the sensitivity of the method frequently used to characterize an amylase, namely, the determination of the amyloclastic to saccharenogenic ratio.[18]

(18) W. J. Whelan and H. Nasr, *Biochem. J.*, **48**, 415 (1951).

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[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Measurements of the Density, Composition and Related Unit Cell Dimensions of Some Protein Crystals[1a]

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Some measurements of composition, density and unit cell size are reported for crystals of β-lactoglobulin, dimer albumin and insulin. These measurements have been made for the "wet," air-dried and vacuum dried states and at other points over a range of relative humidity. The reliability and correlation of the data is discussed. Since the measurements of the composition, density and unit cell volume are all independent, the errors involved in each are unrelated. The partial specific volumes of water and protein in the dimer albumin crystals over the range 13 to 55% water have been derived. These values are not equivalent to comparable data on β-lactoglobulin reported by McMeekin. The significance of this discrepancy is discussed, it has not yet been elucidated.

Introduction

Detailed studies of the properties of protein crystals usually require preliminary measurements of their composition, density and unit cell size. From these data values for the protein molecular weight may be obtained, and the effects of immersion in a variety of media may be calculated. Measurements of protein crystal density and composition present special problems arising from the rapid loss of solvent of crystallization which occurs when the crystals are removed from their mother liquor, and the well known swelling, shrinking and permeability properties in media of different composition.

It is the purpose of this paper to report some studies on single protein crystals in which the only significant components were protein and water. The data are principally for bovine β-lactoglobulin and human serum mercaptalbumin mercury dimer (dimer albumin) crystals. Some incomplete results on acid insulin sulfate crystals also are included. The problems involved in the proper correlations of these measurements and the related concept of "water of hydration" are discussed.

Experimental

Preparation of Crystals.—In this study, large well developed, single crystals of purified protein preparations were required, especially for the composition and density measurements. Techniques were employed and precautions taken to ensure slow crystal growth, and, as far as possible, equant development. The methods used are described below.

Dimer Albumin.—The protein used was a 5 times recrystallized sample of the dimer prepared from a large lot of Squibb 352-RR Fraction V which had been rejected for clinical use because of pyrogen content. The Fraction V had originally been prepared by Method 6 as developed in this Laboratory.[2] The fraction was crystallized as the mercury dimer by the method of Hughes.[3] A sample of the final preparation, dialyzed against cysteine to remove the mercury, indicated an SH content of 0.98 mole of SH/mole of albumin. The dimer appears to be quite stable in a 10% salt-free solution. Thus since the dimer crystals are much less soluble under these conditions than those of the monomer and as the two crystalline forms are quite different, the crystals obtained may be assumed to consist wholly of the dimer form.

Preparative Procedures. (a) **Method of Lewin.**[4]—The technique described by Lewin was employed essentially unchanged. Large crystals were sometimes, but not always, obtained. The results are not predictable. (b) **Vapor Phase Addition of Precipitant.**—A 10% solution of dimer albumin in water was centrifuged to remove any particulate matter. A test-tube containing this clarified solution was placed in a larger tube holding an equivalent volume of aqueous methanol at a concentration somewhat higher than that required to cause crystallization in the protein mixture. The larger tube was tightly stoppered and the whole unit kept at 0° for several weeks. Diffusion of methanol from the outer liquid into the protein solution occurred through the vapor phase, and a gradually changing gradient of precipitant concentration was thus set up. Although small crystals frequently grew at the surface of the protein solution, large single crystals were often obtained in the body of the tube. This method appeared to give more consistent results than method (a). (c) **Use of Gelatin Gels.**—In an attempt to prepare crystalline derivatives of dimer albumin using barium hydroxide, Lewin[4] obtained rigid gels in which particularly well formed single crystals[5] grew, isolated from each other throughout the medium.[6] Comparable conditions were therefore simulated for the pure protein. A solution containing 10% dimer albumin, 1% gelatin and 5% methanol was allowed to stand at 0°. Within 12 hours the mixture was sufficiently rigid to resist pouring. After two weeks, large single crystals appeared throughout the gel. Although showing a marked tendency to twin on the (001) face, the crystals were optically and morphologically identical with those obtained by other methods. The complete absence of gelatin from these crystals has not been demonstrated. They have not, therefore, been used for quantitative measurements in this investigation.

β-Lactoglobulin.—Armour crystalline β-lactoglobulin was used without further purification. This protein has been adequately characterized in the literature and no attempt

(1) (a) This work has been supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University. (b) This work was carried out while one of us (F.M.R.) was an Atomic Energy Commission Predoctoral Research Fellow in the Biological Sciences, and is reported in detail: F. M. Richards, Ph.D. Thesis, Harvard University, 1952.

(2) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(3) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(4) J. Lewin, *ibid.*, **73**, 3906 (1951).

(5) B. W. Low, *ibid.*, **74**, 4830 (1952).

(6) The observation recalls the use of gels (slow diffusion) in the preparation of large single crystals of sparingly soluble inorganic compounds; *cf.* Chamot and Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Edition, John Wiley and Sons, Inc., New York, N. Y., 1947, p. 342.

was made to prepare a more homogeneous material. Samples from Lots No. 0053B and 9053B were recrystallized essentially by the methods of McMeekin and Warner.[7]

Preparative Procedures. (a) **Dilution.**—Saturated solutions of the protein in 0.1 *M* sodium chloride were diluted with 2.5 volumes of water and allowed to stand undisturbed. In no cases were crystals obtained without the concurrent formation of very turbid solutions or rather weak gels. (b) **Slow Dialysis.**—Saturated solutions of the protein in 0.1 *M* sodium chloride were placed in plain glass tubes covered with cellophane at one end and stoppered at the other. These tubes were put in a large volume of water and dialysis was allowed to proceed without stirring. Gels formed near the dialysis membranes and grew slowly up the tube as the salt diffused out. These gels were more rigid than those observed in method (a), and the crystals growing in them were suspended free from each other and the walls of the tube as in the case of the albumin method (c). In this way very perfect specimens in all sizes from 0.1 to 50 mg. were obtained.

