department. Perlmann[4] previously succeeded in preparing a crystalline salt derivative of egg albumin and metaphosphoric acid. McMeekin, *et al.*,[23] have prepared crystalline derivatives of β-lactoglobulin with dodecyl sulfate, and Alderton and Fevold[24] some crystalline salts of lysozyme.

Thus already eight different proteins have been shown to give crystalline salts, five of them—namely, insulin and the four studied in this paper—with a large variety of reagents.

The derivatives described here are distinguished from certain others by the fact: (1) that they may be ascribed to very common functional groups (principally the basic and acidic) of the protein molecule and for these reasons can be prepared from a large variety of proteins, and (2) they generally represent very slight modification of the protein molecule, being obtained under very mild conditions involving little or no danger of denaturation.

The study of these derivatives in this Laboratory is continuing.

Acknowledgments.—The author wishes to express his gratitude to Professor A. Baudouin for help and encouragement during many years; to Professor L. Bugnard for a grant from the French Government Cultural Relations Committee (1947–1948); to Professor E. J. Cohn who suggested this research; to Professor J. T. Edsall and Dr. B. W. Low for many helpful suggestions; to Dr. W. L. Hughes, Jr., who provided the material necessary for these studies; and to Professors J. L. Oncley, G. Scatchard, C. A. Janeway and Dr. D. M. Surgenor for their interest in this work.

BOSTON, MASS. RECEIVED NOVEMBER 29, 1950

(23) T. L. McMeekin, B. D. Polis, E. S. Della Monica and J. H. Custer, THIS JOURNAL, **71**, 3606 (1949).

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

Preparation and Properties of Serum and Plasma Proteins. XXXI. An Optical and Morphological Study of Some Crystalline Human Serum Albumin Preparations and of Their Derivatives[1a,1b]

BY BARBARA W. LOW AND E. J. WEICHEL

A study has been made of the optical and morphological properties of two series of crystalline human serum albumin preparations. Crystals of human serum decanol albumin, mercaptalbumin mercury dimer, and a wide range of the crystalline derivatives, described by J. Lewin in the preceding paper (*q.v.*), of both these protein preparations have been examined. Measurements have been made both on wet, and on air-dried crystals, and observations of changes during drying have been recorded. The morphological and optical constants of the crystals have been measured. Close relationships between the optical and morphological properties of the crystals of the parent proteins and crystals of their derivatives have been established.

Introduction

Measurements of the optical and morphological properties of crystalline preparations are made both for purposes of identification,[2] and also for the valuable light they may throw upon the general features of a crystal structure.[3]

Morphological features and optical constants may provide definite information concerning the shape, orientation and packing of molecules in a crystal structure.

In their X-ray study of methaemoglobin, Boyes-Watson, Davidson and Perutz[4] have deduced the orientation of the heme groups with respect to each other and their general packing direction in the cell, from measurements of the optical properties of the methaemoglobin crystals.

The difficulties involved in measuring the optical constants of protein crystals have been discussed by F. Jones[5] who has studied lysozyme chloride crystals and reported the most detailed measurements of the optical constants of a single protein species so far recorded.[6]

The observations recorded here were made on (1) human serum decanol albumin crystallized at high ethanol concentrations (23–40%) and ionic strengths in the range $\Gamma/2 = 0.05$–0.30[7] and some of its derivatives,[8] and (2) mercaptalbumin mercury dimer, crystallized from ethanol–water mixtures,[9] and some of its derivatives.[8]

The purpose of this study has been to measure the morphological and optical constants of these two series and to investigate the relationships between the crystals of the parent proteins and of their derivatives.

Materials.—With the exception of the mercaptalbumin mercury dimer crystals prepared for us by Dr. W. L. Hughes, Jr., all the crystals used in this study were prepared by Lewin.[8]

(1) (a) This work was supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University. (b) This paper is Number 93 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University.

(2) E. M. Chamot and C. W. Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Edition, John Wiley and Sons, Inc., New York, N. Y., pp. 319–324.

(3) W. H. Hartshorne and A. Stuart, "Crystals and the Polarizing Microscope," 2nd Edition, Edward Arnold and Co., London, 1950, pp. 130–159.

(4) J. Boyes-Watson, E. Davidson and M. F. Perutz, *Proc. Roy. Soc. (London)*, **A191**, 83 (1947).

(5) Francis T. Jones, THIS JOURNAL, **68**, 854 (1946).

(6) For other studies consult the references given by Jones (ref. 5).

(7) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947). This crystal structure is one of two crystallographically quite different crystalline modifications of human serum decanol albumin described by these authors. The range of conditions for these two preparations are wholly different and mutually exclusive. (The second modification is crystallized from water saturated with decanol and at ionic strengths $\Gamma/2 < 0.001$.)

