ing-point method using bromoform as the solvent.[9] The molecular weight of a bis-6-phenylphenanthridine is 508.

Summary

6-Phenylphenanthridine adds phenyllithium to the azomethine linkage giving 6,6-diphenyl-5,6-dihydrophenanthridine. The course of the reaction was demonstrated by showing that 6-phenyl-6-(*p*-tolyl)-5,6-dihydrophenanthridine is formed from 6-(*p*-tolyl)-phenanthridine and phenyllithium as well as from 6-phenylphenanthridine and *p*-tolyllithium. In the latter case there is also formed a bis-6-phenylphenanthridine, in particularly good yields when mesityllithium is used.

(9) The authors are indebted to Dr. H. Shine for assistance.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

X-Ray Molecular Weight of β-Lactoglobulin

BY FREDERIC R. SENTI AND ROBERT C. WARNER[2]

All physical methods of measurement of the molecular weight of proteins in solution are subject to uncertainty because of the tendency of proteins to aggregate or degrade in solution. This uncertainty is minimized or obviated if the protein is examined in the native crystalline state by the X-ray diffraction method. In this method, any denatured protein does not contribute to the sharp interferences of the native protein and therefore may be excluded from consideration.

The X-ray data give directly the volume of an integral number of molecules. This number is restricted to a prescribed set by the space group of the crystal, which is also determined by the X-ray data. Correct choice of this number can be made if an approximate molecular weight is available from other physical measurements, such as osmotic pressure or ultracentrifugal analysis. We may thereby make a precise measurement of the volume of the protein molecule, and this volume, in combination with the density and water content of the protein crystal, yields a value for the anhydrous molecular weight of the protein.

The first X-ray investigation of the orthorhombic modification of β-lactoglobulin was made by Crowfoot and Riley,[3] who reported the unit cell dimensions $a_0 = 63.5$, $b_0 = 63.5$ and $c_0 = 145$ Å. for crystals wet with their mother liquor. Later, Crowfoot[4] revised these values to $a_0 = 67.5$, $b_0 = 67.5$, $c_0 = 154$ Å. Crowfoot and Riley did not calculate the anhydrous molecular weight of β-lactoglobulin from their X-ray data, since they had no direct measure of the hydration of the crystals. McMeekin and Warner[5] have determined the water content of large single crystals of β-lactoglobulin and have also reported a more accurate value for the density of the wet crystals. Using Crowfoot's[4] unit cell dimensions, McMeekin and Warner calculated a value of 33,000 for the anhydrous molecular weight. They computed a value of 35,800 from Crowfoot's unit cell dimensions and their density and water content data for air-dry crystals.

The discrepancy of these molecular weights and the range of values found by other methods (see Table II) made it desirable to obtain X-ray, hydration and density data on crystals from the same preparation.

Experimental

Preparation of Crystalline β-Lactoglobulin.—β-Lactoglobulin was prepared essentially by the method of Palmer as modified by Sørensen and Sørensen.[6] The casein was removed from raw skim milk by isoelectric precipitation. The *p*H of the whey was adjusted to 5.8 to 6.0, and the proteins were fractionated with ammonium sulfate. The protein fraction soluble in 2.2 *M* but insoluble in 3.3 *M* ammonium sulfate was adjusted to *p*H 5.2 and dialyzed. The crystalline lactoglobulin so obtained was recrystallized four times by dissolving in dilute sodium chloride and dialyzing. Two entirely independent preparations (batches A and B) and two preparations of crystals from batch A (A-1 and A-2) were used for the X-ray measurements. Water content and density measurements were made on crystals of batch A only. However, the agreement of the unit cell dimensions and optical properties for batches A and B establishes their identity and justifies the use of the values determined for the crystals of batch A for those of batch B.

