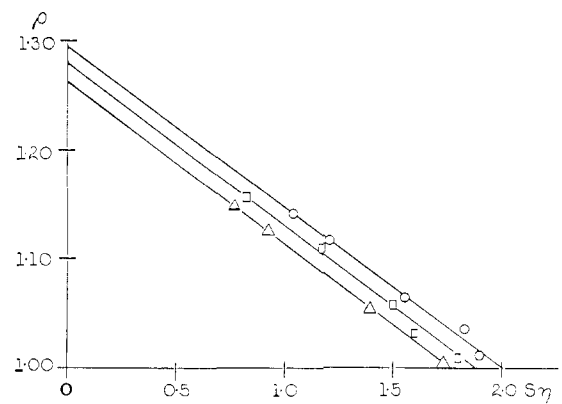

Fig. 3.—Dependence of $S\eta$ on ρ for ribonuclease in KCl at 5°, ○; 25°, □; 40°, △.

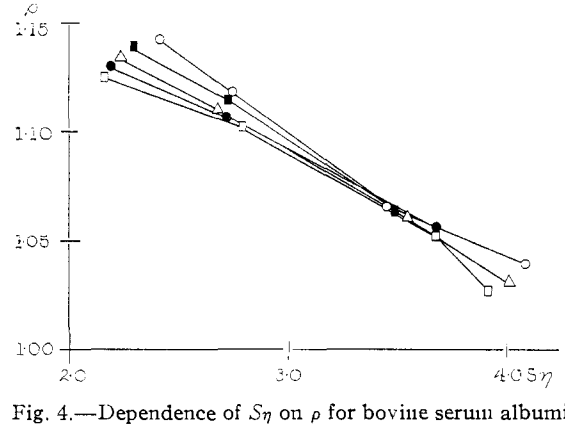

Fig. 4.—Dependence of $S\eta$ on ρ for bovine serum albumin in KCl at 2°, ○; 15°, ■; 25°, △; 35°, ●; 45°, □.

contrast to the results with BSA, the points from the three sets of experiments all fall within experimental error of the same straight line. The plot intercepts the vertical axis at a density of 1.284; taking the partial specific volume as 0.703, this corresponds to a preferential hydration of 0.34 g. per g.

Preferential Hydration as a Function of Temperature.—Figures 3 and 4 show the sedimentation coefficient *versus* density plots for RNase and BSA in KCl at various temperatures. The plots for RNase at each temperature are straight lines; no curvature up or down was observed in any of the experimental plots. The values for the hydrodynamic density at each temperature and the corresponding data for the partial specific volume and preferential hydration are given in Table II. There does seem to be a slight dependence of A on

TABLE II

THE PREFERENTIAL HYDRATION OF RNase IN KCl AS A FUNCTION OF TEMPERATURE

Temp., °C.	$\bar{v}$	ρ_h	A
40	0.715	1.262 ± 0.004	0.37 ± 0.03
25	.706	1.278 ± .004	.35 ± .03
5	.697	1.296 ± .004	.33 ± .03

temperature, the preferential hydration decreasing as the temperature decreases, but the differences are not outside experimental error. It could not

be far wrong to say that the preferential hydration of RNase is independent of temperature over a range of 5–40°.

The data for BSA in Fig. 4 are by no means so clear-cut. At 15° and at 25°, the points fall, within experimental error, on straight lines. In fact, if straight lines connecting the highest and lowest points at each of the temperatures are extrapolated to the vertical axis, the family of plots looks very much like that for RNase. The variation in the apparant hydrodynamic density from 1.25 to 1.30 g./cm.^{3} over the range from 45–2° is, like that for RNase, entirely accounted for by the variation of the partial specific volume. The preferential hydration calculated for each temperature is within experimental error of 0.25 g. of excess water per gram of protein. Unfortunately, however, the data at 2°, 35° and 45° do not describe straight lines. Above 25°, the data curve rather sharply downward, while at 2° the points swerve upward at the lower salt concentrations. The apparent hydrodynamic density increases with salt concentration at 2° and decreases with increasing salt concentration at the higher temperatures.