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

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

In obtaining crystalline derivatives, Lewin most frequently used methanol–water mixtures rather than the ethanol–water mixtures used in preparing the parent protein crystals. Substituting methanol for ethanol the conditions employed by Lewin in his studies of human serum decanol albumin derivatives—methanol (15–33%) and ionic strengths in the range $\Gamma/2 = 0.02$–0.035—are closely similar to those used in the preparation of crystals of the parent protein. They are outside the range of conditions for the preparation of the second modification of Cohn, Hughes and Weare.[7]

Experimental

The optical and morphological examination was largely carried out in a cold room with the temperature thermostatically controlled at 0°. The polarizing microscope was specially lubricated for use at this and lower temperatures. In a few cases where the alcohol concentration in the mother liquor of some mercaptalbumin mercury dimer derivatives was adequate to prevent the crystals from dissolving, preparations were examined at room temperature. These crystals were stable for several days at room temperature. No differences in optical properties were noticed between preparations examined at room temperature and those examined at 0°. The properties of all the preparations will, therefore, be considered together.

In both series optical and morphological measurements were made on only a limited number of the derivatives described by Lewin. The range was wide, however, with respect to the types of reagents used in their preparation. The conclusions may, therefore, be considered general. A study was made both of the "wet" crystals in equilibrium with their mother liquor, and also of some air-dried crystals.

The presence of alcohol in these preparations presented special problems in handling. The mother liquor loses alcohol rather rapidly on exposure to air even at 0°. When a preparation is exposed to the air in a shallow vessel the crystals slowly dissolve. This appears to be due to loss of alcohol from the mother liquor by evaporation, followed by loss of alcohol from the crystals to the mother liquor. While losing alcohol, the crystals dissolve in the alcohol-poor solution.[10]

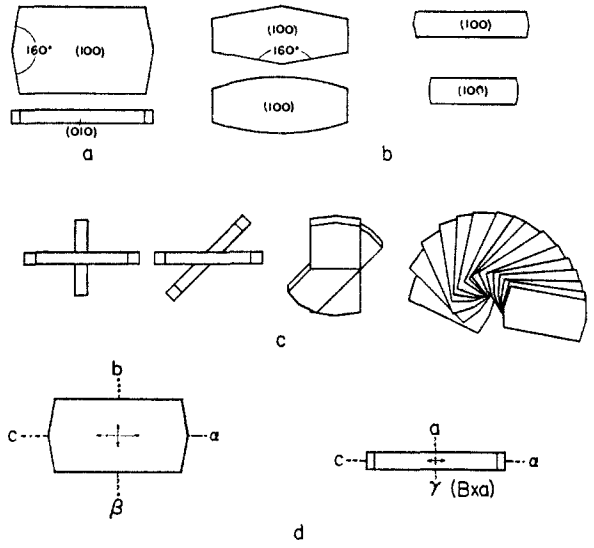

Fig. 1.—Human serum decanol albumin and its derivatives: (a) common habit; (b) modified habits observed in this series; (c) types of interpenetration and whorled growths observed; (d) orientation of the optical indicatrix, in the "wet" crystals.

(10) This explanation is based on the assumption that the crystals contain both water and alcohol of crystallization. The presence of alcohol in the crystals has been shown both by observation of the behavior of crystals in alcohol miscible solvents, and also by density and composition studies (B. W. Low and F. M. Richards, unpublished).

The wet crystals were examined, therefore, while immersed in a few drops of their mother liquor on a slide and covered with a cover slip to prevent evaporation. Welled slides were used when necessary.

Absolute measurements of optical constants were not attempted. The presence of both alcohol and water in the crystals made the use of ordinary immersion liquids impossible.

Changes in the composition of crystals cause variation in the refractive indices. Since the composition of these crystals has not been determined, absolute values for optical constants would have a limited significance. The investigation of the optical constants was limited, therefore, to qualitative estimates of the birefringence and a comparative study of different crystalline derivatives.

Human Serum Decanol Albumin and Its Derivatives

Crystalline derivatives of human serum decanol albumin prepared by Lewin with varying amounts of the following ions have all been examined:

TABLE I

$MoO_4^{=}(Na^+)_2$	$Ag(CN)_2^-(K^+)$	$Mg^{++}(Cl^-)_2$	$Ca^{++}(Cl^-)_2$
$Mn^{++}(Cl^-)_2$	$Sr^{++}(Cl^-)_2$	$Co(NH_3)_6^{+++}(Cl^-)_3$	

The detailed conditions for preparation are recorded by Lewin.[8]

Morphology.—Human serum decanol albumin, when crystallized under the conditions recorded by Cohn, Hughes and Weare,[7] gives six-sided transparent almost colorless orthorhombic laths, described photographically in Fig. 1 in their paper. The crystals are markedly elongated along *c*, flattened on {100} and bounded laterally by the trace of {010} terminated by prism faces. Later preparations of somewhat larger crystals showed minor changes in habit. The preparations examined during this study were six-sided tablets on {100} bounded by {010} and prism faces of the {100} type with an interfacial angle approximately 160° as shown in Fig. 1a. The larger crystals are straw-colored.

