Summary

The solubilities of hexyl- and dodecylammonium chloride have been determined in various mixtures of water and ethanol.

The correlation between solubility and micelle-forming ability is noted.

CHICAGO, ILLINOIS RECEIVED DECEMBER 7, 1945

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

Optical and Crystallographic Properties of Lysozyme Chloride

BY FRANCIS T. JONES

In view of the general interest in antibiotic substances and in crystalline proteins, it seems desirable that the optical and crystallographic properties of lysozyme chloride be recorded in order to facilitate the work of others engaged in research on this material.

Although much work has been done on such crystalline proteins as insulin, hemoglobin, β-lactoglobulin, and the enzymes, all of which is well summarized in books by Schmidt[2] and by Cohn and Edsall,[3] most of the work has been by X-ray methods, and little except qualitative optical data are given. Crowfoot[4] gives index values for insulin, and Fankuchen[5] has recently given some data for β-lactoglobulin. This lack of optical data is due at least in part to the difficulty of obtaining crystals which are constant in composition and which do not disintegrate when exposed to air. It is well known that crystals of some protein substances may lose as much as 30% of their weight when dried, and regain part or all of it, depending on the humidity, when placed in a humid atmosphere. Crystals of some substances, *e. g.*, β-lactoglobulin, may contain salts. The quantity of salt may vary with the composition of the solution from which the crystals separate.[6] Such changes in composition cause changes in density and refractive indices. Values obtained on air-dried crystals will not be constant unless the humidity is constant and unless immersion media that will not affect the composition of the crystals are used. The most useful index values should be those obtained from crystals in equilibrium with the saturated solution from which they separated because they will then have the maximum water content for that preparation and there will be no doubt about the water content or the influence of strain due to partial drying.

The determinations recorded here were made on crystals regarded as lysozyme chloride, grown in acid solutions of *p*H 3–6 by the method of Alderton, Ward and Fevold.[7] These authors point out that crystals that are different from those described here are obtained at the isoelectric point (*p*H 10.8) or when chloride ions are replaced with other anions. In order to test for the presence of sodium chloride in the air-dried crystals several that appeared to be free from encrusting salt were ashed on platinum foil. This treatment produced only a trace of ash which gave a fleeting sodium flame. The crystals evidently contain very little salt, and this is probably occluded or contamination from the film of solution which must dry on the surface of the crystals, leaving sodium chloride crystals. Examination with a dark-field illuminator showed patches of bright particles on the surface of some lysozyme crystals but no appreciable Tyndall effect inside the crystals which were mounted in a liquid of nearly their own refractive index.

Preparation

The preparation was as follows: 4 g. of isoelectric lysozyme, which had been dissolved in dilute acetic acid at *p*H 6 and dried in the frozen state, was dissolved in 60 cc. of 0.2 *M* acetic acid. Saturated sodium chloride solution was then added to bring the composition to 0.86 *M* sodium chloride, and the *p*H was adjusted to 4.5 by adding a few drops of potassium hydroxide solution. Well-defined crystals up to 0.5 mm. in diameter developed in this solution at room temperature during the first twelve hours, and crystallization appeared to be complete within forty-eight hours. Many of the crystals developed at the surface of the solution, hanging from an edge or corner as shown in Figs. 1 and 2.

Crystallographic Description

The crystals of lysozyme chloride are colorless, tetragonal, tabular bipyramids of the first order, usually showing a short prism of the second order (Figs. 2 and 3). In Fig. 1 the prism is absent. Large crystals often develop cracks which radiate from the center. If the crystals are removed from their mother liquor they will dry out quickly and many of them will crack into smaller fragments. Some of the resulting cracks are parallel to the prism faces and appear to be cleavage cracks, but the cracks that develop perpendicularly to the *c* axis are so irregular that the crystals may be said to fracture rather than cleave perpendicularly to the *c* axis. Cracking of the

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

(2) Schmidt, "The Chemistry of Amino Acids and Proteins," C. C. Thomas Co., Springfield, Ill., 1944.

