[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD UNIVERSITY]

Some Studies of Protein Crystals in a Variety of Different Media[1]

BY BARBARA W. LOW, F. M. RICHARDS[2] AND J. E. BERGER

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A study has been made of the effects of immersing β-lactoglobulin crystals in a series of organic media. From the shrinkage and disorientation observed some tentative conclusions can be drawn concerning the nature of the intermolecular association in the crystal lattice. The permeabilities of "wet" crystals of β-lactoglobulin, human dimer albumin and insulin to polysaccharide molecules of graded size have been investigated. X-Ray crystallographic measurements have been used to follow the changes in unit cell size and to observe possible disorientation effects within the crystal lattice. Density measurements have been used to follow the changes in crystal composition. The sucrose contents of some β-lactoglobulin crystals have been directly determined. The anomalous effects reported earlier for sucrose-containing β-lactoglobulin crystals have not been observed. Some observations are reported concerning limitations in the use of the gradient tube method for the measurement of protein crystal densities.

Introduction

Protein crystals in equilibrium with their mother liquor contain considerable amounts of water of crystallization; they are usually soft and fragile. Wet protein crystals are permeable to a wide variety of solute molecules, and they equilibrate rapidly when immersed in media of different composition from that of their mother liquor. When they are removed from solution protein crystals lose some water of crystallization and the crystal lattice shrinks. "Air-dried" crystals are rather hard and horny, and show evidence of intermolecular disorientation. It appears established that the gross features of the intramolecular protein structure remain unchanged when the crystals shrink on losing mother liquor.

The investigations to be reported here are preliminary studies of the swelling and shrinking phenomena. When the aqueous mother liquor surrounding a protein crystal is replaced by a less polar solvent, marked changes in both crystal lattice dimensions and molecular orientation occur. The trend of these changes suggests the nature of the intermolecular association. The water in β-lactoglobulin crystals has been replaced by a series of organic solvents of decreasing polarity and their effects on the crystal lattice and molecular orientation observed. The rapid diffusion of solvents into protein crystals appears to be associated with the presence of layers or channels of loosely bound water. Studies were undertaken in an attempt to describe the limiting size and shape of molecules which can diffuse into crystalline preparations of three different proteins. The permeability of "wet" crystals of insulin, β-lactoglobulin and dimer albumin to uncharged solute molecules of graded size has been investigated.

A detailed study of the permeability of β-lactoglobulin crystals immersed in sucrose solution was undertaken when it became obvious that our preliminary observations differed markedly from those of McMeekin and his associates.[3] The weight fractions of sucrose determined experimentally by McMeekin, *et al.*, are low, and to explain this anomaly these investigators postulated that the unit cell should contract as the sucrose content of the immersion medium was increased. Measurements of unit cell dimensions showed that this was not so and that the lattice tends to expand as the sucrose concentration increases. It appeared therefore that the discrepancy between the two sets of data might lie in the sucrose analyses.

X-Ray crystallographic methods have been used both to follow changes in wet and dry unit cell dimensions after immersion in a variety of media, and also to follow disorientation effects within the crystal lattice. Density and composition measurements have been used to follow material changes in crystal composition. Polysaccharides, the uncharged compounds used, have specific volumes in the neighborhood of 0.65 and replacement of any of the crystal water by these substances should produce a significant increase in density.

Experimental

Materials.—For this study large well-developed protein crystals were required. The preparative procedures used in the preparation of insulin, β-lactoglobulin and human mercaptalbumin mercury dimer (dimer albumin) have been described in detail.[4]

The following non-aqueous solvents were used: methanol, ethanol, propanol, acetone and xylene. All were C.P. grade solvents.

Solute Preparations.—There are unfortunately few well-characterized homogeneous preparations of uncharged water soluble compounds of known structure and graded size. This is especially true in the size range of greatest interest, that is the molecular weight range approximately 1000–4000.

Homogeneous preparations of the mono-, di- and tri-saccharides, glucose, sucrose, raffinose and melezitose,[5] and the cyclic α and γ Schardinger dextrins were used.[6]

Crystalline preparations of Nägeli-type amylodextrins also were used. These amylodextrins are linear starch chains containing between twenty and thirty glucose residues. The amount of more or less highly polymerized material outside this range appears very small, as judged by attempted fractionation.[7]

A standard inulin preparation was used; this probably contained little or no material with less than thirty residues per chain.[8]

Density Determinations.—The densities of the crystals were measured by the two methods developed or extended

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(2) A part of this work was carried out while one of us (F.M.R.) was an Atomic Energy Commission Predoctoral Research Fellow in Biological Sciences.

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

(4) B. W. Low and F. M. Richards, *ibid.*, **76**, 2511 (1954).

(5) We are much indebted to Dr. Hewitt G. Fletcher, Jr., Chief, Section on Carbohydrates, Laboratory of Chemistry, N.I.H., for this preparation of melezitose.

(6) D. French, M. L. Levine, J. H. Pazur and Ethelda Norberg, THIS JOURNAL, **71**, 353 (1949). We are most grateful to Dr. French for giving to us the preparations of α- and γ-dextrin used.