Two methods were found for obtaining large crystals. (1) A saturated solution of lactoglobulin in 0.1 *M* sodium chloride was diluted with about two volumes of water and allowed to stand in a closed vessel for several weeks in the refrigerator without being disturbed. Large crystals formed slowly and grew in some cases to be 0.8 to 1 cm. in the greatest dimension. (2) A saturated solution of lactoglobulin in 0.1 *M* sodium chloride was placed in a small beaker containing a Cellophane tube arranged so that distilled water could be slowly run through the tube. The assembly was placed in the cold room, and the rate of flow of water was adjusted so that crystallization began in about eight hours and was complete in forty-eight hours. It is essential that there be no mechanical disturbance until all the protein has crystallized. Crystals produced by this method ranged in size from 0.1 to 2 mm. in their greatest dimension and were well formed and of sufficient strength to be handled easily without breaking. Crystals used for the X-ray measurements were all made by the second method, since those obtained by the first method were too large.

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(3) D. Crowfoot and D. Riley, *Nature*, **141**, 522 (1938).

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

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

(6) S. P. L. Sørensen and M. Sørensen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **23**, No. 7 (1939).

TABLE I

UNIT CELL DIMENSIONS OF β-LACTOGLOBULIN CRYSTALS

Crystal	Crystal-to-film distance, mm.	Osc. axis	a_0, Å.	σ, Å.	N	b_0, Å.	σ, Å.	N	c_0, Å.	σ, Å.	N
A-1 (wet)	99.78	*b*	69.25	0.08	16	70.46	0.04	7	156.54	0.14	17
A-2 (wet)	99.78	*a*	69.24	.04	10	70.40	.05	14	156.32	.16	10
B-1 (wet)	218.0	*b*	69.32	.03	14	70.41	.03	23	156.48	.08	13
Wet crystal, average			69.29	.03		70.42	.02		156.47	.05	
A-3 (air-dried)	218.0	*b*	60.7	.3	5	61.0	.1	5	112.4	.3	5

TABLE II

ANHYDROUS MOLECULAR WEIGHTS OF β-LACTOGLOBULIN OBTAINED BY DIFFERENT METHODS

Method	Molecular weight
X-Ray, wet crystals (this work)	35,400
X-Ray, air-dried crystals (this work)	35,600
Osmotic pressure[a]	35,050
Osmotic pressure[b]	38,000
Sedimentaion equilibrium[c]	38,000
Sedimentation-diffusion[c]	41,500
Chemical analysis[d]	42,020

[a] H. B. Bull and B. T. Currie, THIS JOURNAL, 68, 742 (1946). [b] H. Gutfreund, *Nature*, 155, 237 (1945). [c] K. O. Pedersen, *Biochem. J.*, 30, 961 (1936). [d] E. Brand, L. J. Saidel, W. H. Goldwater, W. B. Kassel, and F. J. Ryan, THIS JOURNAL, 67, 1524 (1945).

Hydration and Density of Crystals.—The water content of the crystals was determined by the loss in weight of the wet crystals on drying, and the density by flotation in bromobenzene–xylene by the techniques previously described.[5] Mean values and their standard deviations found for the wet crystals were: density, 1.144, $\sigma = 0.002$, water content, 46.2%, $\sigma = 0.45$, in agreement with the previous values[5] of 1.146 and 46%. The density of the dry crystals was 1.259. Since these crystals were examined by X-rays when exposed to the atmosphere, the water content is subject to small variations from day to day. The average value of 9.8% for the water content found previously was therefore used in the calculations.

Optical Properties of Crystals—Crystals of the two batches, although orthorhombic, differed in habit. Those of batch A were thick rectangular tablets, somewhat elongated along [010] and showing (001) dominant. When viewed along [001] they showed low birefringence but gave a well-defined negative biaxial interference figure with the optic axial plane perpendicular to [010]. From the orientation of the optic indicatrix, it follows that α, the lowest refractive index, lies along the crystal thickness, whereas β, the intermediate index, and γ, the highest index, lie along the crystal length and width, respectively. Crystals of batch B occurred as prisms considerably elongated along [010] and usually showing (100) dominant, although (001) was sometimes developed as much as (100). It was first thought that these crystals might be the tetragonal modification described by Crowfoot and Riley[3] as needles, but microscopic and X-ray examination proved them to be orthorhombic. The orientation of the biaxial indicatrix with respect to the crystallographic axes was the same as in crystals from batch A.