(3) Cohn and Edsall, "Proteins, Amino Acids and Peptides," A. C. S. Monograph 90, Reinhold Publishing Corp., New York, N. Y., 1943.

(4) Crowfoot, *Proc. Roy. Soc.* (London), **4164**, 580 (1938).

(5) Fankuchen, THIS JOURNAL, **64**, 2504 (1942).

(6) McMeekin and Warner, *ibid.*, **64**, 2393 (1942).

(7) Alderton, Ward and Fevold, *J. Biol. Chem.*, **157** (1), 43–58 (1945).

Fig. 1.—Lysozyme chloride, *p*H 4.5, 120 ×, prism absent.

sort described is at a minimum in clusters of crystals that are stuck together, and it may be lessened by drying the crystals very slowly. Crystals in contact with their mother liquor are very fragile; even slight pressure during attempts to handle them will cause them to crumble to a fine powder. After the crystals have dried they can be handled with little danger of breakage.

Determination of Optical Properties

The crystals were immersed in liquids of known refractive index until one was found that matched the refractive index of the crystal as indicated by disappearance of the Becke line. The set of immersion liquids used consists of oily organic compounds insoluble in water.[8] Light from a sodium vapor lamp was used for final determinations and the indices were checked on an Abbe refractometer. Examination with white light showed that the dispersion was weak. All determinations were made at a room temperature of about 25°. No crystals were used which showed irregular extinction due to strain produced by drying. Strained crystals showed a diffuse (+) biaxial interference figure of small optic axial angle. *c* axis views of such crystals showed patchy extinction. Crystals that were isotropic in *c* axis views gave uniaxial (+) interference figures.

(8) This set of immersion liquids was purchased from R. P. Cargille, 118 Liberty Street, New York City.

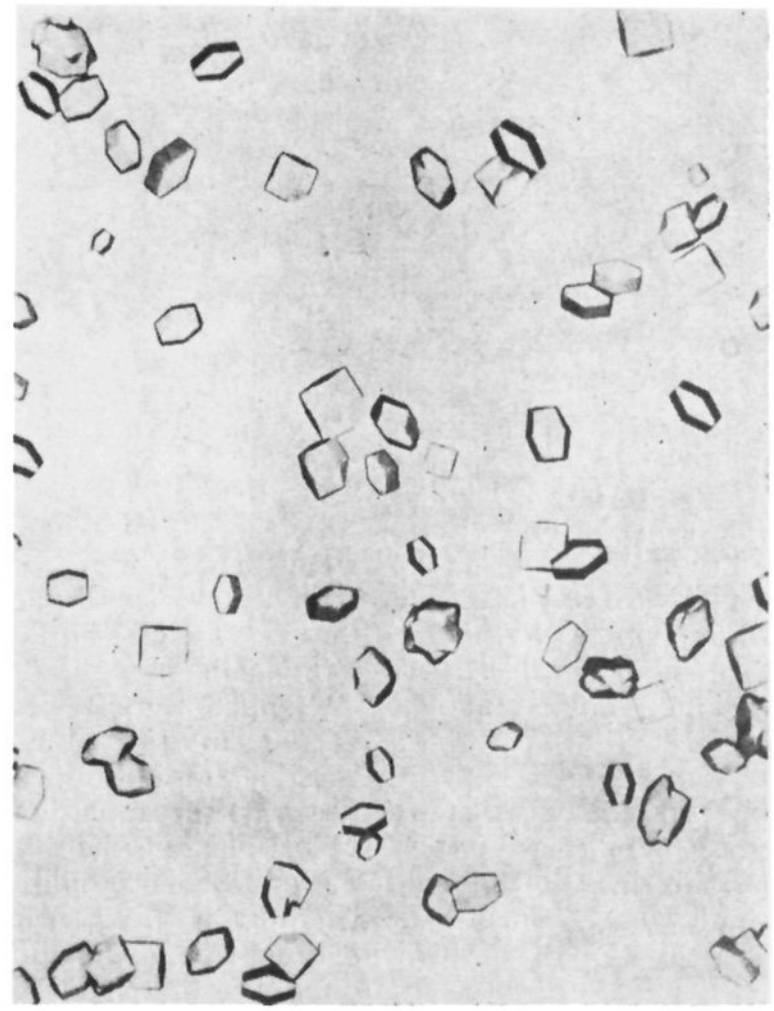

Fig. 2.—Lysozyme chloride, *p*H 4.5, 120 ×, prism present.