(7) D. French, unpublished studies; we are much indebted to Dr. French for this preparation.

(8) J. R. Pappenheimer, E. M. Renkin and L. M. Borrero, *Am. J. Physiol.*, **167**, 13 (1951).

by us to cope with the problems peculiar to the determination of protein crystal densities. The equilibrium microbalance technique[9a,b] was used for the measurements on "wet" crystals at 0°. Observations made on crystals which showed signs of solution were discarded. If the initial density of the crystal was known, the changes in density could be calculated from the observed alterations in volume, apparent weight and known density of the surrounding medium.

The rapid, non-equilibrium, gradient tube technique[10] was also used for crystal density determinations in all states of hydration. Detailed descriptions of these methods are given in the reference cited. For "wet" crystals xylene–bromobenzene gradient columns were prepared both from dry and from water saturated components.

The dimer-albumin crystals contain both alcohol and water. A search was made for column components in which both water and alcohol would be insoluble, but no suitable pair of compounds was found. In the absence of such compounds a study was made of the magnitude of the solubility effect in columns of a type previously employed. A bromobenzene–isoöctane column was set up with components which had been saturated with water before mixing. Four series of salt solutions of different density were prepared. The three members of each series had identical densities (±0.0002 g./ml.), and contained, respectively, 0, 15 and 30% ethanol. Drops of the standard solutions were introduced into the column and their positions measured as a function of time. A column was also prepared in which the two column components had been saturated with a 15% alcohol solution.

Measurements on wet crystals of dimer albumin were all made with the microbalance. In these studies the solutions used all contained about 5% ethanol in an attempt to prevent solution of the crystals. The presence of this much alcohol in the crystal causes no significant change in dimensions. Five per cent. solutions of the dimer containing 3 to 5% ethanol were saturated with the polysaccharides to be studied. In the studies on sucrose a 15% solution of sucrose was used. The balance cell was initially filled with mother liquor (also containing about 3 to 5% ethanol), the crystal mounted, and the area and fiber deflection photographs obtained. With the crystal remaining on the pan, the cell was then flushed out with the polysaccharide solution, and the fiber position observed until no further change occurred. The diffusion was essentially complete in all cases in about 30 minutes (for crystals less than 1 mg.). At this time a second set of photographs was taken.

Microbalance studies were also made of crystals which had been immersed in either alcohol–water or sugar-containing solution for 24 hours or longer. The results of these studies did not differ significantly from results obtained with crystals equilibrated for only 30–60 minutes. On replacing the polysaccharide solution with some of the original mother liquor the crystals returned to their original weight.

Density measurements on both wet and dry β-lactoglobulin crystals were made in the gradient column. Wet crystals were immersed in saturated aqueous solutions of the sugars, equilibrated for an hour or longer and their densities then measured in the gradient column. When removed from the column and re-equilibrated in their mother liquor, the crystal densities all returned to their original "wet" values.

The series of observations on replacement of mother liquor by less polar solvents were made exclusively with β-lactoglobulin. This protein gives crystals which are less fragile and somewhat larger than those of dimer albumin and insulin. The β-lactoglobulin crystals were initially mounted on the microbalance pan immersed in their mother liquor at 0°. This solution in the balance case was then successively replaced by aqueous solutions containing 20, 40, 60, 80 and 100% alcohol. Time was allowed for equilibrium to be established with each change of liquid. In most cases 20 to 30 minutes was adequate. After each equilibration, photographs were taken for the weight and volume determinations; densities of the alcohol solutions were determined pycnometrically. In the propanol study the crystal after final immersion in 100% alcohol was taken back through the same series of solutions to the mother liquor in order to test the reversibility of the changes.

(9) (a) B. W. Low and F. M. Richards, *Nature*, **170**, 412 (1952); (b) F. M. Richards, *Rev. Sci. Instr.*, **24**, 1029 (1953).

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

X-Ray Measurements.—In this investigation the X-ray study of the protein preparations was limited to the measurement of unit cell dimensions and the observation of orientation phenomena. "Wet" and "air-dried" unit cell dimensions of these crystals have been previously reported in the literature.[4,11,12] 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. All the X-ray photographs were taken using Cu $K\alpha$ radiation and a previously calibrated Buerger Precession Camera.

Composition Measurements.—The compositions of wet crystals of both dimer albumin and β-lactoglobulin in equilibrium with their mother liquors have already been determined.[4] This study was not repeated. Large β-lactoglobulin crystals of 3–15 mg. weight were used for the sucrose composition studies. The crystals were analyzed after they had been immersed in sucrose solution at 1.5° for at least 24 hr. The wet weight was determined by the method described elsewhere.[4] Loss of water was found to be a linear function of time for periods ranging up to 5 minutes. The sucrose content of the crystals was determined colorimetrically[13] by the anthrone method which has been described in detail by Dimler[14] and his co-workers. The optical density of anthrone–sucrose solution is directly proportional to the sucrose concentration. The proportionality constant K was determined by measuring five solutions of known sucrose concentration and plotting optical density against sucrose concentration. After weighing, the crystal was put into a flask containing 5 ml. of cold distilled water. The water was heated slowly and maintained just below the point of vigorous ebullience for 5 minutes. The flask was then removed from the heat and distilled water added to a total volume of either 10, 25 or 50 ml. at room temperature. (The crystal volume was never greater than 0.02 ml.—it was therefore neglected.) Duplicate determinations were carried out for each different sucrose solution.

