[CONTRIBUTION FROM WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Molecular Weight of Lysozyme Determined by the X-Ray Diffraction Method

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A method for growing single crystals of the several salts of lysozyme, including the chloride, bromide and iodide, has been described recently.[2,3] Since lysozyme has a comparatively small molecular weight (13,900) and forms chloride, bromide and iodide salts which can be crystallized, it appears probable that a comprehensive X-ray diffraction investigation of these salts may reveal some interesting information about the structure of a native protein molecule. With this end in view a coöperative investigation of the structure of the salts of lysozyme has been started by this Laboratory and the California Institute of Technology. The results of this joint endeavor will be published when available. The present paper is concerned with a preliminary account of the X-ray diffraction results obtained from air-dried lysozyme chloride crystals and the determination of the molecular weight of lysozyme.

Results and Discussion.—Two crystalline modifications of lysozyme chloride have been reported.[2] When the mother liquor has a *p*H between about 3.5 and 6, tetragonal, bipyramidal crystals appear,[2] whereas at a *p*H between about 7 and 11, needle-shaped orthorhombic crystals appear.[3] Both the tetragonal and orthorhombic crystals give rise to a large number of X-ray reflections when in the presence of their mother liquors. When air-dried, however, it has been possible to obtain a diffraction pattern from the tetragonal crystals only. Diffraction spots have been observed for d values down to about 5.7 kX from the air-dried tetragonal crystal as compared to 2.0 kX or less from the wet crystals. The fact that the air-dried tetragonal crystals give a diffraction pattern makes the X-ray method ideally suited for a determination of its molecular weight.

The tetragonal lysozyme chloride crystals used in this investigation were grown by the method of Alderton, Ward, and Fevold[2] at a *p*H of 4.5. Crystals suitable for X-ray diffraction analysis were obtained by allowing the crystals to grow on the side of an Erlenmeyer flask until they had attained a size of from 0.5 to 1.0 mm. on a side. The mother liquor was then decanted and the crystals allowed to dry. Many of the resulting crystals were transparent and had sharp edges and no apparent flaws. The crystals when air-dried are relatively hard and can be scraped from the wall of the flask without difficulty. Suitable crystals were mounted on the ends of thin glass fibers by means of clear shellac.

The optical and crystallographic properties of lysozyme chloride crystals grown at a *p*H of 4.5 have been determined.[4] This microscopic investigation showed that the crystals have positive uniaxial birefringence and appear to be tetragonal, tabular bipyramids of the first order. The tetragonal symmetry has been confirmed by taking several sets of 5° oscillation photographs 90° apart around the c axis. In all cases, these pairs of photographs appear to be identical.

Complete rotation and 5° oscillation photographs through a range of 90° have been taken around the [101], [110] and [001] axes. The unit cell size of the air-dried crystals has been determined from layer line measurements to be a = 71.1 kX and c = 31.3 kX. The volume of the unit cell is, therefore, $V = a^2c = 15.82 \times 10^4$ (kX)^3. The approximately 1200 reflections have been indexed by using reciprocal lattice plots. The only systematic absences appear to be $(h00)$ when $h \neq 2n$ and $(0k0)$ when $k \neq 2n$. These latter restrictions are based on the appearance of only even orders through (14,0,0). No $(00l)$ reflections have been observed from the air-dried crystals in spite of the fact that special care has been taken to observe them. When a tetragonal lysozyme chloride crystal is in the presence of its mother liquor, $(00l)$ reflections are observed only when $l = 4n$. Since reflections are observed from the air-dried crystal only down to d values of about 5.7 kX, it is apparent that if the air-dried crystals, like the wet crystals, have a 4_1 or 4_3 screw axis, the first $(00l)$ reflection that could appear (7.8 kX) would be expected to be weak because of the rapid falling off of intensity with angle. Since no conclusion can be drawn with regard to the c axis in the air-dried tetragonal crystal, the space groups compatible with the X-ray data are D_4^2—$P42_1$; D_4^4—$P4_12_1$; D_4^6—$P4_22_1$; D_4^8—$P4_32_1$; and D_{2d}^3—$P\bar{4}2_1m$. The space group D_{2d}^8—$P\bar{4}2_1m$ can be eliminated because lysozyme is optically active. None of these enantiomorphic space groups is in accord with the external holohedral appearance of the crystals.[4] This discrepancy is probably due to the fact that only the simple prism faces {101} appear. More complicated faces probably would reflect the non-holohedral symmetry.

Following the suggestion of McMeekin and Warner[5] the density of the lysozyme chloride crystals was determined with organic liquids by the suspension method. Since the relative humidity at the time the X-ray photographs were taken was approximately 40%, the crystals to be used for the density determination were first equilibrated in an atmosphere having this relative humidity.

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Gordon Alderton, W. H. Ward and H. L. Fevold, *J. Biol. Chem.*, **157**, 43 (1945).

(3) Gordon Alderton and H. L. Fevold, *ibid.*, **164**, 1 (1946).