The edges are sometimes rounded—a common phenomenon in protein crystals—and parallel overgrowths frequently appear on the main face. The crystals are soft and plastic and apparently do not show cleavage. They crush and break up under slight pressure.

Many of the derivatives examined showed the habit described in 1a. Other derivatives exhibited the modifications illustrated in Fig. 1b. No new forms were observed. Some of the crystal preparations were platy rather than tabular. The photograph in Fig. 3 (Lewin) of the magnesium derivative shows the growth lines commonly found in these crystals. Parallel overgrowths are common in derivative crystals. Interpenetrating growths occur frequently, and some preparations show whorled growths, Fig. 1c. No constant twinning directions have been noted. The fracture of the crystalline derivative is similar to that of the parent protein crystals.

When the cover slip is removed from a slide preparation, the crystals begin to dissolve slowly in their mother liquor, showing rounding-off effects, and parallel dissolution. Narrow, uneven pits develop on the {100} face parallel to *b*, and deepen as the protein appears to go into solution along these parallel troughs. These pits are illustrated diagrammatically in Fig. 2a.

Besides these well-oriented pits, along which cleavage may finally occur, other cracks sometimes appear. These cracks are usually in the direction of elongation, approximately parallel to *c*. They appear to be simply strain line fractures in the crystals.

After removing crystals from their mother liquor, parallel solution pits also appear on the {100} face unless the crystals are wiped quite dry. This solution phenomenon suggests that the alcohol may leave the crystals most easily along certain planes.

Crystals which are dried off by wiping carefully with slivers of filter paper maintain their shape well on further drying and remain clear, although faint surface lines, parallel to *b* and sometimes parallel to *a*, usually appear. The

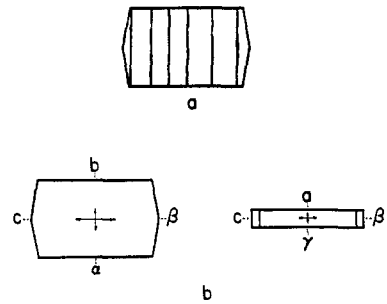

Fig. 2.—Human serum decanol albumin and its derivatives: (a) diagrammatic representation of the orientation of solution pits on (100) during drying; (b) orientation of the optical indicatrix of "dry" crystals.

crystals shrink noticeably upon drying and strain line fractures may occur.

During drying the crystals remain soft and plastic until completely air-dried when they become very brittle and quite hard. They have a glass-like fracture.

Optics.—The birefringence on the main face of the wet crystals is very low, and the crystals appear almost uniaxial $a \doteq \beta$. The orientation of the optical indicatrix ($\alpha \parallel c$, $\beta \parallel b$, $\gamma \parallel a$) is shown in Fig. 1d. The crystals are biaxial positive and $2V$ has a roughly estimated value of approximately 12°.

The optical properties of the crystalline derivatives are similar to those of the parent protein preparation. Slight changes in the value of $2V$ appear probable from observation of the acute bisectrix figure. The crystals appear moderately refracting in their mother liquor. Thick tablets approximately 0.3 mm. in the a direction show only low first order yellow interference colors on the main face {001}. The birefringence of the section (β-α) is, therefore, approximately 0.001, estimated using a Michel-Lévy Chart.[11]

In order to study the optics of the dry crystals, several large crystals wiped dry with filter paper were examined. During the minute or so of time required for this preparation, the crystals lost little apparent birefringence of the (α-β) {100} section.

Drying was allowed to continue while a crystal was kept under observation on a microscope slide. The whole crystal showed increasingly marked disorientation of extinction directions. The extinction directions {001} were parallel to b and c toward the center of the face, but fanned symmetrically outward toward the edges, suggesting crystallite disorientation. The {010} plane extinction directions remained sharp. The birefringence of the section decreased continually until the edges of the crystals were isotropic. As the approximately elliptical isotropic band moved continually inward from the edges, the birefringence about the edges increased. This process continued until the ellipse had contracted to a point at the center of the crystal. The birefringence continued to increase until the whole face, while still showing somewhat disoriented extinction, exhibited a higher birefringence (β-α) than before. The orientation, however, had now changed completely to give $\alpha \parallel b$ and $\beta \parallel c$, as shown in Fig. 2b.

A qualitative idea of the increase in birefringence is given by the change in interference color of a crystal section {100} which showed first order yellow before drying, and changed to first order blue after drying.