For comparison studies, samples of large β-lactoglobulin crystals, prepared in this Laboratory, were kindly given to us by Dr. McMeekin.

Acid Insulin Sulfate.—The protein used was Lilly beef zinc insulin batch number, T 2344.

Preparative Procedure.—The crystals of zinc free acid insulin sulfate were prepared for us by Mr. Henry H. Dix using the method of Oncley, Ellenbogen and Dix[8] who discovered and characterized this new crystalline modification of insulin.

200 mg. of zinc insulin was dissolved in 10 ml. of 0.02 *N* H_2SO_4. If solution was not complete, 0.1 *N* H_2SO_4 was added dropwise until the insulin just dissolved. Six ml. of 0.08 *N* Na_2SO_4 was added and the precipitate was redissolved by adding about 1.5 ml. of 0.1 *N* H_2SO_4. The solution was dialyzed in specially prepared collodion sacs[8] at 5° against 3 changes of 0.003 *N* Na_2SO_4 adjusted to *p*H 1.9 by addition of H_2SO_4. (Under these conditions insulin passes through ordinary cellophane tubing.) The solution was allowed to stand at 5° over a period of several months for crystals to grow.

Composition Measurements.—The dry weight value of a protein preparation is dependent on the drying temperature. Data in the literature[7] on β-lactoglobulin indicate that dry weights determined *in vacuo* at room temperature and at 105° have about the same value (within 1%). As different investigators frequently use different drying conditions, the dependence of the dimer albumin dry weight on temperature was investigated in the following manner.

Two hundred mg. samples of dimer albumin were equilibrated in the absence of air over a sulfuric acid–water mixture giving a relative humidity (R.H.) of about 30%. When the samples had reached a constant weight (1 to 2 days), aliquots were then dried *in vacuo* over P_2O_5 at either 25°, 70° or 100° and weighed after 4- and 24-hour intervals. One sample was dried at 110° in air. Following the final dry weight determination, the samples were re-equilibrated over the 30% relative humidity sulfuric acid solution for 24 hours.

The results for dimer albumin (shown below) and the literature values for β-lactoglobulin indicate that both of these proteins have 70° dry weights within about 0.5% of those obtained under other standard drying conditions *in vacuo*. These differences are less than the other errors in the crystal analyses. The exact drying temperature is thus of minor importance. The dimer albumin and β-lactoglobulin crystals were prepared from three component systems (*i.e.*, protein, water and either salt or alcohol). They were, therefore, thoroughly washed with a saturated aqueous solution of the pure protein before any measurements of composition were made on them. The washes were carried out by allowing the crystals to stand for 24-hour periods in three successive portions of fresh protein solution. The resulting crystals were assumed to contain only protein and water. Analyses showed less than 0.5% salt or alcohol. Composition studies were not carried out on the insulin preparations, the crystals grown so far are rather small (less than 1 mg.), and the salt content introduces special problems.

Drying conditions	Weights as % of initial weight: Equil. 30% R.H.	Drying times: 4 hr.	24 hr.	Re-equil. 30% R.H.	Final color	Soluble in water
25° vac. P_2O_5	100.0	91.6	91.3	100.0	White	Yes
70° vac. P_2O_5	100.0	90.7	90.7	99.0	White	Yes
100° vac. P_2O_5	100.0	90.1	90.1	99.1	White	No
110° in air	100.0	90.5	89.9	96.9	Yellow	No

Analyses were made on single crystals by the method of McMeekin and Warner.[7] The crystals were weighed on a Roller–Smith torsion balance with a 0 to 10 mg. range and a sensitivity of ±0.005 mg. The weighing pans were prepared from aluminum foil and averaged 3 to 4 mg. Weighed crystals were placed in a modified Abderhalden drying pistol arranged for collection of the solvent either over P_2O_5 or in an alcohol Dry Ice trap. The drying was carried out at 70° *in vacuo*. Preliminary trials indicated that 4 hours was sufficient to obtain a constant weight on these small samples. The dry weight under these conditions was considered to represent the protein component.[9] (Other non-volatile components were found to be negligible for purposes of this study.)

"Wet" Crystals.—Crystals were removed from their mother liquor on a small spatula and placed on a piece of hard filter paper previously dampened with a little of the mother liquor. A crystal was turned over on several of its faces with a probe and then transferred to a weighing pan, where its weight was measured as a function of time. Zero time was taken as the moment of placing the crystal on the filter paper. The wiping procedure took about 30 seconds and three or four weights were recorded within the next minute. A value for the "wet" weight was determined by extrapolation to zero time.

"Air-Dried" Crystals.—"Wet" crystals were wiped and allowed to dry in air until they attained a constant weight. This is, of course, only an approximate end-point as the weight depends on the relative humidity. As will be seen, however, it frequently corresponds to a convenient and fairly well defined composition. The additional loss of water on drying *in vacuo* at 70° was then noted.

"Vacuum-Dried" Crystals.—The weights of the crystals after vacuum drying were measured as quickly as possible and recorded as a function of time for approximately 2 to 3 minutes. Extrapolation to zero time gave the dry weight. Water uptake by the dry crystals was much slower than water loss by the wet crystals, and the correction by extrapolation was very small and usually negligible.