(4) F. T. Jones, THIS JOURNAL, **68**, 854 (1946).

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

A crystal was placed in a mixture of toluene and ethylene bromide and the composition of the liquid was quickly adjusted until the crystal remained suspended. A fresh crystal was then added to be sure that excessive dehydration of the original crystal had not occurred. In all cases where the time for adjusting the composition did not exceed a few minutes the second crystal appeared to have the same density as the original. A series of determinations of the density on several different air-dried crystals from two different preparations (both at *p*H 4.5) of tetragonal lysozyme chloride gave an average value of 1.312 ± 0.003 g./cc. at 27°. This value of the density must be corrected because all of the lysozyme chloride crystals have a small amount of sodium chloride associated with them. The sodium chloride results from the evaporation of the mother liquor adhering to the lysozyme chloride crystals after decanting. The major portion of the sodium chloride is probably encrusted on the external faces of the crystals as it can often be seen under the microscope.[4] That the sodium chloride is present as a separate phase is also shown by the fact that lysozyme chloride crystals give, on long exposure, a sodium chloride X-ray pattern.

To determine the correction to be applied to the observed densities, microchemical analyses for sodium and chloride were made[6] on the preparations from which the crystals were obtained for both the X-ray and density measurements. The averaged results, expressed as per cent. of air-dried weight, are shown in columns 1 and 2 of Table I.

TABLE I

ANALYTICAL DATA ON AIR-DRIED TETRAGONAL LYSOZYME CHLORIDE

Na, %	Cl, %	NaCl, %	Cl bound to lysozyme, %	Corrected density (27°C.)
0.50	3.15	1.27	2.38	1.305 g./cc.

The amount of sodium chloride was calculated by assuming that all of the sodium is present as sodium chloride. The result is given in Column 3 of Table I. Column 4 gives the amount of the remaining chloride which is presumably attached to the amino groups of lysozyme as the hydrochloride. On the assumption that the volumes of sodium chloride and lysozyme chloride are additive the observed density was corrected by the formula

$$\rho \text{ corr.} = \frac{m_0}{\frac{100}{\rho_2} - \frac{m_1}{\rho_1}}$$

where m and ρ are the per cent. by weight and density, respectively, and the subscripts 0, 1 and 2 refer to lysozyme chloride, sodium chloride, and the mixture, respectively. The corrected density is given in Column 5 of Table I.

Oncley[7] has determined approximate values for the molecular weight of lysozyme by osmotic pressure (17,500) and sedimentation–diffusion (14,000–17,000). Assuming a value of 16,000 for the molecular weight of lysozyme, the number of molecules in the unit cell can be calculated to be

$$N = \frac{(1.305)(15.82 \times 10^4)}{(1.65)\quad(16{,}000)} = 7.8$$

It is evident, therefore, that there must be eight molecules in the unit cell.

The weight of a lysozyme chloride molecule in the air-dried crystal is then

$$M = \frac{(1.305)(15.82 \times 10^4)}{(1.65)(8)} = 15{,}680$$

This value is an upper limit for the molecular weight and must be corrected for both water of hydration and attached hydrochloric acid.

The weight of a lysozyme molecule, free of hydrochloric acid, can be obtained by reducing the value 15,680 by the amount of hydrochloric acid (2.45%) corresponding to the amount of chloride shown in column 4 of Table I. This leads to a value of 15,300 for the molecular weight of air-dried lysozyme.

The water content of the air-dried crystals was determined by placing weighed amounts of the crystalline material into a 105° vacuum oven for twenty-four hours and determining the loss of weight. Several runs made on different days gave a spread between 8.4% and 9.6% with an average value of 9%. With this value for the water content the molecular weight of dry lysozyme can be calculated to be 13,900.

It is difficult to estimate the probable error of this determination, but it seems reasonable to assume, in spite of the corrections made, that the probable error does not exceed 4% or about 560 molecular weight units.

The value 13,900 is slightly lower than the lower limit (14,000) given by Oncley[7] based on his sedimentation–diffusion measurements. His limits were, however, based on an assumed range of 0.70–0.75 for the partial specific volumes of lysozyme. In any case the lower limit is within the estimated probable error range of the X-ray determination. Oncley's osmotic pressure value of 17,500 is somewhat higher than the X-ray value. This discrepancy may be due to the fact that the membrane used by Oncley was not completely impermeable to lysozyme.

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Summary

Five degree oscillation photographs have been taken of air-dried tetragonal lysozyme chloride. The only systematic absences appear to be $(h00)$ when $h \neq 2n$ and $(0k0)$ when $k \neq 2n$. No $(00l)$ reflections appear. Possible space groups are D_4^2—$P42_12$; D_4^4—$P4_12_12$; D_4^6—$P4_22_12$; and D_4^8—$P4_32_12$.

(6) The authors are indebted to L. M. White of this Laboratory for the microchemical analyses.

(7) J. L. Oncley, private communication to H. L. Fevold.