Calculations of probable crystal sucrose concentrations (qv) were used to determine which of these standard volumes would lead to sucrose concentration in the diluent of between 20 and 35 γ per ml. This is the concentration range which gives maximum sensitivity in the anthrone determination. This procedure was adopted only after the following control experiments had been made: (a) 15 mg. of sucrose-free crystals were extracted by the procedure outlined. The solution showed no apparent sucrose on analysis, (b) 10 mg. of sucrose-free crystals were heated in a (25 γ per ml.) sucrose solution at 100° for 5 minutes. This solution was then analyzed for total sucrose; it was unchanged, (c) for two crystals immersed in a 12.7 weight per cent. sucrose solution the extraction procedure was repeated. The second extracts contained less than 4% of the first extracts. This represents no more than the limiting reproducibility of the method. Only one extraction was therefore carried out.

McMeekin and his co-workers[3] used a slightly different procedure for extraction from that reported here. They determined the sucrose in the extracts by the method of Stiles, Peterson and Fred.[15]

We investigated the McMeekin procedure for extracting sucrose from the crystals. Since the extracted crystals in their study were to be used for protein analysis, care was taken to prevent them from fragmenting during the removal of sucrose. To this end, the crystal to be extracted was dropped immediately into boiling water where it coagulated instantly. It then remained in one piece throughout the extraction. Our own tests showed that coagulation of the protein did not impede extraction.

(11) (a) F. R. Senti and R. C. Warner, *ibid.*, **70**, 3318 (1948); (b) B. W. Low, *ibid.*, **74**, 4830 (1952).

(12) B. W. Low, "The Proteins," edited by H. Neurath and K. Bailey, Vol. 1, Academic Press, Inc., New York, N. Y., 1953, p. 297.

(13) We are indebted to Dr. John L. Hickson of the Sugar Research Foundation, Inc., for suggesting the applicability of this method.

(14) R. J. Dimler, W. C. Schaefer, C. S. Wise and C. E. Rist, *Anal. Chem.*, **24**, 1414 (1952).

(15) H. R. Stiles, W. H. Peterson and E. B. Fred, *J. Bact.*, **12**, 427 (1927).

Estimation of Molecular Sizes.—Molecular dimensions of the diffusing solute are required. Estimations of the polysaccharide size and shape can only be approximate. For the long chain compounds particularly, detailed molecular stereochemistry in solution is not known, and further the homogeneity of these higher homologs is uncertain. The approximate dimensions of the extended amylodextrin chains were calculated from the number of residues and the repeat distance of about 5 Å. for the 1–4 linkage.[16] The length of the extended inulin chain was calculated from a repeat distance of 3.5 Å. for the 1–2 linkage as measured from a scale model of fructofuranose.

Assuming an average specific volume of 0.65 cc./g., the diameter of a sphere of equivalent volume may be computed from the known degree of polymerization of the polysaccharides.

$$\pi d^3 = \frac{6Mn\bar{v}}{N} \times 10^{24} \qquad (1)$$

or

$$d = 6.95n^{1/3}$$

where

d = diameter of sphere in Å.
M = residue mol. wt. = 162
n = degree of polymerization (no. of residues/chain)
$\bar{v}$ = residue specific vol. = 0.65
N = Avogadro's number

By employing Stokes' law,[17] experimentally determined diffusion constants can be related to the diameters of spheres of equivalent diffusion constant where

$$d = \frac{RT}{3\pi\eta ND} \qquad (2)$$

R = gas constant
T = absolute temperature
η = viscosity
D = diffusion coefficient

The appropriate molecular dimensions to be associated with diffusion into protein crystals are unknown. Scale drawings from models of some of the possible molecular shapes and maximum crystal "pore" sizes have been made. From these some estimates of relative molecular size have been deduced.

Results

Density. Gradient Column Limitations.—The effects of the different solubilities of the two components of the drops in the column liquids are shown in Fig. 1. The aqueous standards without alcohol reach and maintain a steady state; the density values obtained by extrapolation (broken line) coincide with the measured values. The drops containing alcohol continued to fall at a rate approximately dependent upon their alcohol content. The density values obtained by extrapolation of the linear part of these curves bear no clear-cut relationship to the initial densities. Thus, for example, a solution of density 1.03 containing 15% alcohol gives an extrapolated density value of 1.036 while a 30% alcohol solution gives an extrapolated value of 1.04. The errors in the extrapolated values are rather large (1%) and cannot be estimated. In a column initially saturated with 15% alcohol, the drops with 15% alcohol come to a steady position; the aqueous drops rise after reaching a maximum value, and the drops with 30% alcohol content fall steadily. Mixed solvents are now frequently used in protein crystallization procedures. It is evident, however, that the gradient column is unsatisfactory for the accurate measurement of the densities of protein crystals when they contain both water and some other liquid of crystallization which is soluble in the column components.