Measurements Over a Range of Relative Humidities.—Measurements were made of the composition of single dimer albumin crystals as a function of relative humidity. Similar studies on β-lactoglobulin crystals have already been reported by McMeekin, Groves and Hipp.[10] "Wet" crystals of dimer albumin were placed in evacuated tubes containing sulfuric acid water mixtures of various relative humidities at 0° (made up according to the data supplied by Wilson[11]). After equilibration for 24 hours, the crystals (run in duplicate) were weighed and their densities measured in bromobenzene–kerosene gradient columns. The crystals were then vacuum dried and reweighed. For the crystals equilibrated in high relative humidities, the problems of handling and analyses were the same as described above for "wet" crystals. The equilibrated weight was obtained by extrapolation to zero time after removal from the controlled atmosphere. The time that the crystal was introduced into the gradient tube was also noted and the composition estimated for this time. Because of the weight loss, the water content of a given crystal was not the same at the isotherm point as for the density determination.

Density Measurements.—The densities of the crystals were measured by the two methods developed or extended by us to cope with the problems peculiar to the determination of protein crystal densities. The equilibrium microbalance technique[12,13] was used for the measurements on "wet"

(7) T. L. McMeekin and R. C. Warner, THIS JOURNAL, **64**, 2393 (1942).

(8) J. L. Oncley, E. Ellenbogen and H. H. Dix, in preparation.

(9) It should be noted that no attempt was made to determine the "true" hydration of the protein: the protein component having a constant weight under the given drying conditions is defined as "anhydrous" protein.

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

(11) R. E. Wilson, *Ind. Eng. Chem.*, **13**, 326 (1921).

(12) B. W. Low and F. M. Richards, *Nature*, **170**, 412 (1952).

(13) F. M. Richards, *Rev. Sci. Instr.*, **24**, 1029 (1953).

crystals. The density of the "wet" crystal weighed in its mother liquor is calculated from the equation

$$d_{(crystal)} = d_{(mother\ liquor)} + \frac{\text{apparent wt. of crystal in g.}}{\text{volume of crystal in ml.}} \quad (1)$$

The density of the mother liquor was measured by a standard method. The apparent weight of a crystal, supported on a balance pan while immersed in mother liquor, is recorded as the deflection of a calibrated fiber. The balance is arranged so that the crystal may be observed in two perpendicular directions and photographs taken of the projected areas. From these the volume of a well-formed regular crystal may readily be computed.

The rapid, non-equilibrium, gradient tube technique[14] was used with crystals in all states of hydration. Detailed descriptions of these methods are given in the references cited. For "wet" protein crystals xylene–bromobenzene gradient columns were prepared both from dry and from water saturated components. The crystals were wiped dry in the manner described (30 sec.) and measurements were made within 5 to 10 minutes[14] of the introduction of the crystals into the gradient tube. For "air-dried" and vacuum-dried crystals dry column liquids were used, and preliminary density readings made 3 to 5 minutes after the crystals were introduced.

X-Ray Measurements.—In this investigation the X-ray study of the protein preparations was limited to the measurement of unit cell dimensions. The "wet" and "air-dried" unit cell dimensions of these crystals have been previously reported in the literature.[6,15] Emphasis here has been laid on dimension changes under various conditions, and particularly on the correlations between density, composition and cell volumes measured on the same preparation.

The "wet" crystals were photographed immersed in a drop of their mother liquor in thin-walled Hysil glass tubes. Air-dried crystals were mounted on glass fibers in the usual manner. For vacuum-dried determinations the air-dried crystals and fiber were put into a glass capillary and the free end of the fiber sealed to the capillary wall with a microflame. After vacuum drying the capillary was quickly sealed with wax before the crystal could equilibrate with the water vapor in the air.

All the X-ray photographs were taken using CuK_α radiation and a previously calibrated Buerger Precession Camera.

Results

Composition.—The results of the analyses of the "wet" and "air-dried" crystals are shown in Table I. Vacuum dried salt-free crystals (by definition) contain only protein. The drying curve for the "wet" protein crystals was linear during the first few minutes, the extrapolation to obtain the "wet" weight could, therefore, be made accurately. It rarely amounted to more than 2% of the total weight, and deviations in the values obtained are probably due to improper wiping.

TABLE I

WEIGHT FRACTION OF WATER IN CRYSTALS EXAMINED

Protein	No. of crystals	Weight fraction of water "Wet"	"Air-dried"
β-Lactoglobulin	3	0.464 ± 0.001	0.112 ± 0.006
Published values		.460[7]	.110[7]
		.462[15]	.099[15]
Dimer albumin	5	.556 ± 0.011	.103 ± 0.010

The crystals of β-lactoglobulin used were rather small (less than 5 mg.), and the marked consistency in the results is therefore largely fortuitous. The agreement with the literature values (measured on larger crystals) is satisfactory indicating a successful wiping procedure. Variability in the dimer albumin values is probably related to the softness of the crystals which made them difficult to wipe properly. More consistent values might have been obtained with larger crystals.

Composition as a Function of Relative Humidity.—The composition of dimer albumin crystals equilibrated at points over a wide range of relative humidities are shown in Fig. 1. Comparable results for β-lactoglobulin obtained by Bull[16] on large samples of packed crystals also are shown. McMeekin, Groves and Hipp[10] have made similar measurements on single β-lactoglobulin crystals which confirm the results on large samples.