The disorientation effects differed between crystals from the same preparation, and occasionally the drying was accompanied by no marked disorientation of any kind. The extent of disorientation on drying is clearly dependent upon the handling of the crystal during preliminary removal from the mother liquor and while the faces are being wiped dry. Strain line fractures may also be due to forces in the preparation during crystallization. A large crystal (about 1 mm. × 0.5 mm. × 0.3 mm.) takes about 10–15 minutes to dry, and the changes appear continuous. The increase in birefringence of the {100} section is accompanied by a marked decrease of the birefringence of the {010} section. The dry crystals are optically positive, $2V$ is much increased in value.[12] Observations on dry crystals in xylene (n = 1.504), immersion oil (n = 1.525) and bromobenzene (n = 1.37)[13] using the Becke line test indicate that α and β for the dry crystals are both slightly less than 1.570. The dry crystals take up mother liquor in the absence of air. The swelling and shrinking appear reversible. A discussion of the significance of the observations and measurements in this series of crystalline derivatives will be given after the properties of the crystals of human mercaptalbumin mercury dimer, and its derivatives, have been described.

Human Mercaptalbumin Mercury Dimer and Its Derivatives

Crystalline derivatives of human mercaptalbumin mercury dimer prepared with varying amounts of the following reagents have all been examined:

TABLE II

$MoO_4^-(Na^+)_2$	$PO_4^-Na^+$	$Fe(CN)_6^{=}(K^+)_4$	$HgI_3^-K^+$
Diiodo-phenyl sulfonate	$Ca^{++}(Cl^-)_2$	$Sr^{++}(Cl^-)_2$	$Ba^{++}(Cl^-)_2$
$Co^{++}(Cl^-)_2$	$Ni^{++}(Cl^-)_2$	$Co(NH_3)_6^{+++}(Cl^-)_3$	$Ba^{++}(OH^-)_2$
$CdCl_2$	$La^{+++}(Cl^-)_3$	$Ag^+NO_3^-$	$Pt(NH_3)_4^{++}(Cl^-)_2$
Orange G	Quinine	Sulfathiazole	Barbital

The detailed conditions for preparation are recorded by Lewin.[8]

Morphology.—Human mercaptalbumin mercury dimer, when crystallized under the conditions described by Hughes,[9] forms colorless diamond shaped orthorhombic plates {001} dominating, and bounded laterally by {110} with an interfacial angle 125° as shown in Fig. 3a. The habit is sometimes modified by the {100} pinacoid to give six-sided plates.

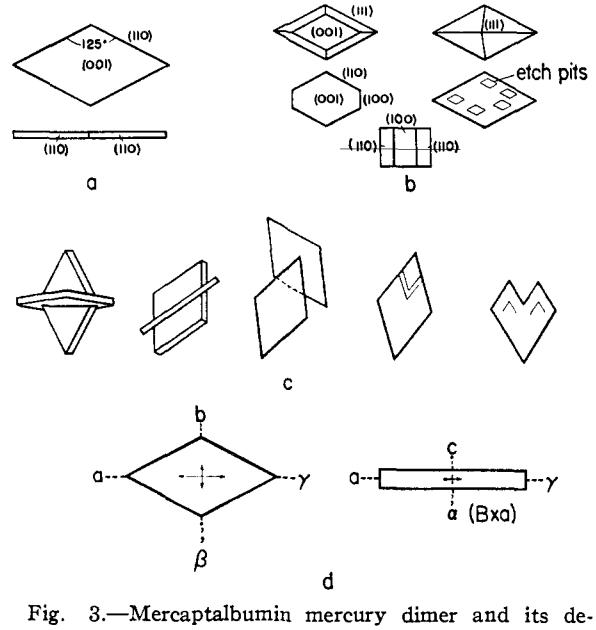

Fig. 3.—Mercaptalbumin mercury dimer and its derivatives: (a) common habit; (b) modified habits observed in this series; (c) types of twinning interpenetration growths observed; (d) orientation of the optical indicatrix, in the "wet" crystals.

(11) A. Michel-Lévy and A. Lacroux, "Les Minéraux des Roches," Plate I, Paris, 1888; *cf.* A. Johannsen, "Manual of Petrographic Methods," McGraw-Hill Book Co., Inc., New York, N. Y., 1918, p. 370.

(12) The observations reported on single crystals were restricted to one preparation of a derivative of human serum albumin. This preparation was the only one available to us which contained crystals large enough to be removed and dried individually. Observations on crystals of human serum albumin and other derivatives exposed on a microscope slide after as much mother liquor as possible had been removed, confirm the general nature of these phenomena.

