latter at the end of the $—CH_2 \cdot CH_2Cl$ group. It is, however, much lower than the static dielectric constant of polyvinyl chloroacetate,[2] although it contains the same kind and number of polar groups. Two reasons for this difference may be advanced: (1) the methyl group in IV inhibits the rotation of the polar ester group, as shown by the comparison of I and II, and (2) the carbonyl group is attached directly to the chain, instead of through an oxygen. There is also a possibility that the side-chain carrying the chlorine is long enough to permit some intramolecular association of dipoles, which reduces the polarization much as intermolecular dipole interaction decreases the polarization of ordinary polar liquids.

A detailed discussion of IV is not possible, because the chlorine analysis and the insolubility suggest that it is somewhat cross-linked, instead of being a simple linear polymer. Other preparations, made under milder conditions, gave higher chlorine analyses, but the polymers were also insoluble in solvents which might be expected to dissolve polymers of the structure IV. However, only a few hundredths of a per cent. of bifunctional polymerant is enough to produce an insoluble polymer. It will be noted that IV, in contradistinction to II, shows the presence of a secondary maximum at low temperatures. Probably the replacement of a methyl hydrogen of II by the $—CH_2Cl$ group changes the spatial relationships so that crystallization becomes possible again; this is indicated by some simple experiments with models. But considerable work remains to be done on the $(—CH_2 \cdot CXY)_n$ polymers before a detailed correlation between structure and mechanical and electrical properties can be made.

Summary

1. The dielectric constants and loss factors of polymethyl acrylate, polymethyl methacrylate (alone and plasticized with 20 and 30% diphenylmethane), polymethyl-α-chloracrylate and polychloroethyl methacrylate at temperatures in the range −70° to +100° and at frequencies from 60 to 8000 cycles have been determined.

2. The preparation of chloroethyl methacrylate (b. p. 170°) is described.

3. Some preliminary results on a correlation between structure and electrical properties for polymers of the type $(—CH_2 \cdot CXY—)_n$ are given.

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The Hydration of β-Lactoglobulin Crystals*

BY THOMAS L. MCMEEKIN AND ROBERT C. WARNER

Knowledge of the composition of protein crystals is of importance in the interpretation of solubility, precipitation and X-ray data on proteins.

Several methods have been utilized in calculating hydration of protein crystals. Sørensen and Høyrup[1] developed the "Method of Proportionality" for evaluating the water content of protein crystals. Adair and Adair[2] applied density determinations to the measurement of hydration. A further method was devised by Crowfoot and Riley,[3] which is based on X-ray measurements of wet and dry crystals. The present report deals with the direct measurement of hydration by the loss in weight of a single protein crystal as well as hydration deduced from density determinations.

β-Lactoglobulin crystals as described by Palmer[4] are particularly valuable for direct study of hydration, since the crystals are quite large and may be prepared in the absence of salt or in the presence of high concentrations of salt.

Materials and Methods

β-Lactoglobulin was prepared from skim milk by the method of Palmer.[4] After several recrystallizations by dialysis, a further crystallization was made by adding concentrated ammonium sulfate solution to the protein solution through a rotating cellophane membrane. When the concentration of ammonium sulfate reached 2.66 molar, needle-shaped crystals appeared. The crystals were separated from the supernatant liquid and dissolved in a small volume of water. Salt was then removed by dia-

* Not copyrighted.

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

(2) Adair and Adair, *Proc. Roy. Soc.* (London), **B120**, 422 (1936).

(3) Crowfoot and Riley, *Nature*, **141**, 521 (1938).

(4) Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

lyzing for two days against distilled water in the presence of toluene. The solution contained 3% protein and had a *p*H of 5.2. This solution, with a small amount of toluene, was stored at 4° in a closed Erlenmeyer flask. After several weeks large crystals of β-lactoglobulin appeared. Both tabular and plate crystals were present; however, only the stable tabular crystals were used in obtaining the reported measurements. Many of the crystals were from 2 to 3 mm. in length and about 1 mm. in each of the other dimensions. The nitrogen content of a single oven-dried crystal was found to be 15.7% in good agreement with Palmer's[5] most recent value of 15.6%.