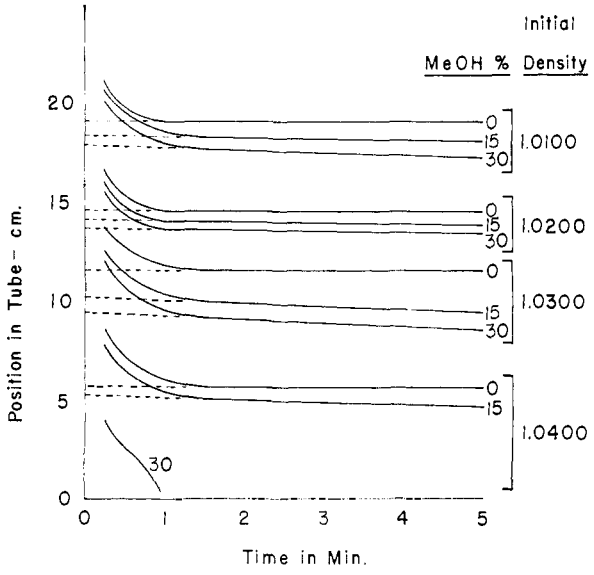

Fig. 1.—Graph showing the effect of sample solubility in one of the components of a gradient column.

Effect of Organic Solvents on β-Lactoglobulin Crystals.—Several changes in physical properties of the crystals were observed. In every case, the crystals became more brittle as the concentration of water decreased. In the pure organic media, the crystals which shrank least were most brittle. In 100% methanol large crystals often disintegrated to fine powders with very slight mechanical jarring. At concentrations of the non-aqueous immersion liquids of between 60 and 80%, a sudden clouding and disorientation regularly took place in the crystals. This effect was less marked in less polar solvents, that is, in the order methanol, ethanol, propanol, acetone. The opacity of the crystals was not removed by returning them to their mother liquor. It should be noted that re-equilibration at 100% relative humidity causes further disorientation in β-lactoglobulin crystals.

Some values for the observed changes in crystal dimensions are listed in Table I, as percentages of the original "wet" values. In order to show the extent of changes in crystal dimensions the corresponding axial lengths in ångströms are given in brackets. They illustrate the type of effects observed. The b and c dimensions shrink at relatively low concentrations of organic solvent. The 60% to 80% concentration range marks the region in which the crystals first begin to shrink along the a-axis.

For the propanol series the final volume in the mother liquor, after a complete cycle, was greater than the initial volume. This volume increase was also observed for methanol and ethanol treated crystals and is undoubtedly a real effect, not one due to experimental error. An attempt was made to show direct swelling effects by immersing vacuum dried crystals in the pure alcohols. The crys-

(16) K. M. Rudall, "Progress in Biophysics," Vol. 1, edited by J. A. V. Butler and J. T. Randall, Academic Press, Inc., New York, N. Y., 1950, p. 63.

(17) H. Mark and A. Tobolsky, "Physical Chemistry of High Polymeric Systems," Interscience Publ., New York, N. Y., 1950, 2nd edition.

TABLE I

CRYSTAL CHANGES IN VARIOUS SOLVENTS (β-LACTOGLOBULIN)

Immersion fluid	Axial lengths and volume as % of natural "wet" *a*	*b*	*c*
Normal mother liquor	100(69 Å.)	100(71 Å.)	100(157 Å.)
20% methanol	100	99	100
60% methanol	100	98	101
80% methanol	98	97	95
100% methanol	98(68 Å.)	96(68 Å.)	92(145 Å.)
95% ethanol	96(66 Å.)	95(69 Å.)	94(148 Å.)
20% propanol	100	99	96
60% propanol	100	98	96
80% propanol	100	98	91
100% propanol	96(65 Å.)	95(67 Å.)	86(135 Å.)
80% propanol	100	99	88
20% propanol	107	105	91
0% propanol	110(76 Å.)	106(75 Å.)	93(146 Å.)
20% acetone	100	100	96
60% acetone	100	96	94
100% acetone	92(64 Å.)	95(67 Å.)	81(127 Å.)
Xylene-dried from water	88(61 Å.)	87(62 Å.)	72(113 Å.)

tals always disintegrated into small chunks and no measurements were possible. Any interpretation of these results must be made in the light of the marked denaturation of the protein which occurs in these concentrated alcohol solutions.[18]