(13) Values of dn/dt for the immersion liquids have been assumed equal to -0.0005. The values given in the text are completed from the literature values given for 20°: xylene $n^{20°}$ = 1.494, immersion oil $n^{20°}$ = 1.515, bromobenzene $n^{20°}$ = 1.56.

The crystals, although soft and fragile, are well formed and show perfect cleavage parallel to the (001) face. Traces of prismatic cleavage are also seen. Many seemingly "single crystals" are laminated by slip along (001), and the {110} faces show the parallel traces of cleavage.

Many of the derivatives have the habit of the parent protein preparation. Other derivatives show modifications illustrated in Fig. 3b. Preparations of large crystals show some new forms. One modified habit is prismatic, elongated along c and bounded by the prism faces {110} and the pinacoid {100}. The edges of the plates may also be modified in larger crystals by the faces of the {111} pyramid face and a few large dipyramids bounded entirely by the {111} faces have been observed. Rounding-off of the prism faces bounding the acute angle has been observed in some preparations. The interfacial angle $\{\bar{1}10\} \wedge \{110\}$ has values which appear to vary between 121–128° in the derivative crystals with 123 ± 2° as the most common value. Some variation in prism angles between derivative crystals is probably real, and outside the inaccuracies involved in their measurement.

Crystals prepared from two reagents—cobaltammine chloride, and barium hydroxide—show diamond-shaped prisms with interfacial angles of 100° and 104 ± 1°, respectively. They appear to be bounded by the {230} prism faces.

Photographs in Fig. 2 and 3 (Lewin) illustrate rather well some of the habits described here. The most common diamond shaped habit (001) bound by {110} is widely represented (*e.g.* the strychnine and platinum ammine derivative crystals). Crystals of the iodo-mercury derivatives show the six-sided plate bounded by {110} and the {100} pinacoid. Crystals of the metaphosphate derivatives show the rounding-off usually associated with re-solution during growth. These photographs illustrate also the occurrence of parallel overgrowths (*e.g.*, Fig. 3, center left, Lewin) and of twinned interpenetration growths (Fig. 2, lower right, Lewin). The top left hand photograph (Fig. 3, Lewin) shows a beautiful example of the growth lines often found in these crystals.

Re-entrant angles and re-entrant growths are among further twinning manifestations recorded, and twinning intergrowths on the {001} face appear. The diagrammatic records of these phenomena are shown in Fig. 3c (this paper). Etch pits on the {001} face have been recorded; these are parallel to zones of growth; they are outlined by the trace of the {110} prism, Fig. 3b. The cleavage of the crystalline derivative is also perfect parallel to {001}. Some of these crystals appear slightly less soft, and more brittle, than the parent protein preparation. The crystals break under strain rather than crush. This increased brittleness is especially marked in crystals prepared with a large number of moles of reagent per mole of protein, where the principal reagent is a large ion.

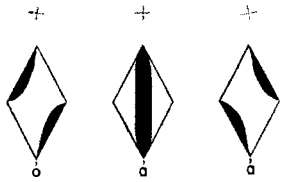

Fig. 4.—Mercaptalbumin mercury dimer and its derivatives. Diagrammatic representation of the disorientation of the extinction directions on drying. At left, the figure shows the position of the brushes at one extreme position for extinction. The center figure shows the position with a and b parallel to vibration directions of the nicols. The right hand figure shows the extinction positions at the opposite end of the angular range (*cf.* text).

When the cover slip is removed from a slide preparation, the crystals dissolve slowly in their mother liquor. As the crystals dissolve, sharp etch pits develop on the {001} face and the crystals dissolve away along the pits. These pits or cracks occur sometimes parallel to b and c, and sometimes parallel to {110}. The surface frequently becomes layered and etched with the trace of fine sharp lines.

Besides these well-oriented pits, along which cleavage finally occurs, other cracks sometimes appear. These are usually approximately parallel to a and they appear to be simply strain line fractures in the crystals.

After removing crystals from their mother liquor, sharp pits appear on the {001} faces unless the crystals are wiped quite dry. This suggests that the alcohol may leave the crystals most easily along certain planes.

Crystals which are dried off by wiping the faces with slivers of filter paper maintain their shape well on further drying and remain clear, although faint surface lines parallel to a and b usually appear. The crystals shrink noticeably upon drying and strain line fractures may occur.

During drying the crystals remain soft and plastic until completely air-dried when they become very brittle and quite hard. They have a glass-like fracture. The (001) cleavage of the wet crystals is absent in the dry crystals.

Optics.—The birefringence on the main face of the crystal is low. The orientation of the optical indicatrix ($\alpha \parallel c$, $\beta \parallel b$, $\gamma \parallel a$) is shown in Fig. 1d. The crystals are biaxial negative, $2V$ is very large, and close to 90° (γ-α) $\simeq 2(\beta$-$\alpha)$.