Fresh crystals growing at the surface were lifted out with a loop of platinum wire, placed on a slide, quickly blotted with bibulous paper, and immediately covered with a drop of the immersion oil. When this was done quickly some crystals could usually be found that were still wet with their aqueous solution on one end and wet with the oil on the other end. Values of the refractive indices for such wet crystals are the lowest values given in Table I. When the crystals were allowed to dry even slightly they showed indices higher than those of wet crystals and often cracked into fragments which, however, were still birefringent.

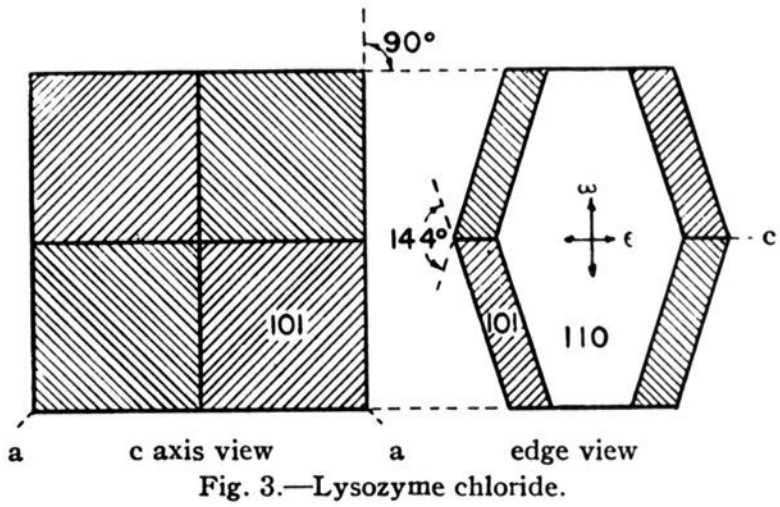

Fig. 3.—Lysozyme chloride.

Observation of the rapid increase in refractive index attributed to loss of water prompted a de-

TABLE I

OPTICAL PROPERTIES OF LYSOZYME

	n_ϵ	n_ω	Optical character	Birefringence
Wet crystals from a soln. at *p*H 3.5	1.495	1.492	Uniaxial (+)	0.003
Av. for air-dried cryst. from a soln. at *p*H 3.5	1.554	1.551	Uniaxial (+)	.003
Av. for air-dried cryst. from a soln. at *p*H 4.5	1.558	1.554	Uniaxial (+)	.004

termination of the water content of the fresh crystals by the method of McMeekin and Warner.[6] The crystals of lysozyme available (grown in a solution of about *p*H 3.5 instead of 4.5) were too small to wipe off individually; hence a small pile of the crystals was blotted to remove excess liquid and immediately placed on the pan of a rapid-weighing micro balance. Readings were taken every half minute. The drying curve obtained was of the same general shape as those obtained by McMeekin and Warner[6] and its extrapolation to the zero point or time of blotting gave the wet weight. When the crystals had reached equilibrium with room air they had lost 40% of their original weight. Drying overnight in a vacuum oven at 70° caused an additional loss of 8% of the air-dry weight. After hanging on the balance five hours the vacuum-dried crystals had regained the 8% of water lost previously. In order to determine whether the mass of wet crystals might have retained some liquid which blotting failed to remove, the balance pan containing the crystals was hung in a closed bottle over a 0.86 *M* solution of sodium chloride. If the crystals had lost nothing but water they should regain all they had lost, since they separated from a 0.86 *M* solution of sodium chloride containing only 0.2 *M* acetate and a little lysozyme. After standing overnight the crystals were quickly reweighed and their drying curve again determined. Their weight, determined by extrapolation, was slightly higher than their original wet weight and their drying curve was similar to that first obtained although the rate of drying was smaller. This evidence indicates that the value used for the wet weight of the sample was not too high as a result of excess liquid, and that the dry lysozyme crystals will reabsorb water.