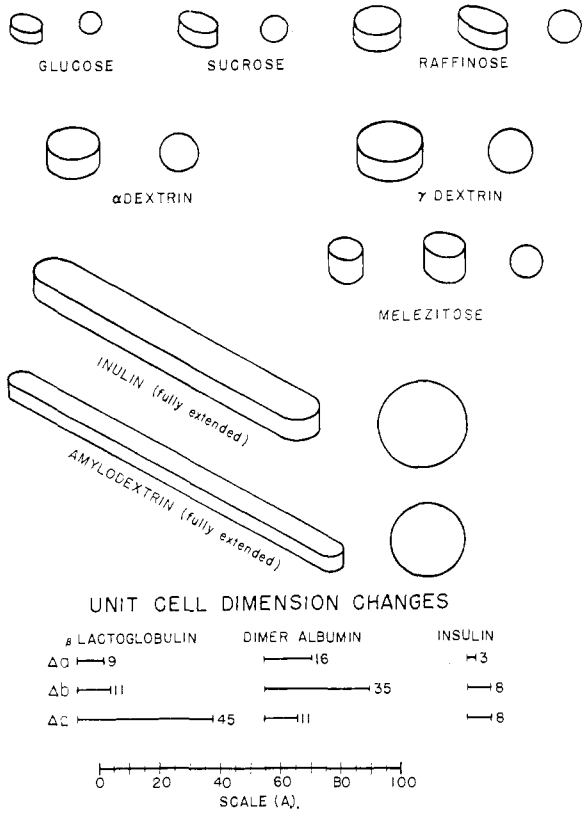

Fig. 2.—Drawings of molecular shapes and limiting dimensions of solutes used in permeability studies. The changes in unit cell dimensions on drying are shown for the protein crystals used.

(18) A sample of β-lactoglobulin treated with 100% methanol became completely insoluble in 0.1 *M* salt at *p*H 5 to 6. In acetate buffer of ionic strength 0.1, solution of the protein occurred at *p*H 4.1. Electrophoretic analysis of this solution showed two components, 66% with a mobility of +5.4 and 34% with a mobility of +3.9, while the original material under these same conditions gave 58% at +5.5 and 42% at +3.9. (The mobilities are given in units of cm.² per sec. per volt × 10^5.) No further tests were made.

Solute Molecular Dimensions.—Values for the molecular shape and size obtained both by calculation and by measurement of scale models are shown in Table II and Fig. 2. The scale drawings represent the limiting envelopes which enclose possible molecular configurations. These do not necessarily enclose identical volumes. Maximum crystal "pore" sizes are represented on the same scale. In an asymmetric molecule the dimension of interest in the study of crystal permeability is unknown. The characteristic dimensions listed in column 6 of Table II indicate the relative molecular sizes of the molecules: they are not critical values.

TABLE II

DIMENSIONS OF POLYSACCHARIDE MOLECULES

Substance	No. of hexose residues *n*	Max. linear dimension	Close packing diameter	Diffusion constant diameter	Probable dimension of interest
Glucose	1	10	7.0	7.2	7
Sucrose	2	13	8.8	8.8	9
Raffinose	3	16	10	11.2	11
Melezitose	3	13	10		11
α-Dextrin	6	15	12.6		13
γ-Dextrin	8	19	14		15
Amylodextrin	25 ± 5	125	20		25
Inulin	30	105	21.6	30	

Unit Cell Dimensions.—The wet, air-dried and xylene-dried unit cell dimensions of some polysaccharide-containing crystals of β-lactoglobulin and insulin are recorded in Table III. The smallest observed spacings shown in column 6 give some indication of the degree of orientation and disorientation in the crystal.

TABLE III

β-LACTOGLOBULIN

Crystal state	Cell dimensions, Å. *a*	*b*	*c*	Vol., A.³	Min. spacing, Å.
"Wet"	69.3	70.4	157.3	767,400	3
with 6.3% sucrose	70.0	70.5	157.7	778,200	3
with 24.3% sucrose	69.1	70.7	155.9	761,600	3
with 34.6% sucrose	70.8	71.5	159.7	808,400	3
with 58.5% sucrose	No pattern obsd.				
Air-dried	62	64	109	433,000	15
with 6.3% sucrose	61	64	...		30
with 34.6% sucrose	65	68	122	539,000	30
Xylene-dried	61	62	113	427,000	8
with 20% sucrose	62	64	119	472,000	25
with 25% melezitose	64	66	124	524,000	25
			INSULIN		
Xylene-dried	45	50	33	64,300	5–7
with melezitose	47	51	37	88,700	5–7
with γ-dextrin	44	50	34	74,800	15

Density and Composition Measurements.—The density data for the crystals immersed in various polysaccharide solutions are shown in Table IV. In Table V both density and composition measurements are recorded for the β-lactoglobulin crystals immersed in sucrose solutions of different composition.

TABLE IV

Solute in immersion liq.	d_c	d_i	Δd_c	Δd_i	R	d_{cl}	$x_{s/cl}$	x_s
β-LACTOGLOBULIN								
Sucrose								
$x_{s/i} = 0.198$	1.145	1.184	0.039	0.080	0.92	1.073	0.182	0.087
Raffinose								
$x_{s/i} = 0.096$	1.147	1.163	.016	.035	.86	1.030	.082	.037
Melezitose								
$x_{s/i} = 0.250$	1.158	1.185	.027	.102	.49	1.050	.123	.059
α-Dextrin								
$x_{s/i} = 0.09$	1.152	1.161	.009	.038	.44	1.017	.040	.019
γ-Dextrin								
$x_{s/i} = 0.125$	1.152	1.163	.011	.054	.38	1.021	.049	.023
Amylodextrin								
$x_{s/i} = 0.071$	1.147	1.155	.008	.025	.60	1.012	.043	.020
Inulin	1.147	1.147	.000	.028				0.000
DIMER ALBUMIN[a]								
Sucrose			0.019	0.059	0.51	1.030	0.080	0.045
Raffinose			.013	.021	.98	1.021	.059	.033
α-Dextrin			.010	.016	.99	1.016	.04	.022
Amylodextrin			.012	.033	.58	1.019	.056	.031
ACID INSULIN SULFATE								
Melezitose								
$x_{s/i} = 0.246$	1.195	1.234	0.037	0.110	0.73		0.180	0.073