The optical properties of the crystalline derivatives are similar to those of the parent protein preparation. Slight changes in the value of $2V$ appear probable. It was not possible in some preparations to demonstrate that $2V$ had not increased in value so that it was greater than 90° and the crystals thus optically positive. The significance of negative and positive birefringence is extremely limited, however, when $2V \simeq 90°$.

The crystals appear highly refracting in their mother liquor. Plates approximately 0.2 mm. thick in the c direction show only low first order yellow interference colors on the {001} face. The birefringence of the section (γ-β) is, from a Michel-Lévy Chart, approximately 0.0015.[11] A Becke line test using immersion oil was made on a preparation of small crystals from which most of the mother liquor had been drawn off. This indicated that the refractive indices γ and β of the wet crystals were both less than 1.525.

In order to study the optics of the dry crystals, several large crystals wiped dry with filter paper were examined. During the minute or so of time required for this preparation, the crystals rapidly lost birefringence. The first order yellow, or blue, of the thickest crystals changed to first order grayish-white.

Drying was allowed to continue while a crystal was kept under observation on a microscope slide. Most of the crystals examined showed marked disorientation of the extinction directions. The extent of this disorientation varied from crystal to crystal and occasionally drying was accompanied by no marked disorientation of any kind.

The complete absence of disorientation on drying is rare. Commonly the crystals show an angular range of extinction directions symmetrically disposed about the a axis line. On rotating a crystal between crossed nicols, the extinction positions on (001) sweep together as two broad dark brushes from opposing edges of the crystal. The brushes join in a wide band of straight extinctions parallel to a and move on and outward as the crystal rotates.

Figure 4 is a diagrammatic attempt to show in three drawings the approximate position of these brushes in the extreme positions and also when the extinction directions are parallel to a and b. The cross above each drawing shows the corresponding vibration direction of the nicols.

The angular range of extinction in each direction about the central a axis line varies from crystal to crystal, values of ±10° and ±5° have been observed. The prism faces of the crystal {110} continue to show well-established and sharp straight extinction with lower birefringence.

After the preliminary loss of birefringence and disorientation further optical changes take place very slowly in the crystal (over a period of several hours). The most curious aspect of the further drying is the marked differences between the behavior of different crystals. The grayish-white birefringence on the (001) face decreases slowly and a few air-dried crystals remained stable for several weeks at this stage. Other crystals showed a continuing decrease in birefringence until the edges of the crystal were isotropic. The isotropic band then moved inward, followed by slowly increasing birefringence with re-orientation of the optical indicatrix ($\beta \parallel a$, $\gamma \parallel b$). The birefringence remained a very low grayish-white after this change. In a few crystals the isotropic band continued toward the center and disappeared leaving the whole crystal with reversed extinction directions. Other crystals, however, appear perfectly stable with only the edges of the crystals showing $\beta \parallel a$, $\gamma \parallel b$. These latter changes were always accompanied by very marked disorientation on the (001) face.

The extent of the disorientation on drying is clearly dependent upon the handling of the crystal during preliminary removal from the mother liquor and while the faces are being wiped dry.

A large crystal approximately 1 mm. × 1 mm. × 0.2 mm. takes several hours to dry. Apart from the sharp change in the first few minutes, drying appears continuous.

Observations on dry crystals in xylene (n = 1.504), immersion oil (n = 1.525) and bromobenzene (n = 1.57)[13] using the Becke line test indicate that γ and β for the dry crystals are both slightly less than 1.570.

The refractive indices of "air-dried" crystals increase after prolonged immersion in xylene. Using the Becke line test and bromobenzene and aniline ($n^{0°}$ = 1.607)[13] the value for γ and β appears to be in the range 1.57–1.60.[14]

Barium Hydroxide Derivatives

In an attempt to prepare crystalline derivatives of mercaptalbumin mercury dimer using barium hydroxide, Lewin recorded pH values in the range 5.12–7.80 for the range-moles of $Ba^{++}(OH^-)_2$ per mole of protein 1–15.

Using one to five moles of barium hydroxide per mole of protein pH values varied between 5.12–6.10. These preparations gave very small well-formed diamond shaped plates with an interfacial angle approximately 104° referred to earlier.

The mother liquors of both crystalline preparations using one or two moles of barium hydroxide were mobile liquids. The preparations of the derivatives with three and four moles of barium hydroxide were gelatinous. The small well-formed crystals described above were embedded in an isotropic gel which showed the loss of rigidity on shaking, characteristic of thixotropy. The gel surrounding the crystals prepared by using five moles of barium hydroxide showed milky-white striations. It was red in transmitted light with a green fluorescence and it showed birefringence between crossed nicols. Preparations containing more than five moles of reagent per mole of protein contained no crystals. These preparations were all gelatinous. The gels, in the test-tubes in which they were prepared, were examined in reflected light, in transmitted light, and between crossed nicols. All the gels were extremely viscous, and opalescent in reflected light.

