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Composition and Hydration of Protein Crystals in Salt Solutions[2]

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Values are reported for the compositions and densities of β-lactoglobulin, hemoglobin and chymotrypsin DP crystals in a number of salt solutions. β-Lactoglobulin crystals suspended in ammonium sulfate solutions differ markedly from hemoglobin and chymotrypsin crystals. The hydration of the protein, as calculated on the assumption that salt in the protein crystal is associated with the same amount of water as in the suspending medium, leads to values for protein hydration which are in good agreement with the relative vapor pressure water content curve except in cases where there is an apparently extensive combination of protein with salt.

The water content of large protein crystals can be determined readily by loss in weight on drying.[3] Estimates of the water in protein crystals have also been made by means of density and X-ray data.[4,5] The results of these measurements show the β-lactoglobulin and horse methemoglobin crystals contain about 0.8 g. of water per g. of protein in the absence of salt. If, however, the water content of these protein crystals is estimated by the method of

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(3) T. L. McMeekin and R. C. Warner, This Journal, 64, 2393 (1942).

(4) D. Crowfoot, *Chem. Revs.*, 28, 215 (1941).

(5) M. F. Perutz, *Trans. Faraday Soc.*, XLIIB, 187 (1946).

proportionality[6] or by the density method[7] in the presence of salts, the values found[3,5] for hydration are much smaller, being only 0.2 to 0.3 g. of water per g. of protein, whereas the total water content amounts to 0.6 to 0.8 g. per g. protein. The low values obtained in measuring hydration by the proportionality and density methods are due to the penetration of protein crystals by salts and other small molecules used in the suspending medium. It has been assumed[5] that there are two kinds of water in the protein crystal: water which is bound to the protein and is not available as a solvent ("bound water"), and water through which salts diffuse freely and reach the same concentration as in the suspending medium. Evidence for the location of salt in the protein crystal is difficult to obtain. It has been reported, however, that the dimensions of the unit cell of β-lactoglobulin, sheep methemoglobin and pepsin crystals change in definite steps during shrinkage or swelling due to changes in vapor pressure,[8] indicating two kinds of water. In order to obtain further information on the nature of the water in protein crystals, the compositions and densities of β-lactoglobulin, hemoglobin and chymotrypsin-diisopropyl phosphate crystals were determined as a function of the concentration of inorganic salts in the suspending medium.

Materials and Methods

β-Lactoglobulin Crystals.—The previously described method for preparing large crystals was used.[9] The water content of the crystals appears to be independent of the method of preparing β-lactoglobulin.

α-Chymotrypsin-diisopropyl Phosphate Crystals.—Unusually large and well-formed crystals of the diisopropyl phosphate derivative DP of chymotrypsin were obtained from Mr. Eugene F. Jansen of the Western Regional Research Laboratory, who has described their preparation.[10]

Hemoglobin Crystals.—Large bipyramidal crystals of human oxyhemoglobin were obtained from Dr. David L. Drabkin of the University of Pennsylvania, who has published their preparation.[11] The crystals were stored at 2° in phosphate buffer at *p*H 6.8. For studies in ammonium sulfate solutions, they were washed repeatedly with ammonium sulfate at *p*H 6.8 until the crystals were free from phosphate. The hemoglobin crystals were investigated over a long period of time after their preparation. No difference was noted in the composition or permeability of these crystals due to age or to a probable change to methemoglobin.

Equilibration of Protein Crystals in Salt Solutions.—Protein crystals were equilibrated at 25° with a large excess of the salt solution adjusted to the isoelectric point of the protein. The time of equilibration was varied from 1 minute to 14 days. From composition studies it was found that equilibrium was reached in 15 minutes. Most of the results reported were obtained after equilibrating for 1 and 2 days. However, there is a tendency for the protein crystals to break up in high salt concentrations on long standing. Consequently, shorter periods of 1 and 2 hours are advantageous, particularly in the case of β-lactoglobulin.