[a] The densities of dimer albumin crystals were measured using the microbalance. The crystals used were chipped and ill-formed; absolute crystal densities were not therefore measured.

The concentration of polysaccharide in the crystal may be calculated from the density measurements if certain assumptions are made concerning the state of the protein, water and sugar in the crystal. For the saccharide-free crystal in its mother liquor, equation 3 expresses the relationship between crystal density, crystal composition, and the partial specific volumes of the two components.

$$\frac{1}{d_c} = \bar{v}_c = x_p\bar{v}_p + (1 - x_p)\bar{v}_{H_2O} \qquad (3)$$

where

d = density
$\bar{v}$ = specific (or partial specific vol.)
x = wt. fraction of component in crystal
y = vol. fraction of component in crystal
w = wt. of component in unit vol.
V = unit cell volume (in Å.[3])

In what follows, subscripts c and i refer to crystal and immersion liquids. Subscripts p, H_2O and s refer to the components protein, water and polysaccharide in the crystal, respectively. The subscript cl refers to the crystal liquid considered as a single component, s/cl to the polysaccharide as a component of the crystal liquid, and s/i to the solute in the immersion liquid. A superscript prime indicates the equilibrium after immersion of the crystal in polysaccharide containing solution where the meaning would be otherwise unclear.

We have generally employed, for reasons discussed later, the following assumptions: (a) that $\bar{v}_{H_2O} = 1.000$ and (b) that the unit cell size is not changed when the crystal is immersed in the sucrose solution. Thus we may write

$$d_c = w_p + (1 - y_p) \qquad (4)$$

$$d_c' = w_p' + (1 - y_p')d_{cl} \qquad (5)$$

where

$$y_p = y_p' = x_p\bar{v}_p d_c \qquad (6)$$

Eliminating w_p from (4) and (5)

$$d_{cl} = \frac{d_c' - d_c}{(1 - y_p)} + 1 = \frac{\Delta d_c}{(1 - y_p)} + 1 \qquad (7)$$

From the density of the crystal liquid a value for the weight fraction of polysaccharide it contains may be obtained from the International Critical Tables for sucrose and raffinose. The use of such tables for this calculation involves the further assumption that the solute in the crystal is in simple solution unaffected by the protein component.

The weight fraction of polysaccharide in the crystal may then be calculated by solving equation 8

$$x_s = x_{s/cl} \times x_{cl} = x_{s/cl}/(1 - x_p') \qquad (8)$$

where

$$x_p' = y_p/\bar{v}_p d_c'$$

From (4) and (5) we may write

$$d_c' - d_c = (d_{cl} - 1)(1 - y_p) \qquad (9)$$

If the concentration of polysaccharide in an aqueous solution is proportional to the density change Δd then if

$$R = \frac{\text{concn. of polysaccharide in crystal liq.}}{\text{concn. of polysaccharide in immersion liq.}}$$

$$R = \frac{d_{cl} - 1}{d_i' - d_i} = \frac{d_c' - d_c}{d_i' - d_i} \times \frac{1}{1 - y_p} = \frac{\Delta d_c}{\Delta d_i} \times \frac{1}{1 - y_p} \qquad (10)$$

The weight fraction of polysaccharide in the crystal liquid may then be calculated independently.

$$x_{s/cl} = Rx_{s/i} \qquad (11)$$

If the unit cell volume should be altered by the entry of sucrose then

$$w_p' = Vw_p/V' \qquad (12)$$

$$y_p' = Vy_p/V' \qquad (13)$$

Thus

$$d_{cl} = \frac{d_c' - V/V'(d_c + y_p - 1)}{1 - y_p'V/V'} \qquad (14)$$

Equation 14 has been used to calculate the sucrose content of crystals immersed in 34.6% sucrose solution, for as Table III shows there is a marked increase in the b and c dimensions at this high sucrose concentration. The calculated values for the weight fraction of sucrose in Table V columns 10 and 11 compare with the experimentally determined values in column 12.

In deriving estimates of the polysaccharide concentration in the protein crystals studied, certain values were assigned to $\bar{v}_p$, x_p and $\bar{v}_{H_2O}$ and some assumptions were made about the effects of polysaccharide on the crystal lattice dimensions. Their

TABLE V

β-LACTOGLOBULIN CRYSTALS

$v_p = 0.752$, $X_p = 0.536$, $y_p = 0.465$, $\bar{v}_{H_2O} = 1.000$.