The tetragonal unit cell of air-dried lysozyme chloride grown at a *p*H of 4.5 has dimensions of $a = 71.1$ kX and $c = 31.3$ kX. There are eight molecules in the unit cell. The density of the crystals was measured by suspension in a mixture of toluene and ethylene bromide. The observed density has been corrected for adhering sodium chloride (1.27%) to give 1.305 ± 0.003 g./cc. at 27°. The corrected density has been used to calculate the weight per molecule in the unit cell. This value was then corrected for moisture (9%) and hydrochloric acid bound to the amino groups (2.45%) to give a value for the molecular weight of dry, chloride-free, lysozyme of 13,900 ± 600.

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Compound Formation between 2,6-Lutidine and Polyhalogenated Methanes

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In phase equilibrium studies recently reported from this laboratory,[1] evidence was obtained for the existence of eight addition compounds of pyridine with various polyhalogenated methanes. Because the structure of all of these compounds could not be explained in terms of a single consistent theory, the present investigation of the systems consisting of 2,6-lutidine with each of these same polyhalogenated methanes was undertaken in the hope that further light might be shed upon the process of addition compound formation in such systems. Selection of 2,6-lutidine was suggested by the following considerations: (1) because of its electronic structure, its nitrogen atom would be expected to exhibit a greater tendency than that of pyridine to act as an electron donor; (2) because of the proximity of the two substituent groups to the nitrogen atom, steric factors might be expected to interfere with the coördination of several lutidine molecules about a single molecule of polyhalogenated methane.

Experimental[2]

Purification of Materials.—The 2,6-lutidine, obtained from the Reilly Tar and Chemical Corporation, was purified by repeated recrystallization of the hydrochloride from concentrated hydrochloric acid. The base was regenerated by means of concentrated sodium hydroxide solution, and, after separation from the aqueous layer, was dried over sodium hydroxide pellets for ten days. Fractional distillation through a five-foot packed column gave pure 2,6-lutidine, b. p. 142.5° at 738 mm., f. p. (from cooling curve) −5.5°. The purified material was stored in a dark bottle and was guarded against contact with moisture and carbon dioxide.

The chloroform, bromoform and carbon tetrachloride were dried over drierite, then fractionally distilled through the column referred to above. The physical constants observed for the pure compounds are as follows: for chloroform, b. p. 60.7° at 745 mm., f. p. (from cooling curve) −62.1°; for bromoform, b. p. 148.2° at 740 mm., f. p. (from cooling curve) 8.3°; for carbon tetrachloride, b. p. 76.1° at 741 mm., f. p. (from cooling curve) −21.8°.

Eastman Kodak Co. iodoform and bromoform were recrystallized twice from ethanol; the former melted at 121.1°, the latter at 90.9°.

Apparatus and Procedure.—The freezing point cell used in the determination of the temperature-composition diagrams was similar to that described in a previous publication from this Laboratory,[3] except that the cell was constructed in two parts joined to each other by means of a ground glass joint. This design makes possible the easy removal of the stirrer. All cooling curves were recorded directly by means of a Brown Electronik Strip Chart potentiometer which had been calibrated at the m. p. of ice, the b. p. of ammonia, and the sublimation point of solid carbon dioxide. Each freezing point was determined at least twice. Data obtained by this method are believed to be correct, even for the steeper portions of the freezing point curves, to 1.5°.

Results

The experimental data are shown in tabular and graphical form below. Compositions, as indicated, are given in mole %.

TABLE I

SYSTEM 2,6-LUTIDINE–CHLOROFORM

Mole % chloroform	Temp., °C.	Mole % chloroform	Temp., °C.
Solid phase C_7H_9N		48.7	−48.3
0.0	− 5.5	49.9	−48.3
5.75	− 8.9	52.1	−49.0
8.10	−10.9	54.3	−49.1
11.1	−11.9	57.2	−51.5
13.4	−13.8	59.8	−53.8
16.3	−15.1	62.8	−56.7
18.4	−17.9	63.3	−57.3
21.1	−20.0	66.9	−62.9
23.4	−21.5	68.2	−64.3
26.0	−24.5	72.9	−72.0
28.2	−27.3	74.5	−74.5
30.5	−30.0	75.9	−77.0
36.2	−40.0	Solid phase $CHCl_3$	
38.4	−44.5	76.5	−76.3
40.4	−48.0	78.5	−74.0
41.2	−49.0	83.0	−71.0
42.4	−51.0	87.3	−67.2
Solid phase $C_7H_9N \cdot CHCl_3$		91.4	−65.0
43.8	−51.5	93.6	−63.2
44.3	−50.5	95.6	−63.0
45.9	−49.5	100.0	−62.1

A. As shown graphically in Fig. 1, curve A, 2,6-lutidine and chloroform form a single stable

(1) Davidson, VanderWerf and Boatright, THIS JOURNAL, **69**, 3045 (1947).

(2) Melting points corrected, boiling points uncorrected.

(3) Davidson, Sisler and Stoenner, THIS JOURNAL, **66**, 779 (1944).