Conditions for Determining Dry Weight.—The determination of protein concentrations in crystals, as well as in solution, is based on dry weight. Proteins may be dried with or without heat coagulation. The influence of heat coagulation and the temperature of drying on the weight of β-lactoglobulin and hemoglobin solutions were determined. A standard β-lactoglobulin solution was found to contain 5.655% protein when freeze-dried over P_2O_5 at room temperature; 5.628% at 70° *in vacuo;* 5.612% at 93° *in vacuo;* and 5.610% *in vacuo* at 105°. Aliquots of the same β-lactoglobulin solution, which were heat-coagulated and dried *in vacuo* at 70°, gave a value of 5.630% protein; 5.634% *in vacuo* at 93°; and 5.625% *in vacuo* at 105°. These results show that the value for the concentration of protein in solution, determined by freeze-drying over P_2O_5, is 0.5% higher than the value obtained by drying at 70° *in vacuo;* that within 0.2% there is no difference in the weight of β-lactoglobulin due to heat coagulation.

Experiments on hemoglobin solutions gave similar results, showing that procedures involving heat coagulation gave the same dry weight at 70° as the non-heat coagulated protein also dried at 70°. As a consequence of these findings, heating to constant weight at 70° *in vacuo* with a small stream of dried air passing through the oven to constant weight was adopted as the best conditions for drying proteins. β-Lactoglobulin, dried at 70° without coagulation, is easily soluble in salt solutions and can be partially recrystallized. The difference in the results obtained between 70 and 105° drying of proteins *in vacuo* amounts to only 0.3% which is usually not significant in measurements on proteins.

Determination of Composition and Density of Crystals.—The water content of protein crystals was determined by the previously described method.[3] Ammonium sulfate was removed from the protein by dropping the dried crystal into about 10 cc. of boiling water, thereby coagulating the protein and extracting the salt. This procedure of washing the coagulated crystals was repeated several times until the salt was completely removed. In some cases the crystals were dissolved in water and the protein precipitated with trichloroacetic acid instead of being heat-coagulated. Values obtained for the concentration of ammonium sulfate in the protein crystal, after removing the protein with trichloroacetic acid, were in good agreement with those obtained by extracting the coagulated crystal with hot water. The amount of ammonium sulfate present was determined by Nesslerization. Although the sulfate ion concentration was not determined in the crystal, the fact that calculated densities agree with the determined values indicates that the sulfate concentration was essentially equivalent to that of the ammonium ion. In the case of lithium salts, the halogen concentration was determined. The weight of protein in the crystal was determined by subtracting the weight of salt in the crystal from the dry weight of the protein plus salt. The weight of the extracted dried protein crystal was also used to determine the weight of the protein in the crystal. Usually the values obtained by these two methods were in good agreement.

For determination of density, the equilibrated crystal was blotted rapidly and dropped into a density gradient tube made with xylene–bromobenzene saturated with water which had been calibrated with salt solutions of known densities.[12,13] After 5 minutes the crystal came essentially to rest and its density was interpolated between 2 drops of standard salt solutions with a slightly higher and lower density. The values obtained for protein crystals by this method are reproducible to ±0.001 in density units.

Results

The composition of β-lactoglobulin crystals suspended in a 3.0 *M* ammonium sulfate solution was determined as a function of time. Figure 1 shows that the rate of loss of water by the crystals in the first 7 minutes is greater than the rate of increase of ammonium sulfate. The water in the crystal under these conditions reaches equilibrium in about 10 minutes compared to 15 minutes for the salt. The values obtained for the composition (0.59 g. water and 0.16 g. salt/g. protein) of these crystals at 15 minutes are in fair agreement with the values obtained by suspending crystals for 24 hours in a

(6) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 169 (1917).

(7) G. S. Adair and M. E. Adair, *Proc. Roy. Soc.* (*London*), **B120**, 422 (1936).

(8) M. F. Perutz, *Research*, **2**, 52 (1949); H. E. Huxley and J. C. Kendrew, *Acta Cryst.*, **6**, 76 (1953).

(9) T. L. McMeekin, M. L. Groves and N. J. Hipp, THIS JOURNAL, **72**, 3662 (1950).