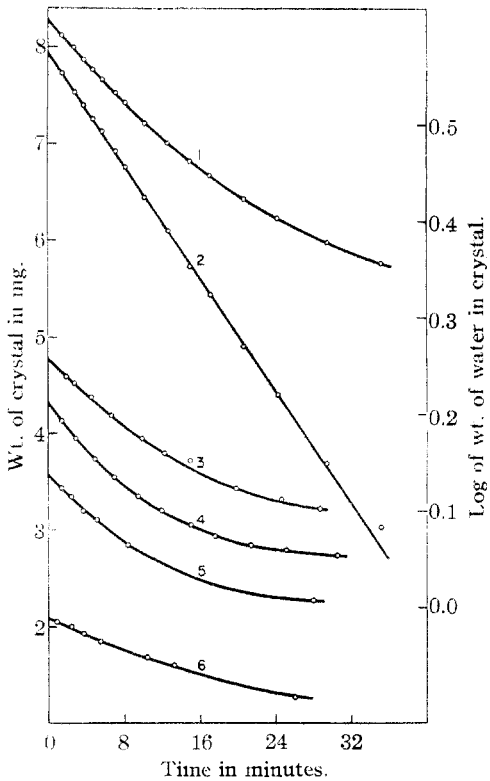

Fig. 1.—Rate of loss of water by β-lactoglobulin crystals at room temperature: Curves 1, 4 and 5, crystals from salt free solution; Curve 3, crystal from ammonium sulfate solution; Curve 6, crystal of $Na_2SO_4 \cdot 10H_2O$; Curve 2, plot of logarithm of weight of water in the crystal shown in Curve 1.

Hydration of the protein crystal was determined by weighing the wiped crystal on a rapid-weighing spring balance sensitive to 0.01 mg. The loss in weight by the crystal from the moment of its removal from the mother liquor until it reached the equilibrium weight obtained after drying in a vacuum oven at 80°, or over phosphorus pentoxide at room temperature was considered to be the water of hydration. The loss in weight with time was plotted (Fig. 1) and the weight of the crystal at zero time was determined by extrapolation. The adhering mother liquor was removed by placing the crystal between the smooth sides of two small pieces of absorbent cotton flannel. This operation required about thirty seconds. The drying crystal was kept on a removable weighing pan during the entire drying and weighing operation. The effectiveness of the method of removing the adhering mother liquor was demonstrated on crystals of ammonium sulfate, sodium sulfate decahydrate and copper sulfate pentahydrate. The crystals of ammonium sulfate or copper sulfate did not lose weight in air after being wiped with the cloth as described. The copper sulfate crystal lost 29.1% of its weight in a vacuum oven at 80°. The theoretical loss in weight is 28.8% when four molecules of water are removed. The amount of water in the hydrated sodium sulfate crystals was found by this method to be: (a) 55.8%, and (b) 57.2%. The theoretical value for 10 molecules of water is 55.9%. While it seems probable that a small amount of mother liquor would adhere to the crystal, these results on crystals with and without water of crystallization indicate that the amount of mother liquor adhering to the crystal is small and within the experimental error of other measurements involved.

The amount of protein in the β-lactoglobulin crystal was determined from the dry weight in vacuum at 80°, or in vacuum over phosphorus pentoxide at room temperature. In the case of the experiments with added ammonium sulfate, the weight of ammonium sulfate was deducted from the total dry weight to give the weight of the protein. Ammonium sulfate was determined by direct nesslerization in a volume of 50 ml. The presence of the protein during the development of the color with ammonia and Nessler reagent caused a small increase in the color, amounting to 3.5%. Correction for this effect has been applied to the measurements.