Unit Cell Dimensions.—X-Ray measurements were made on both wet and air-dried crystals of batch A, but only on wet crystals of batch B. Wet crystals produce much superior diffraction patterns, and the lattice parameters can be determined with greater precision than those of dry crystals. Crystals (0.3 to 0.5 mm. in diameter) were maintained wet during exposure by mounting them in thin-walled Pyrex capillaries in contact with their mother liquor. Patterns were recorded on flat film 99.8 mm. from the crystal, with a 3.25 × 4-inch cassette, and at a distance of 218.0 mm. with a 5 × 7-inch suction-back cassette. We determined film-to-crystal distances from powder patterns of calcium sulfate dihydrate, taking Larsson's[7] value of 7.5793 kX for d_{020} and using 1.5387 kX as the wave length of CuK_α[8] radiation. All measurements were made with a vernier scale graduated to 0.02 mm. The average of ten or more settings was taken to determine the film coördinate of a reflection.

For crystals A-1, A-3 and B-1, b_0 was computed by the formula

$$b_0 = \frac{k\lambda}{\sin\left(\tan^{-1}\frac{y}{D^2 + x^2}\right)}$$

where x and y are the film coördinates of a reflection in the k^{th} layer line and D is the crystal-to-film distance. The distance $2y$ was measured between symmetrical reflections in upper and lower layer lines. On patterns symmetrical with respect to the meridian, *i. e.*, taken with the beam parallel to *a* or *c* at the midpoint of the oscillation range, $2x$ was also measured between symmetric reflections. On other photographs, reflections were chosen for which x was small, as compared with D, and the error in the measurement of x was not significant. The same method was used to evaluate a_0 from the patterns of crystal A-2.

The lattice parameter, c_0, was determined from equator reflections of crystals A-1, A-2 and A-3. In crystal B-1, one oscillation range was symmetrical about *a*, and reflections lying on layer lines as well as on the equator were used since both $2x$ and $2y$ could be measured with precision for these reflections. For oscillation ranges not symmetrical about *a*, equator reflections having indices h and k small, as compared with l, were selected in order that the errors in the values of a_0 or b_0 (taken from layer line measurements) would not contribute significantly to the error in the value of c_0. In a similar manner, a_0 and b_0 were determined from oscillation patterns symmetrical or nearly symmetrical with respect to *b* and *a*, respectively.

Discussion

The results of the unit cell determination on the

(7) A. Larsson, Uppsala Univ. Årsskrift (1929), Mat. och Naturv. I.

(8) E. A. Wood, *Phys. Rev.*, 72, 436 (1947).

three wet crystals and the one air-dry crystal are presented in Table I. Mean values of a_0, b_0 and c_0 as calculated from the measurement of N pairs of reflections, and the standard deviation of the mean, σ, are expressed in Å. units. Comparison of the external and internal consistency of the parameters derived from the three wet crystals shows it improbable that error is present due to incorrect crystal-to-film distance in any of the three crystals. Accordingly, we have computed an average value (Table I, line 4) for a_0, b_0 and c_0, assigning weights to the individual crystal values on the basis of their probable errors. Although the standard deviations indicate considerable precision in the measurements, the accuracy is less, for no correction has been made for absorption in the crystals. Since absorption displaces the center of intensity of reflections to larger angles, the unit cell dimensions reported are minimum values. In view of the low absorption of lactoglobulin crystals, the error in each dimension is probably no greater than 0.5%.