(10) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(11) D. L. Drabkin, *ibid.*, **185**, 231 (1950).

(12) K. Linderstrøm-Lang, *Nature*, **139**, 713 (1937).

(13) R. F. Boyer, R. S. Spencer and R. M. Wiley, *J. Polymer Sci.*, **I**, 249 (1946).

TABLE I

COMPOSITIONS AND DENSITIES OF PROTEIN CRYSTALS IN SALT SOLUTIONS AT 25°

Suspending medium, mole/l.	Composition of crystal: g. H_2O / g. protein	Composition of crystal: g. salt / g. protein	Density	Hydration (h) g./g. protein	Salt/water (inside) / Salt/water (outside)
		Chymotrypsin DP			
Ammonium sulfate					
1.14	0.63	0.06	1.210	0.26	0.58
1.32	.63 (0.62–0.63)	.07 (0.066–0.075)	1.213	.26	.58
1.50	.61 (.60– .62)	.08 (.070– .089)	1.217	.24	.59
2.20	.59	.10	1.231	.30	.50
2.70	.57 (.56– .58)	.13 (.129– .143)	1.240	.27	.52
3.00	.55	.15	1.245	.25	.55
3.50	.53	.17 (.174– .175)	1.254	.25	.52
4.00	.51	.20	1.264	.24	.53
		Hemoglobin			
2.36	0.58	0.10	1.209	0.31	0.46
2.84	.56	.11	1.218	.31	.42
3.22	.54	.14	1.222	.29	.47
3.92	.51 (0.49–0.53)	.18 (0.16–0.19)	1.234	.26	.49
		β-Lactoglobulin			
0.00	0.85 (0.83–0.87)	0.00	1.151	..	..
2.20	1.02 (.91–1.20)	.25 (0.22–0.28)	1.185	.29	0.72
2.70	0.68 (.63– .76)	.17 (.15– .19)	1.209	.29	.57
3.00	.62 (.59– .71)	.19 (.18– .22)	1.218	.24	.61
3.50	.55 (.50– .60)	.20 (.16– .23)	1.231	.22	.59
4.00	.52 (.50– .55)	.22 (.20– .24)	1.240	.22	.57
Zinc sulfate					
2.31	1.02 (0.92–1.08)	0.29 (0.25–0.35)	1.296	.27	0.74
2.66	0.80 (.78– .82)	.23 (.22– .24)	1.305	.28	.65
2.99	.66 (.62– .70)	.22 (.18– .25)	1.316	.22	.66
3.35	.55 (.52– .60)	.21 (.18– .27)	1.315	.18	.67
Lithium bromide					
12.0 (sat.)	.32 (.31– .34)	.61 (.56– .66)	1.62	– .08	1.25
Lithium chloride					
13.7 (sat.)	.21 (.18– .23)	.28 (.26– .30)	1.32	– .13	1.60

3.0 M ammonium sulfate solution (Table I). The density of the β-lactoglobulin crystals equilibrated in 3.0 M ammonium sulfate for 16 minutes was found to be 1.217 and its protein content to be 57.2%. Since the salt-free crystal has a density of 1.151 and a protein content of 54.0%, it was calculated that the crystal contracted 0.17 cc. per g. protein, or about 11% on equilibrating in 3.0 M ammonium sulfate.

The effect of salt solutions on the composition and density of β-lactoglobulin, chymotrypsin DP and hemoglobin crystals is shown in Table I. The composition values are averages, and the variations of individual determinations are shown in parentheses. Many more measurements of composition were made on β-lactoglobulin crystals since they showed greater variations. The effect of the concentration of salt in the suspending medium on the water content of β-lactoglobulin is greater than on chymotrypsin DP and hemoglobin, Fig. 2. This difference in behavior of β-lactoglobulin is particularly marked in 2.2 M salt where its water content of 1.02 g. per g. protein is considerably greater than the value of 0.85 in the absence of salt.