Air-dried crystals were obtained by pouring off the aqueous solution and allowing the crystals which stuck to the container to dry slowly. The best crystals could then be picked off the sides of the dish with the aid of a greenough binocular microscope.

The value of the index ω was found to change slightly from one day to another and the value of ϵ changed correspondingly, maintaining a constant difference. The variation from day to day is attributed to changes in humidity of the room air, because the indices were lower on a rainy day than on a clear day; further, as shown above, the wet crystals have much lower indices than the dry crystals and the crystals absorb water from a humid atmosphere. The lowest values found for air-dried crystals from a preparation at *p*H 3.5 were $n_\epsilon = 1.551$, $n_\omega = 1.548$; the highest values found were $n_\epsilon = 1.557$, $n_\omega = 1.554$. On a preparation at *p*H 4.5 the lowest values found were $n_\epsilon = 1.554$, $n_\omega = 1.551$, and the highest values were $n_\epsilon = 1.562$, $n_\omega = 1.558$. The lowest values for these two preparations were measured on the same day. Since the difference between corresponding indices measured on the same day is only 0.003, it seems probable that a change of *p*H from 3.5 to 4.5 does not cause much difference in the composition of the crystals.

In spite of care to select strain-free crystals it was found that values of n_ϵ varied about 0.002 from the lowest to the highest observed in any one preparation on the same day. Values of n_ω varied within the same range on the same crystals. The values reported in Table I are the averages of observations made on different days on about 50 crystals in each case. Any one determination could be made with a precision of 0.001 index unit.

Birefringence was determined on one crystal by measuring its thickness and determining its retardation. The crystal was cemented with shellac to the end of a wire which could be rotated about its axis in a stage goniometer. An eye-piece micrometer was used to measure the thickness, then the crystal was rotated 90° for determination of its retardation by means of a graduated quartz compensator. The average of five measurements gave a birefringence $B = 0.0035$, which agrees with the value given by the difference between the measured indices.

Birefringence measurements made on crystals immersed in their mother liquor gave values for different crystals ranging from $B = 0.0027$ to $B = 0.0032$. The crystals used showed only first order white polarization colors for the edge views which gave the maximum birefringence. The thickness of any one crystal was measured by means of the fine adjustment, focusing first on the bottom of the solution filled cell in the depression slide and then on the top of the crystal. The apparent thickness thus obtained by averaging several determinations was multiplied by the refractive index of the saturated solution in contact with the crystals to obtain the true thickness. The refractive index of the solution was n_D^{25} 1.344. The apparent variation in birefringence is attributed chiefly to the difficulty of measuring the thickness accurately by the method used. A better value could have been obtained if thicker crystals had been available or if the cell had been shallow enough to permit using an objective of smaller depth of focus than the 16 mm. objective used.

Discussion

Abraham and Robinson[9] have published a

(9) Abraham and Robinson, *Nature*, **140**, 24 (1937).

photomicrograph of a crystalline preparation described as lysozyme but which might be lysozyme acetate, since it was obtained by evaporation of a solution in 0.05 *N* acetic acid. Although crystallographic examination of the material was to have been undertaken, no publication of such results has been found. The shape of the crystals pictured appears to be that of a rhombic dodecahedron and the face angle, measured as carefully as possible on the picture, is 110°, which is correct for that form. The corresponding angle, measured on the crystals of lysozyme chloride, is 144°; thus the two preparations must be different, although they do look somewhat similar. Since the refractive index measurements reported here were made, a preparation of lysozyme chloride containing hydrochloric acid in place of the acetic acid used in the original preparation has been examined. The crystals have the same shape as those grown in the acetate-containing solution. The face angle is the same and the optical properties are the same, so far as has been determined, although the refractive indices were not measured because the preparation was not suitable for that purpose. Attempts to prepare lysozyme acetate have not yielded a crystalline product suitable for crystallographic examination.