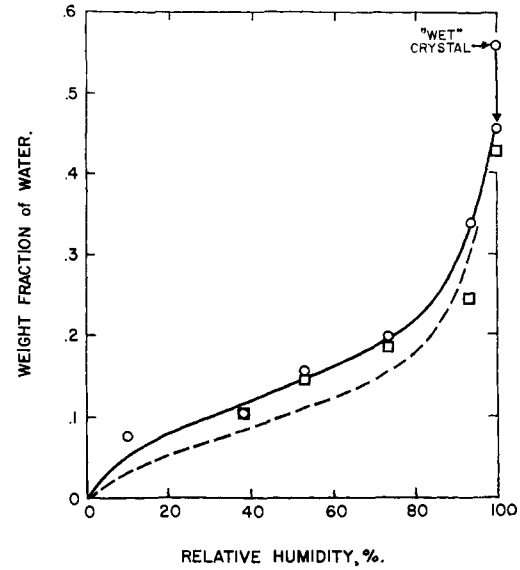

Fig. 1.—Absorption isotherms: (a) solid line, dimer albumin; (b) broken line, β-lactoglobulin data from Bull[16]: O, determined on dehydration cycle; □, determined on hydration cycle.

The reversibility of the isotherm was checked by replacing the vacuum-dried crystals in the same relative humidity as in the initial experiment. In most cases the hysteresis was very small. The discrepancy at 93% relative humidity may be an experimental error and most probably does not represent true equilibrium. The most striking effect is the loss of water in going from mother liquor to vapor of 100% relative humidity. It has not been demonstrated that the vapor surrounding the crystals during all these measurements was really saturated. The isotherm is very steep at high humidities and a 1 or 2% decrease from complete saturation would explain the observed effect.

Density. "Wet" Crystals.—"Wet" crystal density values are listed in Table II. Control studies have shown that the densities of the "wet" protein crystals removed from their mother liquor are the same, within the limits of experimental error recorded, as those measured on crystals from which all salt or alcohol had been removed by the equilibration procedure described earlier. The "wet" crystal density was only 10 to 20% greater than the density of the mother liquor. Thus, for the microbalance determinations, a 5% error in the correction term of equation 1 resulted in less than a

(14) B. W. Low and F. M. Richards, THIS JOURNAL, **74**, 1660 (1952).

(15) F. R. Senti and R. C. Warner, *ibid.*, **70**, 3318 (1948).

(16) H. B. Bull, *ibid.*, **66**, 1499 (1944).

TABLE II

DENSITIES OF SINGLE CRYSTALS[a]

No.	State	Observed densities, g./ml. Gradient tube	Microbalance	Comments	Literature values
			β-Lactoglobulin		
1	"Wet"	1.147 ± 0.003	1.144 ± 0.006[b]	Main batch soon after prepn.	1.146 ± 0.002[b] 1.144 ± .002[c] 1.153 ± .002[d]
2	"Wet"	1.157 ± .003		Sample 1, 2 years after prepn.	
3	"Wet"	1.148 ± .003		Sample prepared by Dr. McMeekin	1.149 ± .001[e]
4	Air-dried	1.257 ± .003			1.260[7]
5	Xylene-dried	1.255 ± .005			1.260[7]
6	Vacuum-dried	1.25 ?[f]			1.25[10]
			Dimer albumin		
7	"Wet"	1.135 ± 0.005	1.135 ± 0.004	Main batch soon after prepn.	
8	"Wet"	1.145 ± .005		Sample 7, 2 years after prepn.	
9	Air-dried	1.288 ± .003			
10	Xylene-dried	1.285 ± .005			
11	Vacuum-dried	1.31 ?[f]			
			Acid insulin sulfate		
12	"Wet"	1.200 ± 0.005[g]	1.190 ± 0.005[h]		
13	"Wet"	1.195 ± .005[i]		Sample 12 after 8 months	
14	"Air-dried"	1.320 ± .003			

[a] All the values recorded are averages for 4 to 6 crystals with the observed variation indicated. [b] The limits of error as reported for this value earlier[12] contain an uncorrected typographical slip. The limits are ±0.006 as given here. [c] F. R. Senti and R. C. Warner, THIS JOURNAL, **70**, 3318 (1948). [d] T. L. McMeekin, M. L. Groves and N. J. Hipp, *ibid.*, **72**, 3662 (1950). [e] Private communication from T. L. McMeekin. [f] This value discussed in text. [g] Crystals measured in dextran gradient column (see text). [h] One poorly formed crystal gave a density of 1.175 g./ml. This value was not included in the average. [i] Crystals measured in a bromobenzene–xylene gradient column.

1% error in the computed density. The β-lactoglobulin values were most accurately determined as both the albumin and insulin crystals were much softer and correspondingly more difficult to manipulate without damage. Chips, cracks and rounded edges all complicate the estimation of crystal volume.

The use of the gradient tube technique for the measurement of "wet" protein crystal densities has been discussed in detail elsewhere.[14] It depends for its success on the rapidity with which crystals can be wiped free of adhering mother liquor and transferred to the gradient column. Studies[14] have shown that an increase in the wiping time from 30 to 60 seconds or the use of crystals weighing less than 2 mg. leads to a marked increase in the recorded density. The water loss during wiping must produce a negligibly small density change during the time interval involved. The values cannot in this case be extrapolated to zero time as this would demand further assumptions about the relation between crystal density and water content. In this study, for measurements made within 5 to 10 minutes after introduction of the crystals into the gradient tube, there was no significant difference between bromobenzene–xylene tubes prepared from dry components and those prepared from water saturated components. After 10 minutes, however, a perceptible increase in density was observed in almost all cases. Within 24 hours β-lactoglobulin crystals in the dry columns had attained densities of about 1.26 g./ml. while those in the water-saturated ones gave values of about 1.16 to 1.17 g./ml.