The salt content of protein crystals increases with an increase in salt concentration in the suspending liquid when calculated on the basis of the water content of the crystal. In the case of ammonium sulfate, the ratio of the salt per g. water in the crystal to that of the salt per g. water in the suspending liquid is essentially constant for each of the proteins, with the exception of β-lactoglobulin in 2.2 M salt (Table I). This ratio of salt to water

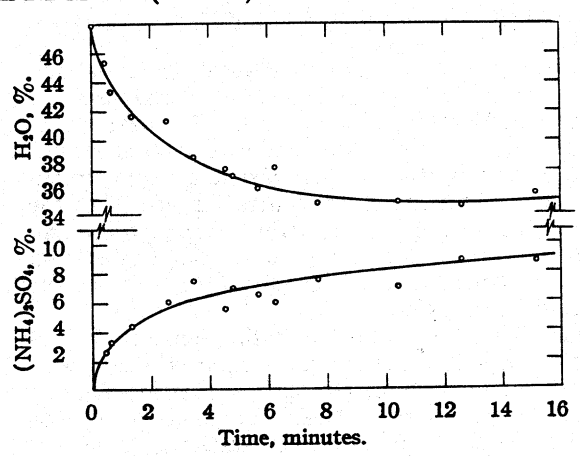

Fig. 1.—Loss in water and gain in ammonium sulfate by β-lactoglobulin crystals suspended in 3.0 M ammonium sulfate as a function of time.

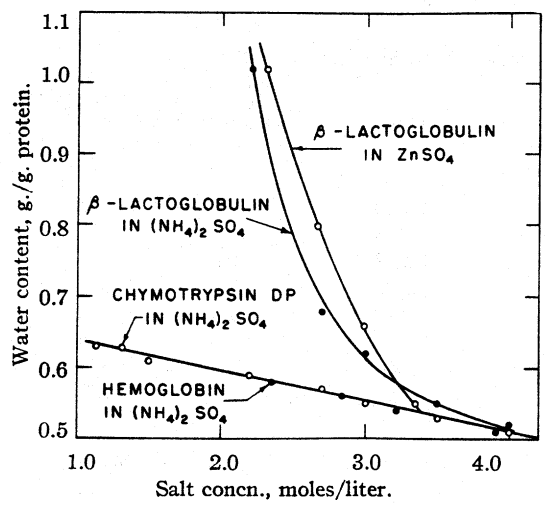

Fig. 2.—Water content of protein crystals as a function of salt concentration.

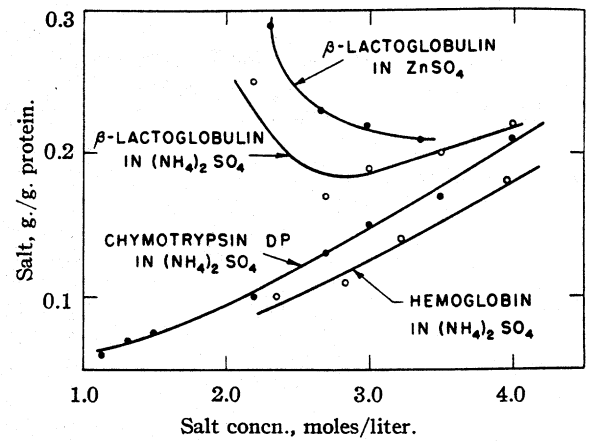

Fig. 3.—Salt in protein crystal as a function of the salt concentration in the suspending medium.

is inversely related to the percentage protein in the crystal. Thus the average value for this ratio for hemoglobin is 0.46; chymotrypsin, 0.54; and β-lactoglobin, 0.59; as compared with average per cent. protein values of 59.3, 58.8 and 55.9, respectively. The application of this principle is particularly apparent in the β-lactoglobulin composition data where the percentage protein varies considerably. For example, the crystal in 2.2 *M* ammonium sulfate contains only 44% protein and the salt–water ratio is 0.72; while in 2.7 *M* ammonium sulfate, the crystal contains 54% protein and the salt–water ratio is reduced to 0.57. The values for the salt–water ratio of β-lactoglobulin crystals suspended in zinc sulfate solutions are similar to those obtained in ammonium sulfate though slightly higher. The values for the salt–water ratio for β-lactoglobulin crystals suspended in lithium salt solutions, however, are markedly different, being greater than unity. This finding is good evidence that both lithium chloride and lithium bromide combine with β-lactoglobulin in the crystal. It is of interest to note that the β-lactoglobulin crystals were no longer soluble in dilute salt solutions after being immersed in concentrated solutions of lithium salts, showing that the β-lactoglobulin was denatured.