Preferential Hydration as a Function of *p*H.—Figure 5 shows the variation of the sedimentation coefficient with density for BSA and for RNase in citrate buffer at *p*H 4.7, phosphate buffer at *p*H 6.7 and borate buffer at *p*H 9.2. All the runs were done at 20°, and KCl was the density-increasing solute. The sedimentation coefficient of RNase is not at all affected by such a wide variation in *p*H, but the behavior of BSA is once again extremely variable. The apparent hydrodynamic density of RNase does not vary beyond experimental error. The average value, 1.285 g./cm.^{3}, falls neatly between the previously determined hydrodynamic densities at 5° and 25°. The preferential hydration of RNase is not a function of *p*H. The values of ρ_h for BSA are different in the three buffers. The hydrodynamic density is 1.26 g./cm.^{3} at *p*H 4.7, 1.28 at *p*H 6.7 and 1.30 at *p*H 9.2. Since only two points were taken at each *p*H, it cannot be said whether the data show a curvature similar to that found in the temperature study.

Sedimentation of BSA and RNase in Deuterium Oxide.—The sedimentation coefficients of BSA and RNase in D_2O were measured to provide a check against any large systematic error in the plotting method which might throw the hydration measurements into question. Svedberg and Erikssen-Quensel[24] suggested that $S\eta$ *versus* ρ plots with D_2O as the density-increasing material should, on extrapolation, intercept the density axis at the reciprocal of the partial specific volume of the macromolecule. Their results with hemocyanin were in good agreement with pycnometric values of $\bar{v}$. The method has been used repeatedly to obtain $\bar{v}$'s when too little material was available for a standard pycnometric determination. Precise determination of $\bar{v}$ requires, however, that the sedimentation coefficient be corrected for the effect of deuterium exchange on both the molecular weight and the partial specific volume of the macromolecule. In Table III appear the results of the experiments with BSA and RNase. The sedimentation coefficients in water are taken from the previous experiments in KCl–water solutions. All the runs were done at 20°. For the deuterium exchange corrections, the molecular weight of RNase was taken at 13,680[28] and that of BSA as 65,400.[29] The agreement between $\bar{v}$ values obtained by sedimentation in D_2O and those measured by the pycnometric method is quite good. The sedimentation values are the lower of the two in both cases, but the uncertainty of the deuterium exchange correction on the one hand and the moisture content of the protein samples on the other is such that the differences between the data are not considered significant. It does not seem that the pycnometric method has any great advantage in precision over the sedimentation technique, and the latter method requires far less material. A complete set of measurements in triplicate can be done on 10 mg. of material, as compared to the several hundred milligrams required for pycnometric determinations of equal precision.

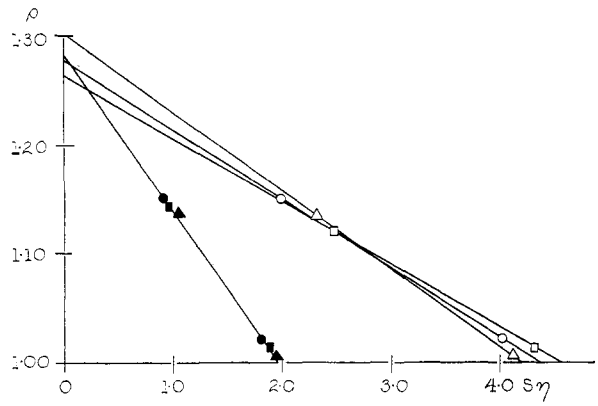

Fig. 5.—Dependence of $S\eta$ on ρ for ribonuclease (filled points) and bovine serum albumin (open points) on *p*H at 20°: *p*H 4.7 (citrate, □; *p*H 6.7 (phosphate), O; *p*H 9.2 (borate), Δ.

TABLE III

DETERMINATION OF PARTIAL SPECIFIC VOLUMES BY SEDIMENTATION IN D_2O AND H_2O

Protein	S	#D's exchanged	S^{D_2O} (corr.)	S^{H_2O}	$\bar{v}$	$\bar{v}$ (pycnom.)
RNase	1.59	230	1.48	2.00	0.693	...
RNase	1.56	230	1.45	2.00	.705	...
RNase	1.60	230	1.48	2.00	.693	...
Average	..	..	..	..	.697	0.703
BSA	3.38	1040	3.11	4.44	.723	...
BSA	3.38	1040	3.11	4.44	.723	...
BSA	3.35	1040	3.08	4.44	.727	...
Average	..	..	..	..	.724	0.734

In regard to the purpose of the experiments in this context, the $S\eta$ *versus* ρ plotting method as it has been applied here seems to have no large systematic errors built into it. It seems legitimate to interpret the depression of the apparent hydrodynamic density below $1/\bar{v}$ as being due to preferential hydration.