Densities were determined in mixtures of bromobenzene and xylene and, in some cases, in solutions of saturated ammonium sulfate containing added sodium sulfate or in solutions of sucrose. The crystals were removed from the mother liquor on the end of a thin strip of paper, pressed against a piece of cotton flannel and then placed in a test-tube containing the flotation medium in a water-bath at 25°. The composition of the solutions was adjusted so that the crystals just sank in one solution and just floated in another. The densities of the two solutions, referred to water at 4°, were then determined pycnometrically at 25°. The density of the crystal was taken as the mean of the two values.

Hydration of β-Lactoglobulin at *p*H 5.2 in the Absence of Salt.—The loss of water by single protein crystals at room temperature and at 80° *in vacuo* was determined. The amount of water present in the crystal was independent of the temperature at which the crystal had been stored. Thus, the amount of water in a crystal kept at 4° was the same as that of a similar crystal stored at 25°. The results of the measurements on the rate of evaporation at room temperature and total amount of water present in β-lactoglobulin crystals are shown in Fig. 1 and Table I. The average values of 0.66 g. of water per g. of air-dried protein and 0.84 g. of water per g. of vacuum-dried protein are of the same order as the value of 0.54 g. of water per g. of air-dried protein calculated from the data of Crowfoot.[6] The rate of evaporation follows a first-order equation, the rate of loss of water being proportional

(5) M. Sørensen and Palmer, *Compt. rend. trav. lab. Carlsberg*, **21**, 283 (1938).

(6) Crowfoot, *Chem. Rev.*, **28**, 215 (1941).

TABLE I

HYDRATION OF β-LACTOGLOBULIN CRYSTALS AT *p*H 5.2 IN THE ABSENCE OF SALT

Wet crystals (extr. to zero time), mg.	Air dry crystals, mg.	Water per g. of air dried protein, g.	Oven dry crystals, mg.	Water per g. of oven dry protein, g.
1.80	1.07	0.68	0.96	0.87
3.10	1.82	.70	1.64	.89
3.12	1.92	.62	1.71	.82
2.64	1.58	.67	1.48	.78
2.95	1.79	.65	1.55	.90
3.59			2.03[a]	77
1.07			0.57[a]	.88
1.41			0.77	.83
8.27			4.56	.81
4.32	2.60	.66	2.35	.84
	Average	0.66 ≈ 40% water		0.84 ≈ 46% water

[a] Dried over phosphorus pentoxide at room temperature.

to the amount of water remaining. This is shown by Curve 2 in Fig. 1, in which the logarithm of the weight of water in the crystal is plotted as a function of time. A straight line is obtained up to the point at which about 70% of the water has been lost. Beyond this the rate falls below that calculated from a first-order equation. It might be inferred from this calculation that the vapor pressure of water in the crystal does not change while most of the water is being lost. The first-order constant obtained for the rate of loss of water increases as the size of the crystal is decreased, presumably because of the increase in surface per volume ratio. Ferry and Oncley[7] analyzed dielectric dispersion curves of β-lactoglobulin solutions and compared the results with those obtained from ultracentrifuge, diffusion and viscosity measurements. The data are interpreted in terms of an elongated ellipsoidal molecule and hydration of 0.3 g. of water per g. of protein. Since their measurements were made on solutions, it is not necessarily to be expected that such hydration would be the same as that of the crystal.

Hydration of β-Lactoglobulin in the Presence of Concentrated Ammonium Sulfate Solutions.—β-Lactoglobulin crystals freed from adhering solution were placed into 28.9, 30.2 and 31.6% by weight solutions of ammonium sulfate. All of the crystals floated at first and later sank to the bottom, the time of sinking increasing with increase in concentration of salt. The crystals and salt solutions were allowed to equilibrate for two days at 25° and then the crystals were removed and their surfaces freed from adhering liquid. The amounts of water and ammonium sulfate in the crystal were determined as previously described. The results are recorded in Table II.

The amount of water associated with 1 g. of protein remained unchanged when the salt-free crystals were placed in concentrated salt solutions. However, ammonium sulfate diffused into the crystal and, on the assumption that ammonium sulfate goes into the water of the protein crystal rather than becoming attached to the protein, the concentration of salt in the water of the protein crystal

(7) Ferry and Oncley, THIS JOURNAL, **63**, 272 (1941).