If the salt content of these crystals were referred to the weight of protein, the individual proteins differ considerably (Fig. 3). In the case of β-lactoglobulin the amount of salt associated with a gram of protein does not increase in a regular manner with an increase in concentration of salt in the suspending liquid. In contrast, hemoglobin and chymotrypsin crystals show a regular increase in salt per g. protein with an increase in salt in the suspending medium.

Since zinc and other metallic ions bind proteins,[14,15] it was of interest to compare the composition of β-lactoglobulin crystals suspended in zinc sulfate solutions with their composition in ammonium sulfate. The results indicate (Table I, Figs. 2 and 3) that the behavior of β-lactoglobulin crystals is essentially the same in zinc sulfate as in ammonium sulfate. These results do not, however, preclude the possibility of zinc ions combining in a unique manner with imidazole groups, since the total of such an ion combination would be small as compared to the total amount of zinc present.

The complexity of the relation of the composition of β-lactoglobulin crystals to the composition of the salt solution in which it is suspended, as compared to chymotrypsin DP and hemoglobin, is also apparent when its densities are plotted as a function of the ammonium sulfate concentration (Fig. 4). The effect of the specific volume of the protein on its crystal density is reflected by the difference between the densities of hemoglobin (specific volume 0.749) and chymotrypsin (specific volume 0.736) crystals, since their water contents are the same (Fig. 2) and their salt contents not too different (Fig. 3).

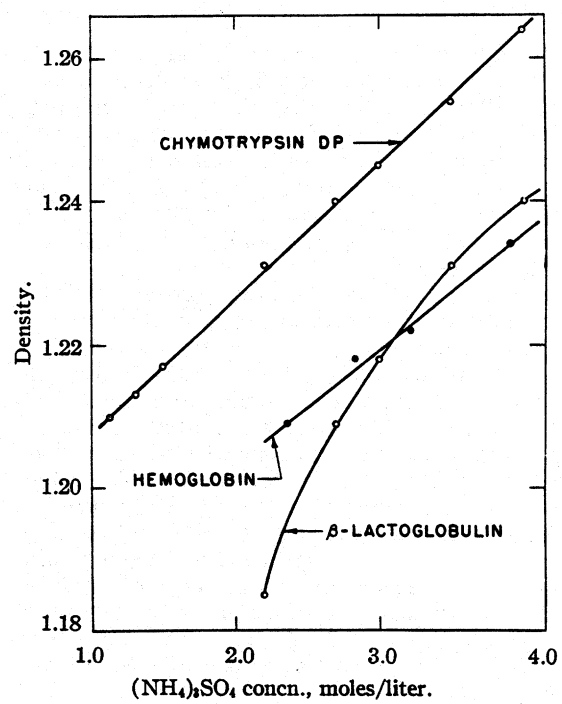

Fig. 4.—Density of protein crystals as a function of the concentration of ammonium sulfate.

(14) C. Tanford, This Journal, **74**, 211 (1952).
(15) F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952).