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(29) J. M. Creeth, *Biochem. J.*, **51**, 10 (1952).

Discussion

Comparison of the experimental results obtained with BSA and RNase shows clearly that the sedimentation behavior of the two proteins in concentrated salt is different in every respect.

RNase, at any given temperature, shows the same behavior at all the values of *p*H and in all the salts that have been examined. The variation of the plots with temperature is entirely accounted for by the measured variation of the partial specific volume. Under all conditions studied, the sedimentation *versus* density plots are linear. The preferential hydration of RNase is constant over a temperature range from 5–40° and at *p*H values from 4.7 to 9.2. At 20°, the preferential hydration is the same in sodium chloride, potassium chloride and ammonium sulfate. The interpretation of the data for RNase in terms of preferential hydration seems unambiguous.

On the other hand, the sedimentation *versus* density plots for BSA vary with every parameter examined: temperature, *p*H, and the species of salt present. Wherever complete series of points have been taken, the plots are curved to some extent, and the data do not give an unequivocal measure of preferential hydration. The curvature of the plots indicates that one or more of the molecular constants of equation 3 varies with salt concentration: molecular weight, partial specific volume, frictional coefficient or preferential hydration. Where the plot curves upward, as in lithium chloride or ammonium sulfate, or in potassium chloride at 2°, a variation in molecular weight—aggregation—may well be the cause of the deviation from linearity. But the downward curvature observed in other experiments must be due to something else, since it may be assumed for these proteins which contain a single polypeptide chain that a decrease in molecular weight is unlikely with increasing salt concentration. In potassium chloride, at two temperatures, the partial specific volume of BSA is unaffected by the presence of a high concentration of salt; $\bar{v}$ may, of course, change in other salts, but there is no particular reason to believe that it does.

An increase in the fractional coefficient with salt concentration at high temperature and a decrease in f with increasing ρ at low temperature would explain the data in Fig. 4 very well. Since a shape change in the molecule should involve a sizable entropy change, such a change would be expected to be variable with temperature. On the other hand, changes in α with salt concentration could also account for the curvature of the data in Fig. 4. Since the preferential hydration is a function of the *difference* between the amount of salt bound and the amount of water bound, a variation in α would indicate a change in the relative amounts of salt and water adsorbed to the protein. In fact, an increase in the preferential hydration will be found whenever the proportion of salt in the bound solvent increases more slowly than the proportion of salt in the medium. Where considerable amounts of salt are bound at low salt concentrations then the preferential hydration will change with the salt concentration, even if the absolute amounts of salt and and water bound do not change. Such an effect would cause a downward curvature of the $S\eta$ *vs.* ρ plot and therefore is not adequate to explain the upward curvature of the BSA data at 2°.

Klotz has compared the affinity of various proteins for binding a number of anions.[30] Of all the proteins listed by Klotz, serum albumin is by far the most voracious ion-binder; ribonuclease, on the other hand, is practically inert in binding anions. It might be reasonable to suppose, therefore, that the variability of the observed preferential hydration for BSA as contrasted with its constancy for RNase could be due to a difference in their behavior toward salt ions, rather than in their affinity for water.

On the other hand, the differences between the behavior of the two proteins could as easily be due to a differing tendency to change shape under varying conditions. If such deformations as a function of *p*H and temperature were found for BSA and not for RNase, it would be expected that the sedimentation coefficients of the two would vary in quite different ways with changes in the experimental conditions.

The most significant conclusion to be drawn from this work is that the preferential hydration of RNase does not change under a rather wide variety of conditions. Since RNase binds little, if any, of the salts used in these experiments, it seems likely that the absolute amount of water bound does not vary.

(30) I. M. Klotz, *Cold Spring Harbor Symposium for Quantitative Biology*, **14**, 97 (1950).