Gels containing more than eight moles of barium hydroxide per mole of protein appeared flocculent and fibrous. The color in transmitted light varied from red to yellow with increasing barium hydroxide concentrations.

Between crossed nicols the gels appeared birefringent, and most of the tube preparations showed considerable orientation with the slow ray parallel to the length of the tube. The flecks seen in reflected light appeared as patches of birefringence between crossed nicols differently oriented from their background. In some tubes these random striations are oriented parallel to each other and to the length of the tube. In this orientation they extinguished sharply in the 45° position. In other preparations the "fibrous" strips are less well oriented. The "flocculent" or "fibrous" appearance of the gel corresponded to the oriented accumulation of the striae in one part of the tube.

The birefringence of the "background" gel was largely concealed by the yellowish-orange color of the gel, and by its strong light absorption. When a little of the gel was drawn up into a capillary tube, 1–2 mm. in diameter, the orientation of the matrix gel was almost perfect with the slow ray parallel to the length of the capillary. Such tubes showed low first order birefringence colors. The "flecks" appeared either to be disoriented, or to show a tendency to orient themselves perpendicular to the capillary length.

The gels are all thixotropic and liquefy upon shaking. On resetting, the gels in some preparations showed a curious reorientation phenomenon. A plane through the center of the test-tube divided an oriented portion of the gel which had the slow ray parallel to the test-tube length, from an equally well-oriented half with the fast ray parallel to the length of the tube. Most of the gels reset without disorientation of the matrix, but with some disorientation of the "flecks." When the gel was sucked gently up and down the capillary, the orientation of the striae perpendicular to the tube was in some cases improved.

(14) The refractive index calculated for completely anhydrous human serum albumin from refractive index increments in aqueous solutions is 1.598. (*cf.* S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. M. Hasson, THIS JOURNAL, **69**, 1747 (1947).

Discussion

The optical and morphological study of these two series of preparations establishes the isomorphism of the crystalline derivatives and their parent protein preparations. In this context, "isomorphous" or "isostructural" indicates that the essential molecular packing is determined by the shape and size of the protein molecules, and by the intermolecular forces between them, rather than by the effect of ions and molecules of various size.

X-ray studies of the swelling and shrinking of protein crystals at different stages of drying have demonstrated the existence of layers, channels, or cage-like enclosures of liquid between the protein molecules in "wet" protein crystals. On drying, the shrinkage of protein crystals has been shown to be due to the loss of aqueous solution from these channels and the consequent moving together of the protein molecules. Further it has been shown that "wet" protein crystals are permeable to salts,[15] sugars[16] and dyes,[15] etc.

These observations lead to the conclusion that the intermolecular spaces in wet protein crystals are either large enough to permit molecules greater than 10 Å. in diameter to pass between them, or that the crystal lattices swell to accommodate large ions and molecules.

The slight changes in interfacial angle recorded for some of the crystalline derivatives examined are explicable in terms of the accommodation of nonbonded molecules or ions of various shapes and sizes in the "free" liquid intermolecular layers or channels. If the reagents used by Lewin in his study are bound at specific sites on the protein molecule, the isomorphism shows that this binding does not interfere sterically with the intermolecular protein packing, nor alter markedly the orienting forces between protein molecules.

These optical and morphological studies do not, however, permit a distinction between the two possibilities.[17] Further study is needed to determine the nature both of the intermolecular spaces, (whether these are continuous layers, channels, or cage-like enclosures) and of the intermolecular protein bonds which can maintain rigid crystals with such marked permeability.

In this context, it is interesting to note that human serum albumin crystallized without ethanol, from water saturated with decanol, and at very low ionic strengths (< 0.001) gives a different crystal structure from that described here. Similarly, in the presence of barium ions at $\Gamma/2$ near 0.01 and at pH values greater than about 6.1, human mercaptalbumin mercury dimer no longer crystallizes but gives oriented gels.

Thus, both large changes in ionic strength and also increased pH in the presence of barium ions have a more marked effect upon interprotein packing forces than do the presence of large molecules

(15) For a general discussion of the swelling and shrinking of protein crystals and their permeability, *cf.* M. F. Perutz, *Research*, **2**, 52 (1949).

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

(17) Preliminary X-ray investigations (Barbara W. Low, Unpublished Studies) are equally ambiguous. Further X-ray and density studies (with F. M. Richards) for the purpose of resolving this problem are in progress.

or ions within narrow ranges of *p*H and ionic strength.