The values for the density of human hemoglobin crystals recorded in Table I are in good agreement with the values reported by Perutz[5] for horse methemoglobin and are consistent with the results obtained by Drabkin[11] for human hemoglobin suspended in phosphate solutions. The extrapolated values for the density, 1.16, and water content, 42.2%, of salt-free crystals of human hemoglobin are also in better agreement with values for horse hemoglobin[5] than with the reported value of 41.4% for the volume of the liquid in human carboxyhemoglobin crystals.[16] It is of interest to note that the value obtained for the density of chymotrypsin in the absence of salt by extrapolating the density curve to zero salt concentration on a weight per cent. salt basis is 1.183. The water content in the absence of salt is estimated to be 40.9% by a similar extrapolation of Fig. 2. From these data, the specific volume of chymotrypsin DP is calculated to be 0.736, in agreement with the observed value of 0.736 for α-chymotrypsin in solution.[17]

The effect of pH on the density and composition of β-lactoglobulin crystals was determined by equilibrating the crystals in 2.7 M ammonium sulfate solutions adjusted to a series of pH values by the addition of ammonium hydroxide or sulfuric acid. The results, as given in Table II and Fig. 5, show that from pH 3 to 8 there is a small but definite change in the composition and densities of the crystals. From measurements of densities of β-lactoglobulin solutions in 0.8 M ammonium sulfate over the same pH range, it was found that the apparent specific volume of β-lactoglobulin did not change; consequently it appears probable that the change in density of β-lactoglobulin crystals with pH is due to changes in the salt and water contents of the crystals rather than to changes in specific volume of the protein.

TABLE II

EFFECT OF pH ON COMPOSITION OF β-LACTOGLOBULIN CRYSTALS IN 2.7 M $(NH_4)_2SO_4$

pH	Total water, g. H_2O/g. protein	G. $(NH_4)_2SO_4$/g. protein	Hydration (h), g. H_2O/g. protein	Density
7.9	0.77	0.22	0.27	1.203
5.1	.71	.19	.27	1.214
3.3	.66	.18	.25	1.221

No significant change in the bound water as a function of pH was found. In the case of methemoglobin crystals, Perutz[5] found that "bound water" was a maximum near the isoelectric point, and decreased when the suspending salt was made more acid or alkaline.

The protein crystal data of Table I have been used to calculate "bound water" or hydration (h) per g. protein, using the equation[18]

$$h = 1/P(1 - C_T/C_u)$$

in which P is the amount of protein per g. total water of the crystal, C_T is the concentration of salt per g. of total water of the crystal, and C_u is the concentration of salt per g. water in the suspending liquid.

(16) J. Boyes-Watson, E. Davidson and M. F. Perutz, *Proc. Roy. Soc. (London)*, **A191**, 83 (1947).

(17) G. W. Schwert and S. Kaufman, *J. Biol. Chem.*, **190**, 807 (1951).

(18) D. M. Greenberg and M. M. Greenberg, *J. Gen. Physiol.*, **16**, 559 (1933).

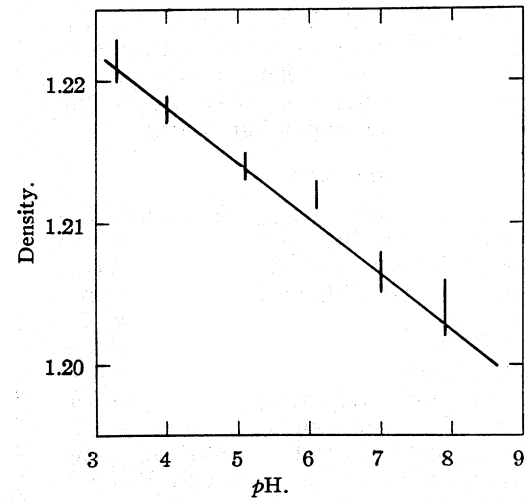

Fig. 5.—Effect of pH on the density of β-lactoglobulin crystals suspended in 2.70 M ammonium sulfate.

Another method for calculating (h) g. water per g. protein which is usually more convenient, is given by the equation

$$h = W_T - \left[S \times \left(\frac{100 - C}{C}\right)\right]$$

where W_T is the total water per g. protein, S is the salt content of the crystal in g. per g. protein and C is the weight per cent. of salt in the suspending medium. The values for hydration as recorded in Table I show considerable variation, reflecting the sensitivity of the calculation. The negative values for hydration in lithium salts indicate the binding of salt and are a consequence of the greater apparent concentration of lithium salts in the crystal water rather than in the suspending liquid, as previously noted. The values for hemoglobin and β-lactoglobulin vary significantly in the direction which would be expected from the vapor pressures of the suspending solutions. The values for hydration of chymotrypsin appear, however, to be independent of the salt concentration.