TABLE II

COMPOSITION OF β-LACTOGLOBULIN CRYSTALS IN THE PRESENCE OF AMMONIUM SULFATE SOLUTIONS AT *p*H 5.2 AND 25°

Am. sulfate in filtrate, wt. %	Hydrated crystal (extr. to zero time), mg.	Dry crystal, mg.	Am. sulfate in crystal, mg.	Am. sulfate in crystal water, wt. %	Concn. am. sulfate in crystal expressed as % of concn. in filtrate	Water per g. dry protein, g.
28.9	(a) 3.30	1.97	0.415	23.8	82.3	0.86
	(b) 5.85	3.55	.719	23.8	82.3	.82
30.2	(a) 7.80	4.82	.915	(23.5)[a]	(77.8)[a]	(.76)[a]
	(b) 2.64	1.62	.328	24.3	80.4	.79
31.6	(a) 4.78	2.88	.683	26.4	83.4	.86
	(b) 6.55	4.00	.910	26.3	83.2	.82
				Average	82.3	.83

[a] Not included in the average.

amounts to about 82% of the salt concentration in the surrounding liquid. This finding is confirmed by density determinations as recorded in the next section.

Direct measurements were made of the length and width of a crystal by means of a micrometer at low magnification before and after placing it in the concentrated salt solution. No change in crystal dimensions was noted. However, the method was sensitive to only about 2% and the calculated increase in the linear dimensions of the crystal due to the presence of ammonium sulfate would be about 2%, assuming that the partial specific volumes of the constituents remain unchanged in the presence of salt. The protein crystals break more easily in the presence of salt than in its absence. This may be an indication of an increase in volume of the protein crystal due to the added salt.

When a β-lactoglobulin crystal, in equilibrium with a 30.2% by weight solution of ammonium sulfate of *p*H 5.2, was heated in boiling water for thirty minutes, the crystal became opalescent and insoluble in water without obvious loss of form. The heat-coagulated crystal contained less water than the uncoagulated crystal, the water content being 0.53 g. per g. of protein. However, the salt concentration calculated on the basis of the water present in the coagulated crystal was found to be the same as in the uncoagulated crystal. When the method of proportionality is applied to the data obtained on the heat-coagulated β-lactoglobulin crystal, a value of 0.15 g. of water per g. of protein is obtained. Adair and Adair[2] obtained values of 0.17 to 0.19 for coagulated egg albumin by means of the method of proportionality.

Density of β-Lactoglobulin Crystals.—The data obtained on density determinations are recorded in Table III. The "density difference" given in the table is the difference between the densities of the solutions of higher and of lower density than the crystal.

The wet crystals were found to increase gradually in density after immersion in the bromobenzene-xylene mixture. There was essentially no change for about five minutes and the densities recorded are based on the initial behavior of the crystals on being placed in the solutions. Since this behavior was independent of the size of the crystal over a range of more than tenfold in weight, it is improbable that the results are appreciably influenced by occlusion of air or other possible surface effects. When the wet crystals had stood for as long as twenty-four hours in

TABLE III

DENSITY DETERMINATIONS ON β-LACTOGLOBULIN CRYSTALS

Preparation	Flotation medium	Density	Density difference
Wet crystals	BBX[a]	1.146	0.004
Dry crystals	BBX	1.260	.003
Wet crystals after standing 24 hrs. in BBX[a]	BBX	1.260	.001
Crystals equilibrated with 30.2% $(NH_4)_2SO_4$	BBX	1.214	.005
Wet crystals	Satd. $(NH_4)_2SO_4$ + Na_2SO_4	1.240	.007
Wet crystals	Sucrose	1.256	.008

[a] BBX refers to mixtures of bromobenzene and xylene.

the flotation medium, their density had increased so that it was precisely equal to that of the dry ones. The water in the crystal had thus evidently been removed, leaving only the anhydrous protein. Crystals which were dried over phosphorus pentoxide did not change in density following immersion and were not influenced by placing the system in a vacuum.