The gels obtained with barium hydroxide are highly oriented. The specificity of the barium ion for their formation has not been demonstrated. The divalence of the barium ion and its size may both be important in the formation of oriented chains or networks of protein molecules. This possible explanation has been suggested by Dr. John T. Edsall (private communication).

In both series of crystalline preparations the birefringence of the crystals ($\gamma - \alpha$) decreases on drying. The general decrease in birefringence is accompanied by a marked increase in the absolute values of the refractive indices of the dried crystals.

A similar increase in refractive indices on drying has been reported by Jones[5] for lysozyme. The increase in the absolute values for the refractive indices of both series of dry protein crystals is to be expected on the basis of the calculated value for the refractive index of anhydrous human serum albumin.[14] The presence of water ($n^{20°} = 1.333$) and methanol ($n^{20°} = 1.328$) in the crystals would be expected to reduce the refractive indices of the crystal below that of the anhydrous protein molecule.

The intrinsic optical anisotropy of the serum albumin molecule may be represented by the optical anisotropy of the dry crystal. The optical anisotropy of the wet crystals may reflect the distribution of the alcohol–water layers in the crystal structure. The dilution effect in both series of serum albumin crystals is itself anisotropic, and appears to conceal and reverse the anisotropy of the molecule. It thus differs from the dilution effects assumed in methaemoglobin crystals.[4]

The behavior of these crystals upon drying indicates that at a certain stage the edges of the crystal bounding the main face are "dry" before the center of the crystal has reached equilibrium. Subsequent liquid loss must take place, therefore, at the air–crystal interface of the plate.

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The Action of Amylo-glucosidase on Amylose and Amylopectin

BY R. W. KERR, F. C. CLEVELAND AND W. J. KATZBECK

The hydrolysis of corn amylose by an *Aspergillus niger* enzyme preparation was studied by determination of total reducing substance, of glucose and of the change in wave length for peak light absorption of the iodine–hydrolysate complex. Two actions were apparent; an endwise attack on some amylose molecules to produce relatively large percentages of glucose early in the reaction, and a random hydrolysis of the others. By treatment of the enzyme preparation with acid at lower *p*H levels, the α-amylase function was inactivated more in proportion than the amylo-glucosidase; at a level of *p*H 2.2, substantially only amylo-glucosidase activity remained. Amylo-glucosidase was found to produce glucose at the same rate, in weight per unit time, from equimolar solutions of amyloses differing widely in original chain length. The unconverted polymer residue at different times during the first half of the hydrolysis was found not to change materially in DP_n, as measured by iodine-spectrophotometry, and glucose was the only sugar found in the hydrolysate by paper chromatography. At equal in weight concentrations, the initial rate of hydrolysis for corn amylopectin (B-fraction) was found to be 4.5 times the initial rate for corn amylose (A-fraction), but the rate for the amylopectin decreased abruptly to a lower value at a level of 60% hydrolysis to glucose. The hydrolysis of a highly linear substrate, corn crystalline amylose, was found to be a first order reaction. Amylo-glycosidase action can be interpreted by assuming a terminalwise hydrolysis of starch molecules to glucose and, in the case of linear ones at least, according to the "single-chain" mechanism. The wave length for peak light absorption by the iodine complexes of a series of amyloses was correlated against DP_n.

The α-glucosidases, as a class, are able to hydrolyze α-glucoside linkages in glucosides, producing glucose. Members of this class of enzymes which are able to operate on higher polysaccharides were recognized in early work by Kerr and co-workers[1,2] because of their ability to produce large yields of glucose from starch and starch products. One fungal preparation at least was found able apparently to hydrolyze even the limit dextrins remaining after an extended hydrolysis of starch by malt diastase, materially increasing the glucose yield and decreasing the percentage of residual dextrins.[2] Glucosidases which split 1–6 α-glucoside linkages have been called limit dextrinases.[3,4] The α-glucosidases have also been grouped under the general heading of maltases.[5] Other names have been proposed and of the names suggested it would now appear appropriate, following Cori and Larner,[6] to designate all of these starch-splitting, glucose-producing enzymes by the general group name of amylo-glucosidases.

Work in recent years with very pure enzyme preparations has shown, however, that the α-amylases are also able to produce glucose from starch. Thus, Bernfeld and Studer-Pecha,[7] in a study of the action of crystalline α-amylases, believe that the final limit of hydrolysis of amylose is attained in a prolonged second phase of the reaction with complete conversion of substrate to glucose and mal-

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(5) J. Corman and A. F. Langlykke, *Cereal Chem.*, **25**, 190 (1948).
(6) Gerty Cori and Joseph Larner, *Federation Proc.*, **9**, 163 (1950).
(7) P. Bernfeld and H. Studer-Pecha, *Helv. Chim. Acta*, **30**, 1895 (1947).