Calculations based on the densities and compositions of the protein crystals in Table I show that the volume of the crystals per g. protein decreases when the concentration of salt increases with the exception of β-lactoglobulin in 2.2 M ammonium sulfate. Thus the volume of a salt-free β-lactoglobulin crystal is 1.61 ml. per g. protein, as compared to 1.92 ml. in 2.2 M salt, giving an increase of 0.31 ml. per g. protein, or an increase of 19% in volume. In 4 M salt, however, the volume of β-lactoglobulin crystal is 1.40 ml. per g. protein, resulting in a contraction of 0.21 ml. based on its volume in the absence of salt, a contraction of about 13%. Similar calculations show that hemoglobin crystals contract 0.02 ml. per g. protein or 1.4% between 2.4 and 3.9 M ammonium sulfate, and chymotrypsin DP crystals contract 0.04 ml. per g. protein or 3.1%, in going from 1.1 to 4 M ammonium sulfate. If the volume of the hemoglobin crystal were calculated in the absence of salt, how-

ever, from the density of 1.162 and a water content of 42% obtained by extrapolating the salt data to zero concentration, a value of 8.6% is obtained for the total contraction when a salt-free hemoglobin crystal is placed in 3.9 *M* ammonium sulfate.

Discussion

The distribution of salt in a protein crystal suspended in salt solutions has been used by Perutz[5] to determine the hydration of the protein molecule on the assumption that all the salt is dissolved in a portion of the water of the crystal and that the concentration of the salt solution in the crystal is the same as that of the suspending medium. There is no unique way for determining the state of salt in protein crystals. The probability that protein molecules in the crystal combine with salt is indicated by the finding of Sørensen and Palmer[19] that β-lactoglobulin crystals suspended in very dilute ammonium chloride solutions contain a greater amount of ammonium chloride based on the water content of the crystals than does the suspending liquid. Adair and Adair[20] have also found that in carboxy hemoglobin crystals suspended in 0.01 *M* ammonium phosphate, the distribution of water and salts was such that the ammonium phosphate concentration was greater in the hemoglobin crystal than in the suspending liquid. The results reported in Table I, on the composition of β-lactoglobulin suspended in saturated solutions of lithium salts, are strong evidence for salt–protein combinations. It may be expected that salt–protein combinations will vary with the protein and the salt. Thus Davis and Dubos[21] reported that serum albumin and β-lactoglobulin are unique among proteins in binding long chain fatty acids in solution. It is also well known from the studies of Pfeiffer[22] that lithium salts form complexes with amino acids. It seems probable that the marked difference between the composition of β-lactoglobulin compared with hemoglobin and chymotrypsin, as given in Table I and illustrated in Figs. 2 and 3, is due to a greater degree of combination of β-lactoglobulin with salt.

Previous results on the composition of β-lactoglobulin over the range 28.9–31.6% ammonium sulfate, reported by McMeekin and Warner,[3] showed that the water content of the crystal was essentially the same as in salt-free crystals. The present results, as illustrated in Fig. 2, though slightly different from the previous results due to improved analytical methods, confirm the previous results and illustrate the marked difference between the behavior of β-lactoglobulin and hemoglobin which has been remarked upon by Perutz.[5]

There are several obvious possible ways in which salt can be distributed in a protein crystal. It may be combined to the protein and not dissolved in the water of crystallization, or it may be dissolved in a portion, or in all of the crystal water, or in combinations of these ways. Since the specific volume of ammonium sulfate varies from 0.565 in the solid state to 0.410 in dilute solution, it is possible that the state of ammonium sulfate in the crystal would be reflected in its density. Calculations were made on the density of β-lactoglobulin crystals equilibrated in several concentrations of ammonium sulfate, assuming combination of the salt with the protein or distribution of the salt in a portion or in all of the crystal water, and compared with the experimentally determined density. When the partial specific volume of 0.751 was used for β-lactoglobulin and 1.003 for water, the calculated density agreed well with the experimentally determined density when the specific volume of 0.565 for solid ammonium sulfate was used. Density calculations, based on the salt solution being the same as the suspending solution and also the salt being distributed in all the water, differed significantly from the observed values. These calculations on density favored the idea that the salt is combined with the protein. However, when the more probable values of 0.772 for the specific volume of β-lactoglobulin and 0.983 for water[23] are used in the calculation of the densities of the crystals containing salt, the calculated results for density do not differ significantly from the observed density regardless of the hypothesis for the distribution of the salt in the crystal.