The density of 1.260 for the anhydrous crystal is in good agreement with determinations on other dry proteins. The specific volume of 0.794 for the crystal is thus much higher than the partial specific volume of 0.751 determined in dilute solution.[8] Cohn[9] has pointed out that this difference cannot be accounted for by electrostriction of the solvent as is the case with amino acids.

The density of 1.146 found for the wet crystals is lower than any density previously reported for protein crystals. It is, however, consistent with the high fraction of water found in the crystal. Using the partial specific volume of lactoglobulin $v_p = 0.751$ determined by Pedersen,[8] and assuming that of water to be $v_{H_2O} = 1$, the specific volume of the crystal, $\bar{v}$, can be calculated from the formula $\bar{v} = v_p X_p + v_{H_2O} X_{H_2O}$, where X_p = fraction of protein = 0.54, and X_{H_2O} = fraction of water = 0.46. The density $(1/\bar{v})$ is found to be 1.155. The partial specific volumes of proteins have been found in general to be independent of concentration over a range up to 10 or 15% protein. It is probable that this constancy does not extend to a system which has the composition of the crystals and that v_p is higher than the value assumed above (and v_{H_2O} consequently lower). Any change of this sort would tend to give a better agreement with the observed value.

The higher values reported in the literature for the density of wet protein crystals have all been obtained in aqueous media. In view of the results reported above on the salt content of crystals in ammonium sulfate solution which demonstrate the ease with which salt exchange takes place with the environment, it can be concluded that the density of a protein crystal in equilibrium with its mother liquor cannot be determined by immersion in an aqueous medium of different composition. This is well shown by the density determinations in aqueous media given in Table III. The crystals which had a density of 1 146 in bromobenzene–xylene increased to 1.240 in $(NH_4)_2SO_4$ + Na_2SO_4 and to 1.256 in sucrose because of the diffusion of salt or sugar into the water in the crystal. The difference between the densities in the two aqueous media is similar to that found by Adair and Adair[2] and is evidently due to a difference in the distribution of salt and sugar between the inside and the outside of the crystal. The fact that the observed density in the sugar solution approaches the value for the density of the anhydrous protein indicates that the distribution must be nearly equal in this case.

The density of crystals equilibrated with 30.2% ammonium sulfate was determined in bromobenzene–xylene to be 1.214. The analytical results on such crystals (Table II) show the concentration of ammonium sulfate in the water of the crystal to be 24.4% and the composition of the crystal to be 48.7% protein and 51.3% salt solution. The ammonium sulfate solution in the crystal would have a specific volume of 0.877. From these data and the assumption that the partial specific volume of the β-lactoglobulin is 0.751, the density of the crystal can be calculated to be 1.225 by the method used above for the salt-free solution. The error in the calculation is probably referable to the value assumed for the partial specific volume of the protein.

The use of organic solvents for density determinations on wet crystals has been criticized because of the possibility of irreversible changes caused by the solvents. We have found that crystals which have stood for several days in the bromobenzene–xylene are completely soluble in dilute salt solutions, indicating the absence of denaturation. Any error incurred by the use of such solvents is certainly less than that inherent in the use of salt solutions which yield densities bearing little relation to the density of the crystal in its mother liquor.

Discussion

The hydration of proteins as determined by the "method of proportionality" of Sørensen and Høyrup[1] involves the determination of the nitrogen factor (x) as calculated from the equation $x = 100/P_b[1 - a_b/a_f]$ where a_f is weight per cent. of ammonia nitrogen in the filtrate, a_b is weight per cent. of ammonia nitrogen in the crystal and adhering mother liquor, and P_b is weight per cent. of protein nitrogen in the crystal. The hydration value is obtained by comparing the nitrogen factor of the wet crystals with the nitrogen factor of the anhydrous salt-free protein. Adair and Adair[2] have derived a formula to give the same information from density determinations as is obtained by the "method of proportionality" from analytical data.