Immersion liq.		d_c	Δd_c	d_i	Δd_i	R[a]	d_{cl}[b]	$x_{s/cl}$[c]	$S_{s/cl}$[d] (ICT)	x_s[e]	x_s[f]	x_s(exp)
Normal mother liquor		1.154	...	1.000	...	...	1.000	...	...	...	...	...
Sucrose soln., $x_{s/l}$ =	0.063	1.164	0.010	1.023	0.023	0.813	1.019	0.051	0.053	0.024	0.025	0.023
	.127	1.173	.019	1.049	.049	.725	1.036	.092	.095	.044	.045	.043
	.243	1.194	.040	1.100	.100	.748	1.075	.182	.186	.088	.090	.083
	.346	1.208	.054	1.149	.149	.745	1.111	.258	.266	.126	.130	.135
	.585	1.270	.116	1.277	.277	.783	1.217	.458	.477	.235	.245	.273

[a] Calcd. from eq. 10. [b] Calcd. from eq. 7 or 14. [c] Calcd. from eq. 11. [d] Calcd. from d_{cl} and ICT. [e] Calcd. from $X_{s/cl}$ and eq. 8. [f] Calcd. from $x_{s/cl}$(ICT) and eq. 8.

validity is considered below, before the more general aspects of the study are discussed.

The most recent value reported for the weight fraction of protein in β-lactoglobulin is $x_p = 0.536$.[4] The β-lactoglobulin crystals used in the study of immersion liquids containing sucrose had a density $d_c = 1.154$. The change in crystal densities of protein preparations on long standing has been noted elsewhere.[4] This effect could arise either from the loss of water and crystal shrinkage, or from slow packing changes in the crystals which would lead to lower partial specific volumes for the two components. It is possible to distinguish between these two alternatives, as the unit cell volume changes involved are very different. If the density increase in the "aged" crystal were attributed to water loss (where $\bar{v}_p$ and $\bar{v}_{H_2O}$ are considered constant), then the weight fraction of protein in the crystal would be much increased, and the new unit cell volume would be $0.95\ V$ where V is the crystal volume at $d_c = 1.144$. Alternatively if the change in density were associated with more economical packing of the crystal components relative to each other, then a new protein partial specific volume can be determined from equation 4 if x_p and $\bar{v}_{H_2O}$ are constant.[19] This gives values of $\bar{v}_p = 0.752$ and $y_p = 0.465$ for the crystals of density 1.154. The unit cell volume of the aged crystal can be calculated and is 0.4% smaller than that of the fresh crystal. Although the excellent agreement between this calculated cell volume change and that observed is probably largely fortuitous, the observed effects clearly correspond to intermolecular packing changes. If the water molecules are initially incorporated into the lattice in non-equilibrium positions, equilibrium may be attained rather slowly. The equilibrium position in the aged crystals is closely similar to the state in dilute solution. The partial specific volume of the protein component is 0.752, a value which is close to that for the protein in dilute solution.[20] In freshly grown crystals it is 0.763.

For dimer albumin crystals the protein partial specific volume of 0.732 was associated with crystals of density $d_c = 1.135$.[4] The weight fraction of protein in these crystals is 0.444.

(19) The assumption that x_p is constant appears reasonable. Studies of the weight fraction of β-lactoglobulin in crystals of different age give values close to that for freshly prepared material. The assumption that $\bar{v}_{H_2O}$ is constant and equal to 1.000 is arbitrary. Obviously a more compact arrangement of water with respect to the protein is indistinguishable from a more compact packing of protein with respect to water.

(20) K. O. Pedersen, *Biochem. J.*, **30**, 961 (1936).

Comparison of the calculated and measured weight fractions of sucrose in β-lactoglobulin crystals provides some check on the assumptions made in using equations 8, 10 and 14. Table V shows that the calculated values for the weight fraction of sucrose in the crystals—columns 10 and 11—agree rather well with the measured values (column 12). For crystals equilibrated with 34.6% sucrose there is an increase in cell volume and allowance was made for this in calculating R and d_{cl}. After immersion in a 58.5% sucrose solution β-lactoglobulin crystals became opaque, and gave no diffraction pattern. If the swelling observed with 34.6% sucrose solution increases with increasing sucrose concentration, the crystal lattice probably becomes severely disoriented and the crystal a pseudomorph. The calculated weight fraction values for sucrose are based on a normal unswollen lattice. The 20% apparent discrepancy is not therefore surprising. Thus it appears that once appropriate allowances are made for any concomitant changes in cell volume the polysaccharide content of protein crystals can be estimated with reasonable accuracy by the method outlined.

The values of R shown in Tables IV and V are always less than one. For hemoglobin crystals immersed in salt solution Perutz[21] has reported that the concentration of salt in the crystal liquid is less than in the immersion medium. To account for this he proposed that the water in protein crystals is of two kinds—"bound" and "free." "Bound" water is the water of hydration; "free" water is in dynamic equilibrium with the immersion fluid. On this basis, the concentration of the solute in the free water is equal to that in the immersion fluid. The concentration of solute is calculated or measured for the crystal liquid as a whole. If the simple free and bound water concepts are adequate here, there should be a single constant value for R which, as equation 10 shows, will be less than one for each crystalline protein. The R values determined however change with both solute concentration and solute identity.