We have recently attempted to eliminate the wiping step in the gradient tube procedure by the use of aqueous gradient columns containing dextran. Crystals and their adhering mother liquor may be introduced directly into these columns. The heavy component was about 50% dextran (Commercial Solvents clinical dextran Lot No. NA647) and the light component about a 20% dextran solution. The medium is so viscous that centrifugation of the tubes is essential to get reasonable settling rates even for large crystals. The results for β-lactoglobulin and insulin were within 0.5% of the best values determined by other methods. However, the dimer albumin value was uncertain and much too large. The amount of low molecular weight material in this sample which might diffuse into the crystal has not yet been determined, nor are the osmotic effects of such concentrated solutions yet known. It is felt that this modification shows considerable promise particularly for those protein preparations where only very small crystals are available and a wiping procedure is impractical.

"Air-Dried" Crystals.—The density determinations on "air-dried" crystals, Table II, were all made in gradient columns composed of dry bromobenzene with either xylene or kerosene. Since the crystals were by definition in equilibrium with the atmosphere of the room, the problem of rapid changes on transfer did not occur. The only systematic error might have been the interchange between the remaining water and the column components after immersion. Such a replacement would be expected to be very slow as was indicated by the "wet" crystal measurements, further the remaining water in an "air-dried" crystal is much more tightly bound than that coming off initially from a "wet" crystal. The very marked difference between the polar nature of the solvents and of water would further discourage replacement. Indeed, it appears that the gradient tube is very well adapted

for measurements on these crystals. All the crystals used settled rapidly to an equilibrium position and remained stationary for many hours.

Vacuum-Dried Crystals.—The first determinations on vacuum-dried β-lactoglobulin crystals appeared to give quite satisfactory results. The crystals were removed from the drying chamber and introduced into a column. They settled quite rapidly to an equilibrium value which changed only slowly over a period of a few hours. When similar measurements were made on dimer albumin crystals, however, no satisfactory equilibrium position was reached in the first 30 minutes. Typical density–time curves for the two proteins are shown in Fig. 2. There is no part of the albumin curve in the early time period which can be extrapolated with confidence. A close examination of the β-lactoglobulin curve does show that the time required to reach equilibrium is somewhat longer than that shown for a crystal which undergoes no change in composition (*e.g.*, an air-dried sample of the same size and shape).

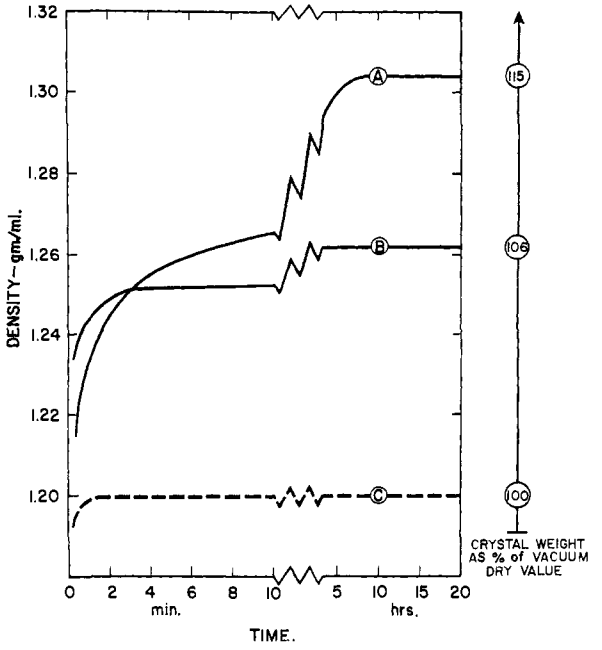

Fig. 2.—Apparent densities of vacuum-dried crystals as a function of time in bromobenzene–isoöctane columns: A, dimer albumin; B, β-lactoglobulin; C, hypothetical crystal of constant composition.

The composition changes during the time required to introduce the crystals into the gradient column were very small as was demonstrated in the composition measurements. The rate of change of the density was much too large to be explained simply by the uptake of water from the column liquids. The proper explanation appears to involve the uptake of the column liquids themselves, and this was demonstrated by measurements in isoöctane–bromobenzene columns. After immersion for an hour the crystals were removed, wiped, weighed and extracted with pure isoöctane for several hours. The increase in crystal weight over the vacuum-dried value demonstrated penetration of the crystal lattice by the column liquids. The absorption of the isoöctane extract at 264.5 mμ indicated the presence of considerable amounts of bromobenzene in this penetrating liquid. Thus the true density of vacuum-dried protein crystals cannot be determined by the gradient column technique as described.

Density as a Function of Relative Humidity.—The densities of the crystals equilibrated at various relative humidities were all measured in a gradient column using bromobenzene and xylene. Crystals equilibrated below 30% relative humidity showed the same anomalous behavior in the gradient tube as has already been described for vacuum-dried crystals. The density data recorded for these crystals are simply values at a standard time (3 minutes) after introduction into the column. The data for dimer albumin are shown in Fig. 3 with the specific volume of the crystals, $1/d_{\text{crystal}}$, plotted as a function of water content. This method of plotting the results, first employed for proteins by McMeekin and his associates,[10] gives a straight line here over the range 15 to 55% water.