Sørensen and Høyrup[1] found the nitrogen factor (x) to be independent of the concentration of salt in the presence of varying concentrations of ammonium sulfate. They considered this to be evidence that salt did not go into the crystal. Table IV shows the results obtained when our data are calculated by means of the proportionality formula.

The nitrogen factor here is found to be independent of the concentration of salt. Direct analysis of these identical crystals shows a con-

(8) Pedersen, *Biochem. J.*, **30**, 961 (1936).
(9) Cohn, *Ann. Rev. Biochem.*, **4**, 93 (1935).

TABLE IV

COMPOSITION OF β-LACTOGLOBULIN CRYSTALS IN THE PRESENCE OF AMMONIUM SULFATE SOLUTIONS AT *p*H 5.2 AND 25° CALCULATED ACCORDING TO SØRENSEN AND HØYRUP

NH_3-N in filtrate (a_f), wt. %	NH_3-N in crystal (a_b), wt. %	Protein N in crystal (P_b), wt. %	Wt. of protein containing 1 g. N (x)
6.13	(a) 2.66	7.35	7.70
	(b) 2.60	7.55	7.63
6.40	(a) 2.49	7.80	7.83
	(b) 2.63	7.64	7.71
6.70	(a) 3.02	7.17	7.68
	(b) 2.94	7.36	7.64
		Average	7.70

centration of salt in the crystal which is apparently a constant ratio of the concentration of salt in the surrounding liquid. If the total water in the crystal and the ratio of the concentration of salt inside the crystal to that outside are independent of the salt concentration, it follows that the nitrogen factor (x) will show a variation of about 0.5% for the salt concentration range used in our experiments. Thus the constancy of the nitrogen factor is not a measure of the freedom of the crystal from salt, and the value of this factor is not related to the total amount of water associated with the protein crystal. This view, that the salt penetrates the protein crystal and that the concentration of the salt in the crystal is about 82% of the salt concentration in the surrounding liquid, independently of the concentration, is strengthened by the results of Chick and Martin[10] on egg albumin in ammonium sulfate solutions. Chick and Martin found that the water in pressed crystalline egg albumin contained less salt than the filtrate. In three experiments the salt present in the pressed crystals amounted to 17.6, 22.1 and 22.4% by weight of ammonium sulfate, while the corresponding filtrates contained 26.9, 27.2 and 28.1% by weight of ammonium sulfate. The percentage ratio of salt inside of the crystals to that of the filtrate is 65.4, 81.2 and 80.0 which compares with our value of 82.3% for β-lactoglobulin.

The finding that ammonium sulfate diffuses into the protein to the extent of 82% of the outside concentration on the basis of water of crystallization may be considered to indicate that the water of crystallization is bound in two different ways, as is the case of water in copper sulfate crystals.[11] The present measurements do not furnish evidence of the distribution of salt in the crystal, although in the calculation of a numerical value for the ratio of the concentration of salt in the crystal to the outside salt concentration, the assumption is tacitly made that the salt is equally distributed in the water of the crystal. Following the formula of Sørensen and Høyrup,[1] the water of hydration for β-lactoglobulin may be calculated as $(7.70-6.41)/6.41 = 0.20$ g. of water per g. of protein, in contrast to the direct experimental finding of 0.83 g. of water per g. of protein.

Adair and Adair's[2] formula may be applied to the experiment in 30.2% ammonium sulfate for which density determinations on the crystals were made. Their formula requires the density of the crystal to be equal to that of the salt solution in which it is suspended and hence it is not directly applicable to the case considered above. However, by the use of the additional analytical data available, a value for the water of hydration of 0.294 g. water per g. of protein can be calculated by their method as compared with the above value of 0.20 g. The application of the method of proportionality to β-lactoglobulin in very dilute salt solutions led M. Sørensen and Palmer[5] to conclude that β-lactoglobulin does not contain "surplus water but, on the contrary, surplus ammonia or ammonium chloride."