Acknowledgment.—Thanks are due Mr. Gordon Alderton for making the lysozyme preparations used in this work, and to Mr. L. M. White for the moisture determinations.

Summary

The optical and crystallographic properties of single crystals of lysozyme chloride have been determined on air-dried and on wet crystals. The refractive indices have been found to vary with moisture content, but the birefringence remains constant.

ALBANY, CALIFORNIA RECEIVED DECEMBER 31, 1945

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Peptic Digestion of Human Gamma Globulin

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It has been shown by a number of workers, chiefly Pope,[2] that when horse diphtheria antitoxin is acted upon by pepsin at *p*H 4.0, the product may be separated into a more soluble portion containing the antibody activity and a less soluble portion that is inactive. It was later demonstrated in this Laboratory by sedimentation and diffusion constant measurements[3] that the active portion consists of half the original antibody globulin particle, which has been split in a plane normal to its long axis. Similar results have been obtained with tryptic digestion.[4] In a study of the effect of pepsin on bovine serum globulins[5] it was found that at hydrogen ion concentrations in the neighborhood of *p*H 4.0 the splitting stops with halves of the original particle, while in more acid solutions (*p*H 2) digestion proceeds farther to quarter particles and a much larger proportion of small (dialyzable) fragments.

In the present study human serum globulin has been digested by pepsin. The degree of splitting attained has been followed by ultracentrifugal analysis, and the products have been characterized physico-chemically. The effect of digestion on antibody activity also has been determined.

Methods.—The globulin used was the "Fraction II"[6] obtained by the low temperature alcohol fractionation of human plasma.[7,8,9] Sample II-55 contained 6% albumin on electrophoretic analysis; II-37 contained 10% albumin and 3% beta globulin. Some experiments were also done on electrophoretically pure gamma globulin (99%) obtained from Fraction II by two precipitations of the crude globulin with 40% saturated ammonium sulfate. This has been designated F II-40.

The pepsin used for the digestions was crystallized by the method of Philpot[10] from Parke-Davis or Armour 1:10,000 pepsin, and stored in the cold in acetate buffer at *p*H 5. Its activity was determined by the hemoglobin method of

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(2) C. G. Pope, *Brit. J. Exptl. Path.*, **19**, 245 (1938); **20**, 132, 201 (1939).

(3) M. L. Petermann and A. M. Pappenheimer, Jr., *J. Phys. Chem.*, **45**, 1 (1941).

(4) J. H. Northrop, *J. Gen. Physiol.*, **25**, 465 (1941-1942); A. Rothen, *ibid.*, p. 487.

(5) M. L. Petermann, *J. Phys. Chem.*, **46**, 183 (1942).

(6) Supplied by Dr. E. J. Cohn of the Department of Physical Chemistry, Harvard Medical School. The preparation of this fraction was developed in the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University (*cf.* Ref. 7).

(7) (a) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H., Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944); (b) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, in preparation. The preparations of Fraction II used in the present studies were prepared by the method denoted as Method 2 in the paper of Oncley, Melin, Richert, Cameron and Gross. Some of the later methods described by these authors yielded gamma globulin preparations of 99% purity or better.

(8) J. F. Enders, *J. Clin. Invest.*, **23**, 510 (1944).

(9) J. W. Williams, M. L. Petermann, G. C. Colovos, M. B. Goodloe, J. L. Oncley and S. H. Armstrong, Jr., *ibid.*, **23**, 433 (1944).

(10) J. St. L. Philpot, *Biochem. J.*, **31**, 54 (1937).