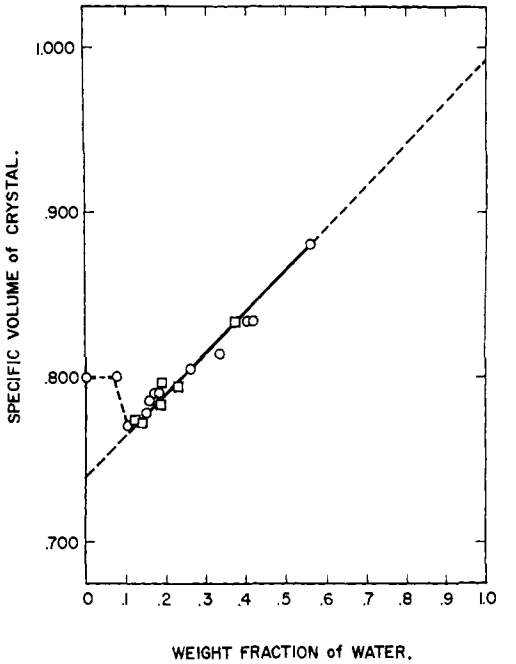

Fig. 3.—Dimer albumin plot of specific volume of the crystal $1/d_{\text{crystal}}$ as a function of water content: O, determined on dehydration cycle; □, determined on hydration cycle; solid line region between 13 and 55% water content.

Unit Cell Dimensions.—The unit cell dimensions measured for the two crystalline preparations in various states are listed in Table III. The crystals of both proteins are orthorhombic. The probable range of error is tabulated after each value. This error was principally dependent upon the maximum number of observed orders of reflection, and on the sharpness of the individual diffraction maxima recorded on the photographs. The smallest spacings observed are indicated in column 5. These values are some measure of the degree of order in the crystals, the smaller the observed spacing, in general, the higher the degree of order. Since the unit cells of both proteins are orthorhombic, in each case the cell volume, V, is equal to abc, the

TABLE III

UNIT CELL DIMENSIONS

Crystal state	a, Å.	b, Å.	c, Å.	Minimum spacing, Å.	Cell volumes, Å.[a]
		β-Lactoglobulin crystals			
"Wet"	69.3 ± 0.1	70.8 ± 0.1	156.8 ± 0.5	3	769,000 ± 4,000
Air-dried	60.1 ± .5	60.5 ± .4	111.5 ± 1.0	11–15	405,000 ± 10,000
Xylene-dried	60.9 ± .3	61.8 ± .3	113 ± 0.7	8	425,000 ± 7,000
Vacuum-dried	59.3 ± .4	60.6 ± .3	112 ± 0.7	8	402,000 ± 8,000
100% R.H. 5 hr.[a]	67.0 ± .4	70.6 ± .4	123 ± 1.0	10	582,000 ± 11,000
100% R.H. 24 hr.[a]	70 ± 2.0	71 ± 1.0	142 ± 5.0	15–20	706,000 ± 50,000
		Most recent published values[15]			
"Wet"	69.29 ± 0.03	70.42 ± 0.02	156.47 ± 0.05		763,600 ± 800
Air-dried	60.7 ± 0.3	61.0 ± 0.1	112.4 ± 0.3		416,000 ± 4,000
		Dimer albumin crystals			
Xylene-dried	150.7 ± 1.0	48.7 ± 0.2	52.6 ± 0.2	9	386,000 ± 5,000
Vacuum-dried	149 ± 1.0	48.1 ± .2	52.0 ± .2	9	373,000 ± 5,000
40% R.H. 24 hr.[a]	151.2 ± 1.0	48.5 ± .2	51.7 ± .3	9	379,000 ± 6,000
100% R.H. 5 hr.[a]	165.5 ± 2.0	52.0 ± .4	59.7 ± .4	9	514,000 ± 13,000
100% R.H. 24 hr.[a]	175 ± 2.0	62.5 ± 1.0	63.4 ± 1.0	15–20	693,000 ± 30,000
		Most recent published values[5]			
"Wet"	165	83	63	3	863,000
Air-dried	148	50	51.5	7–5	381,000

[a] Crystals equilibrated at given relative humidity at 0° after vacuum drying.

product of the three axial lengths (column 6 in each table). Discrepancies with the literature values shown are probably within experimental error.

It was found, as McMeekin[7] has observed, that "wet" crystals immersed in dry xylene for about 24 hours appeared to approach the composition of the air-dried ones while maintaining a more perfect form and optical clarity. Diffraction patterns of such crystals confirm this observation by showing reflections to much shorter spacings than those of the "air-dried" material. The cell dimensions were very similar. For this reason, the vacuum-dried specimens were prepared in each case from crystals dried slowly in xylene. The shrinkage in going from the "air-dried" to the vacuum-dried state is very small.

Discussion

The measurements of composition, density and unit cell volume are all independent, and the errors involved in each are not related. The internal consistency of the results may be checked in many different ways; the following scheme was chosen. With the "wet" state as a reference point, the densities to be expected for the air-dried and vacuum-dried states may be computed from the measured changes in their composition and unit cell size. Thus if:

d_1 = "wet" crystal density
d_2 = crystal density in state 2
W_2 = crystal weight in state 2 as % of "wet" weight
V_2 = crystal volume in state 2 as % of "wet" volume

then

$$d_2 = d_1(W_2/V_2) \qquad (2)$$

Density values obtained in this way are listed in Table IV. It is seen that for the air-dried state the calculated and observed densities check reasonably well. For the vacuum-dried crystals, however, the correlation is very poor. The volume and composition measurements on these crystals are accurate to about 1 to 3%. The apparent densities of the vacuum-dried crystals are shown to be incorrect as we suggested from the behavior of the crystals in the columns, since the non-polar liquids of the column appear able to enter the crystals.