Our results for wet crystals differ significantly from Crowfoot's,[4] who reports $a_0 = 67.5$, $b_0 = 67.5$, and $c_0 = 154$ Å. for wet, tabular orthorhombic crystals of lactoglobulin. Our values for the unit cell dimensions of air-dried crystals may be compared with Crowfoot's[4] values $a_0 = 60$, $b_0 = 63$, $c_0 = 110$ Å., and the values $a_0 = 60$, $b_0 = 62$, $c_0 = 111$ Å. found by Fankuchen.[9] In view of the uncertainty in the water content and the error in measurement of patterns of air-dried crystals, these results are in reasonable agreement.[10]

An anhydrous molecular weight of 35,400, $\sigma = 400$, was computed from our unit cell, density, and water content data on wet crystals and a choice of eight molecules per unit cell, whereas the corresponding data for air-dried crystals give a value of 35,600. No standard deviation is given because the water content of the air-dried crystals was not known with sufficient precision. In computing these values, no correction for crystal absorption has been applied, and the molecular weights are correspondingly minimum values. It is unlikely, however, that absorption would introduce a correction exceeding 1.5%.

The orthorhombic space group[3] $P_{2_12_12_1}$ permits a multiple of four asymmetric molecules per unit cell. A choice of eight molecules to the cell gives a molecular weight in best agreement with those determined from osmotic pressure and sedimentation–equilibrium measurements, shown in Table II. Assuming six molecules per cell instead of eight increases the X-ray molecular weight to 47,000. This, however, makes no significant improvement in the agreement with the values 42,020 and 41,500, obtained by chemical analysis[11] and sedimentation-diffusion, respectively. Moreover, it requires that two of the lactoglobulin molecules possess twofold screw symmetry and be crystallographically different from the other four. We therefore consider 35,400 to be the best X-ray value for the molecular weight. This value is in good agreement with Bull and Currie's value, 35,050, determined by osmotic pressure measurement.

(9) I. Fankuchen, THIS JOURNAL, **64**, 2504 (1942).

(10) Although Crowfoot and Fankuchen reported unit cell dimensions in Ångstrom units, actually the kX unit was used (private communication), and their results should be multiplied by 1.00202 in order to be comparable with our values.

Summary

A molecular weight of 35,400, $\sigma = 400$, was computed for β-lactoglobulin from the density, hydration and unit cell data for the orthorhombic crystals wet with mother liquor. A value of 35,600 was derived from the corresponding data for air-dried crystals.

Dimensions of the orthorhombic unit cell found for the wet crystals are $a_0 = 69.29$, $b_0 = 70.42$ and $c_0 = 156.5$ Å., and those for the air-dried crystal are $a_0 = 60.7$, $b_0 = 61.0$ and $c_0 = 112.4$ Å. Crystals saturated with mother liquor had a water content of 46.2%, $\sigma = 0.45$, and a density of 1.144, $\sigma = 0.002$. Corresponding values for the air-dried crystals were 9.8% and 1.259, respectively.

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(11) Recent work on the methods of analysis of proteins for tryptophan,[12] cysteine,[13,14] and cystine,[14] and recent determination of isoleucine[15] and alanine[16] in β-lactoglobulin indicate that the composition on which the chemical molecular weight was based is in question. This may explain the discrepancy between the chemical molecular weight and the X-ray and osmotic pressure molecular weights. It is important also that β-lactoglobulin is electrophoretically inhomogeneous,[17,18] at *p*H 4.8 consisting of two components. It is not yet known whether the components differ significantly in composition or molecular weight.

(12) J. R. Spies, Abstract, 113th Meeting, American Chemical Society, Chicago, 1948, p. 48C.

(13) M. Halwer and G. C. Nutting, *J. Biol. Chem.*, **166**, 521 (1946).

(14) H. S. Olcott and H. Fraenkel-Conrat, *ibid.*, **171**, 583 (1947).

(15) E. L. Smith and R. D. Greene, *ibid.*, **172**, 111 (1948).

(16) A. S. Keston, S. Udenfriend and R. K. Cannan, THIS JOURNAL, **68**, 1390 (1946); S. Udenfriend, Dissertation, New York University, 1948.

(17) C. H. Li, THIS JOURNAL, **68**, 2746 (1946).

(18) T. L. McMeekin, B. D. Polis, E. S. Della Monica and J. S. Custer, *ibid.*, **70**, 881 (1948).