In considering the distribution of salt and water in protein crystals, Alexander and Johnson[24] expressed the view that it is not unreasonable to consider that some of the water is associated with the salt rather than with the protein. In view of a lack of evidence for the distribution of salt in the crystal water, the simplest assumption is that the amount of water associated with the salt in the crystal is the same as that associated with the salt in the suspending liquid. In calculation, this idea amounts to the same result as the "bound water" calculation. However, it does not imply the existence of "bound" and "free" water as pre-existing in the crystal in the absence of salt. Briggs[25]

TABLE III

RELATION OF HYDRATION OF PROTEIN CRYSTALS IN AMMONIUM SULFATE SOLUTIONS TO VAPOR PHASE HYDRATION

R.H., %	Hydration in $(NH_4)_2SO_4$ soln. (*h*), g. H_2O/g. prot.	Vapor phase absorption, g. H_2O/g. prot.	Difference
	β-Lactoglobulin		
90.2	0.29	0.34[23]	+0.05
87.8	.29	.30	+ .01
86.3	.24	.28	+ .04
84.0	.22	.25	+ .03
81.7	.22	.23	+ .01
	Hemoglobin		
89.4	0.31	0.30[a]	−0.01
87.1	.31	.28	− .03
85.3	.29	.26	− .03
82.0	.26	.23	− .03

[a] Crystalline horse oxyhemoglobin. Data of J. R. Katz, *Koll. Beihefte*, 9, 32 (1917).

(19) M. Sørensen and A. H. Palmer, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **21**, 283 (1938).

(20) G. S. Adair and M. E. Adair, *Biochem. J.*, **28**, 1230 (1934).

(21) B. D. Davis and R. J. Dubos, *J. Exptl. Med.*, **86**, 215 (1947).

(22) P. Pfeiffer, *Z. angew. Chem.*, **36**, 137 (1923).

(23) T. L. McMeekin, M. L. Groves and N. J. Hipp, *J. Polymer Sci.* Jan. (1954).

(24) A. E. Alexander and P. Johnson, "Colloid Science," Oxford University Press, London, 1949, p. 144.

(25) D. R. Briggs, *J. Phys. Chem.*, **36**, 367 (1932).

has clearly defined the relation between vapor pressure and the degree of protein hydration.

A comparison of protein hydration in crystals suspended in ammonium sulfate solutions (data from Table I) with the total water content of salt-free crystals equilibrated at the same relative humidity is shown in Table III.

It may be noted that the values obtained by these methods essentially agree and also that they vary in a consistent manner with relative humidity as would be expected. Adair and Adair[7] and Adair and Robinson[26] have reported a similar relation for hemoglobin. Results for the hydration of β-lactoglobulin in sucrose as obtained by this calculation[9] are considerably less than would be expected from the vapor phase measurements of the water content of β-lactoglobulin crystals at the same relative humidity. Thus the hydration or "bound water" for β-lactoglobulin crystals in 34.7% sucrose with a relative humidity of 96.8% was found to be 0.32 g. of water per g. of protein, as compared to a value of 0.64 g. of water from vapor phase measurements. This difference between these two values may be attributed to the binding of sucrose by the protein or to the difficulty of making accurate vapor phase measurements at high humidities.

It is apparent from these results on the composition of protein crystals suspended in various media that the calculation for hydration or "bound water" varies with the method chosen for the determination.

(26) G. S. Adair and M. E. Robinson, *J. Physiol.*, **72**, 2P (1931).