TABLE IV

CORRELATION OF CRYSTAL MEASUREMENTS

Crystalline state	Weight % of "wet"	Volume % of "wet"	Density Calcd.	Density Measured
		β-Lactoglobulin		
"Wet"	100	100	.....	1.145
Air-dried	60.4	54.2	1.27 ± 0.02	1.260
Vacuum-dried	53.6	52.3	1.17 ± 0.02	1.25[a]
		Dimer albumin		
"Wet"	100	100	.....	1.135
Air-dried	49.5	44.4	1.27 ± 0.02	1.288
Vacuum-dried	44.4	43.2	1.17 ± 0.02	1.25[a]

[a] The errors involved in the accurate measurement of the density of vacuum-dried protein crystals are discussed in the text.

Flotation or gradient tube procedures thus appear ill-adapted for measurements of the true density of crystals equilibrated at relative humidities lower than that corresponding to the air-dried state. Indeed the final equilibrium density value for vacuum-dried dimer albumin in a bromobenzene–xylene column is about 1.31 g./ml. as compared to the value of 1.29 g./ml. for the air-dried crystal. The density computed for vacuum-dried β-lactoglobulin using equation 2 corresponds to a specific volume for the crystals of about 0.855 cc./g., considerably larger than the value (0.802 cc./g.) calculated by McMeekin, *et al.*, using his measured gradient column value for the density of the vacuum-dried crystals.

Preliminary measurements of density and unit

cell size are often used in the determination of protein molecular weights. If the composition of the protein crystals is also measured, the protein molecular weight may be derived unambiguously. Without data on the crystal composition, the weight fraction of protein may be calculated only if assumptions are made about the partial specific volumes of the protein and the liquid of crystallization in the crystal.

If the partial specific volume of dimer albumin in the crystal is equated to the monomer dilute solution value of 0.733 cc./g. reported by Oncley, Scatchard and Brown,[17] and the partial specific volume of the liquid of crystallization is taken as 1.0 cc./g., then the calculated weight fraction of protein is 44.6%, in good agreement with the measured value of 44.4%. For β-lactoglobulin crystals the weight fraction of protein calculated in this way using 0.753 for the partial specific volume is 51.0%, quite different from the measured value of 53.6%. The significance of this observation will be referred to again. It is important to emphasize, however, that X-ray molecular weights obtained without measurements of protein crystal composition may be seriously in error if the assumptions valid for dimer albumin crystals are made in every case.

A further possible source of error appears in the correlation of data on protein crystal density, composition and unit cell size. In Table II several different values for the density of "wet" crystals of β-lactoglobulin are given, many of which lie outside the range of experimental error for the individual determinations. The values of McMeekin and his associates show this same drift. Their most recently published value of 1.153 ± 0.002 g./ml. was obtained by flotation methods. The density of β-lactoglobulin crystals grown by Dr. McMeekin has been measured by him in his laboratory and by us, both groups using the identical gradient column technique.[14] Here, as the table shows, both groups of workers obtained values which agree within the limits of error of the method. It appears from these observations that small, real differences in density do exist between different crystalline preparations even though the preparative procedures employed were formally identical. Further, as the values for samples 1 and 2 and 7 and 8 show, the densities of crystals in a single batch appear to change with time. The existence of these unexplained differences emphasizes the necessity for attempting a careful correlation only of measurements made on the same crystal batch at the same time.

Unit cell size determinations were more limited in number than the corresponding composition and density studies, and, in particular, observations on cell dimension changes and degree of order in the crystal have not been fully correlated with the desorption and absorption parts of the hydration cycle. The usual shrinking phenomenon of "wet" protein crystals is observed on drying and the crystals swell again on absorbing water. Completely reversible stages of swelling and shrinking have not been observed. The partial swelling during the first 5 hours produced very little change in order compared to the air- or vacuum-dried states observed. Marked disorientation as shown by the increase in minimum spacing, Table III, did take place during the next 20 hours. For both proteins the axial length showing the greatest change on drying did not regain its "wet" length on re-equilibration at 100% relative humidity, and the increase along this axis was slower than in the other two directions. The cell dimensions of the dimer albumin crystals equilibrated at 100% relative humidity after the complete cycle show another change from the normal "wet" dimensions. One axial length has increased from 165 to 175 Å. Neither the increase nor the decrease in length on rehydration can be accounted for satisfactorily at this time.

The disordered state of air-dried and vacuum-dried crystals and their even more marked further disorientation on water absorption both suggested marked changes in the intermolecular binding on drying. The nature of the protein–protein and protein–water interactions in crystals is unknown. There is some evidence that the shrinking of a "wet" protein crystal corresponds largely to unchanged protein molecules moving closer together rather than sponge-like shrinking of the molecules themselves.[18] It is known that there are spaces in "wet" protein crystals large enough to permit the passage of molecules at least 10 Å. in diameter.[19] A simple model for protein crystals suggests that in the "wet" state neighboring protein molecules (of extremely complex and knobby surface structure) are either not in contact at all or only at a few points. The air-dried state then corresponds to protein molecules in close contact, with probable local side group entanglement and intermolecular holes, or voids, filled by water molecules.[20] Vacuum drying would then remove water without markedly changing the unit cell dimensions now dependent on protein–protein packing. This would explain the absorption of solvent molecules by vacuum-dried crystals in the gradient tube, and the difficulty of measuring the real density of these crystals. In that humidity range where the unit cell volume is independent of the water content, nothing useful can be said about the specific volume of the bound water from these gross crystal measurements since the apparent specific volume of the water would be zero regardless of its actual state.