Density determinations on protein crystals have been made in connection with X-ray measurements. Crowfoot and Riley[3] thus report values for β-lactoglobulin of 1.257 for the wet crystal in sugar solutions and 1.27 for the dry crystal in organic solvents. These values are in agreement with those found here under similar experimental conditions. Crowfoot realized that the value for the wet crystal was an upper limit, but nevertheless discarded the value for the dry crystal because it was so close to that for the wet crystal. She assumed 1.31 (dry density of insulin) in making her calculations when it was the wet density that was in error. A recalculation from the data of Crowfoot[6] using the density 1.146 for the wet β-lactoglobulin crystal gives a value for the wet molecular weight of 61,100 and on a dry basis (46% water) a value of 33,000.

The molecular weight for an air-dried crystal calculated from the data of Crowfoot,[6] using a density of 1.26, is 39,700. The data in Table I show that our air-dried crystals contained 9.78% water. Applying this correction, an anhydrous molecular weight of 35,800 is obtained.

(10) Chick and Martin, *Biochem. J.*, **7**, 392 (1913), and correction on page 548 of the same volume.

(11) Beevers and Lipson, *Proc. Roy. Soc.* (London), **A146**, 570 (1934).

Crowfoot[6] also has given the dimensions of the unit cell in wet and in air-dried β-lactoglobulin crystals, and the following shrinkages on drying can be calculated for the various dimensions: $a = 11.1\%$, $b = 6.7\%$, and $c = 28.6\%$. In order to correlate the unit cell shrinkage with the macroscopic behavior of the crystal, we have observed the change in dimensions of the crystal on drying in the air in a low power microscope with a micrometer eyepiece. The shrinkages observed in two experiments were: length, 8.5% and 9.9%; height, 6.8% and 7.8%; width, 29.5% and 26.4%. These changes are of the same order as those in the unit cell. The shrinkage of the crystal was also measured in a mixture of bromobenzene–xylene over a period of twenty-four hours. The change in dimensions observed was 9.8% and 31.5% in the length and width, respectively. This confirms the conclusions from density determinations that a dehydrated crystal is obtained after contact with bromobenzene–xylene for twenty-four hours. It also indicates that the water in the crystal is not replaced by the organic liquid. In order to check this point a crystal which had been dehydrated in xylene was wiped and weighed by the technique used previously. There was no change in weight after putting the crystal on the balance or after placing the crystal in an oven at 105°. These measurements, together with the consideration that the densities of vacuum-dried crystals and crystals which stood in bromobenzene–xylene are the same, indicate that in the dry crystal there is no space occupied by air.

The dehydrated crystals obtained with bromobenzene–xylene had good form and a high degree of birefringence as compared with the air-dried crystal. This technique might be used with advantage to obtain completely dehydrated crystals for X-ray examination. In any such application xylene alone should be used as the dehydrating agent because of the tendency of the crystal to become discolored after long standing in the presence of bromobenzene.

It seems likely that previous estimates of the water of hydration of protein crystals are entirely too small, because of the effect of diffusion of salt into the crystals. It may be calculated that the cell shrinkage reported by Crowfoot[6] for crystalline hemoglobin amounting to 46.6% would lead to a hydration value nearer to 0.9 g. of water per g. of protein than the value of 0.18 to 0.30 g. calculated by Adair and Adair.[2,12]

Summary

1. β-Lactoglobulin crystals were found to contain 0.83 g. of water for each g. of anhydrous protein over a wide range of temperature in the absence of salt or in the presence of high concentrations of ammonium sulfate.

2. Ammonium sulfate diffuses rapidly into the β-lactoglobulin crystal; however, the concentration of ammonium sulfate on the inside of the crystal reaches only 82.3% of the salt concentration in the surrounding liquid.

3. The density of wet β-lactoglobulin crystals was measured in organic liquids and was found to be 1.146. This value is consistent with the high water content of the crystal. Values for the density of β-lactoglobulin crystals under various conditions are reported. It is shown that the method of measuring the density of wet crystals in aqueous media is subject to large errors.

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(12) Since the preparation of this paper, Bailey (*Trans. Faraday Soc.*, **38**, 186 (1942)), has reported that crystalline edestin contains about 0.67 g. of water per g. of protein.