Discussion

In wet protein crystals the specific protein–protein binding may be of three possible types: (1) charged molecules separated by solvent; (2) hydrogen bonding of specific groups mediated by arrays of oriented solvent molecules; (3) direct ionic or hydrogen bonds between specific groups.

If the charged molecules are separated by solvent

(21) M. F. Perutz, *Trans. Faraday Soc.*, [XL] **11B**, 187 (1946).

(Type 1) unoriented except at the molecular surface, then the dielectric constant of the medium should determine the equilibrium distance. The crystal shrinks relatively little in 100% methanol but it is highly disoriented. Further in considering the series, methanol, ethanol, propanol, acetone, only slight changes in dielectric constant of the medium are accompanied by marked increased shrinkage. In the case of hydrogen bonding through oriented solvent molecules the extent of the bonding will depend on the nature of the solvent, and behavior in the series of solvents enumerated suggests that type (2) interactions may be of importance.

Gross denaturation of the protein molecules accompanied by unfolding and changes in molecular shape could account for the open lattice of the protein crystal in 100% methanol, and the apparent solvent effect could be an artifact. In this connection it is interesting to note that the onset of disorientation as observed usually corresponds to the most marked shrinkage stage. The permeability of protein crystals to a variety of solutes suggests that the presence of many direct protein–protein bonds (Type 3) is most unlikely.

A simple model for a wet protein crystal would involve neighboring protein molecules (of extremely complex and knobbly surface structure, *e.g.*, protruding side-chains) separated by water molecules either completely or at all but a few points of direct protein–protein interaction. Considerations of this model suggest appropriate modifications of the bound and free water hypothesis. Even after a layer of water is "bound" at the molecular surface there will be very many indentations on the molecular surface. The channels between protein molecules will also vary in diameter along their length. The regions of "free" occluded water between molecules may be too small to accommodate a single molecule of some solutes, they may be able to accommodate one but not quite two, or two but not quite three molecules of some smaller solutes. On this picture we might expect R, the partition ratio, to decrease as the concentration of a given solute increased. At very high solute concentration, dilation of the channels by swelling of the crystal lattice will reduce the amount of water "bound" by stereochemical effects; R should then increase. In the studies of β-lactoglobulin crystals immersed in sucrose solution the partition ratio R does go through a minimum as this hypothesis would require.

The variation of partition ratio with solute is most marked. In general the partition ratio decreases with increasing solute size. The changes in unit cell dimensions between the wet and dry state do give some indication of probable limiting size of protein intermolecular channels. Thus, for example, inulin does not appear to enter crystals of β-lactoglobulin. In comparing different protein preparations striking differences also appear: for example, the partition ratio for melezitose in insulin is much higher than in β-lactoglobulin even though the melezitose molecule is large compared to the change in the c dimension of insulin crystals on drying.

These observations all suggest that the intermolecular packing is highly complex with interconnecting channels of varying width and cross-section.

BOSTON 15, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Spectroscopic Study of the Monomer and the Dimer in Nitrosobenzene Derivatives

BY KAZUO NAKAMOTO[1] AND R. E. RUNDLE

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I. Infrared Spectra.—The infrared spectra of seven nitrosobenzene derivatives have been measured both in the crystalline state and in solution. From a comparison of these spectra, the absorption bands of the nitroso group characteristic of the monomer and the dimer forms have been found. Studies of the spectra of nitrosomesitylene at various concentrations and of *p*-bromonitrosobenzene in mixed crystals render the above results more certain. Assignment of the characteristic bands of the monomer and the dimer have been made, and compared with that of other investigators.

Introduction

Most organic nitroso compounds exist as colorless or pale yellow dimers in the crystalline state, and green or blue monomers in the liquid state.[1a] It is known, however, that nitrosomesitylene is colorless in solution, while *p*-dimethylamino- and *p*-iodonitrosobenzene are green even in the solid state. The latter compound is especially peculiar because its analogs, *p*-chloro- and *p*-bromonitrosobenzene, are colorless crystals. However, a literature survey indicates that, thus far, no attempt has been made to discuss the relation between the spectra and dimerization in the solid state as well as in solution.

In order to discuss the above relation, it is first of all necessary to establish a method for determining whether a compound is monomeric or dimeric in either state. Although cryoscopic and ebullioscopic measurements can tell the degree of association in solution, these methods are evidently not applicable to the crystalline state.

Association problems, such as that above, are particularly amenable to spectroscopic study. However, there is little literature on the infrared spectra of the nitroso compounds, and the characteristic bands of the nitroso group have not yet been definitely established. For the nitroso stretch-

(1) On leave from the Department of Chemistry, Osaka University, Osaka, Japan. Corn Industries Research Foundation Postdoctoral Fellow.

(1a) For example, see N. V. Sidgwick, "Organic Chemistry of Nitrogen," Oxford Press, London, 1942, p. 204.