The data on β-lactoglobulin reported by McMeekin, *et al.*,[10] in the range between 14 and 46% water are sufficiently precise that there seems little doubt that the partial specific volume of the water is 0.983 cc./g. and the protein 0.772 cc./g. in this region. Unfortunately, because of the errors brought about by small size and softness of the crystal, the equivalent data on dimer albumin are not so precise. In the region between about 13% and 55% water the partial specific volume of the water is between 0.992 and 1.000 cc./g. while that of the protein is 0.737 to 0.739 cc./g. The partial specific volume of dimer albumin in dilute aqueous solution has not yet been determined, but that for the

(17) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(18) D. Crowfoot and D. Riley, *Nature*, **144**, 1011 (1939).

(19) M. F. Perutz, *Research (London)*, **2**, 52 (1949).

(20) The geometry of the protein–protein interactions has been investigated using solute molecules of various shapes and sizes. This will be reported shortly.

monomer is 0.733 cc./g. Thus in the region of interest, the protein specific volume is only 0.005 cc./g. greater than the probably dilute solution value for dimer albumin, whereas from the data of McMeekin it is 0.021 cc./g. greater for β-lactoglobulin.

The close agreement between the partial specific volume of dimer albumin in the "wet" crystal and its probable value in dilute solution appears to suggest that, in spite of the relative proximity of neighboring protein molecules, protein–protein interactions are not strong in the wet dimer albumin crystals. The crystals are certainly very soft. Stronger interactions should lead to harder crystals, as in β-lactoglobulin, and some concomitant changes in partial specific volume.

It is difficult to understand the physical significance of the actual values obtained by McMeekin for β-lactoglobulin. It would seem reasonable that the water first absorbed by the dry protein at low relative humidities should be attached to the charged groups on the side chains, with electrostriction causing a considerable shrinkage in the volume of protein plus water on a molecular level[21] (although this would not show up as appreciable changes in the macroscopic crystal volume for the reasons discussed above). The water bound at higher relative humidities is held by much weaker forces, the electrostriction being small or negligible, so that the partial specific volume of this water should be nearly that of the pure solvent. There are no obvious forces which could be responsible for the contraction in solvent volume observed for β-lactoglobulin. The discrepancy between the β-lactoglobulin and dimer albumin results, in this respect, points up the desirability of further studies of this type on a variety of crystalline proteins.

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(21) A discussion of the significance and magnitude of electrostriction effects is given by E. J. Cohn and J. T. Edsall, in "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, Chap. 7, p. 155.

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Vibrational Spectra and Possible Structures of the Dimers of Cyclooctatetraene[1]

BY R. C. LORD AND R. W. WALKER

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Two dimers of cyclooctatetraene, one a liquid with two double bonds (m.p. 14°) and the other a solid with three double bonds (m.p. 43°), discovered by Reppe, Schlichting, Klager and Toepel, have been studied by means of infrared and Raman spectra. A 1:3 mixture of the dimers was produced by heating the monomer for some hours at 160°. Presence or absence of oxygen seemed to have little effect on this ratio, but the solid dimer was found to convert quantitatively to the liquid dimer on simple heating above 200°. Of the two structures proposed by Reppe and co-workers for the liquid dimer, one is supported by the spectroscopic evidence and the other seems definitely excluded. Two structures for the solid dimer are proposed, neither of which is entirely in accord with the observed spectra.

The dimerization of cyclooctatetraene was discovered by Reppe and his co-workers.[2] They reported the existence of two dimers, a solid melting at 43° and containing three double bonds, and a liquid containing two double bonds. Apart from boiling points at various low pressures, no other physical data on the dimers were given by them. Reppe, *et al.*, pointed out that the small number of double bonds in the dimers implies the formation of many new saturated rings, since no evidence for aromatic rings could be found. They also suggested that the dimers were formed by diene condensation through the intermediate of the bicyclo[4.2.0]octa-2,4,7-triene isomer of cyclooctatetraene. Two structures suggested by them for the liquid isomer (two double bonds) are shown in Fig. 1 (I and II). They suggested no structure for the solid dimer (three double bonds) because diene condensation of the bicyclooctatriene cannot result in an odd number of double bonds.

These proposed structures do not lend themselves readily to elucidation by either physical or chemical methods. It seemed worthwhile, however, to investigate the infrared and Raman spectra of the compounds, and to interpret the spectra in terms of the proposed and other plausible structures, with the objective of drawing as detailed conclusions as possible.

Reppe, *et al.*,[2] reported that only the liquid dimer is formed by dimerization in an atmosphere of nitrogen. We were unable to confirm this, and believe that their preparation of the pure liquid isomer under nitrogen resulted from the high temperature (230°) attained at one stage of the preparation. In our experience the ratio of liquid to solid dimer depended on the temperature of the dimerization rather than the presence or absence of oxygen. In the course of six dimerizations under different atmospheric conditions ranging from pure nitrogen to laboratory air, the solid:liquid dimer ratio (as shown by infrared spectra) was approximately constant at 3:1 as long as the temperature stayed at about 160°. However, if the dimerization was

(1) Based on the thesis presented by R. W. Walker to the Graduate School of the Massachusetts Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1952.

(2) (a) W. Reppe, O. Schlichting, K. Klager and T. Toepel, *Ann.*, **560**, 1 (1948). (b) J. W. Copenhaver and M. H. Bigelow, "Acetylene and Carbon Monoxide Chemistry," Reinhold Publ. Corp., New York, N. Y., 1949, pp. 210–212.