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Insulin ... Preliminary X-ray Studies of Two Types of Sulphate Crystals

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Preparations of beef insulin sulphate crystals yield two crystalline forms. Both are orthorhombic, space group $P2_12_12_1$, and both usually give the same air-dried form. The morphology, optics, and unit-cell dimensions are described. The two-dimensional Patterson projections for both forms are presented.

Introduction

Insulin crystallizes on the acid side of the iso-electric point in the pH range 2·0–4·0 as sulphate, selenate, citrate, formate and acetate. Insulin was first crystallized as the sulphate by Oncley & Ellenbogen (Ellenbogen, 1949). Preliminary analytical studies (Ellenbogen, 1949) suggested that the crystals were zinc-free. Further, Tanford & Epstein (1954) have interpreted their study of the hydrogen ion titration curve of crystalline zinc insulin in terms of the dissociation of zinc from insulin in solutions more acid than pH $\sim$ 4.

No attempt has been made in this paper to correlate our results with the results of the X-ray studies of Crowfoot on wet rhombohedral zinc insulin crystals grown at pH $\sim$ 5·2 and $\sim$ 6·2 (Crowfoot, 1939; Wrinch, 1948). Earlier some comments were made concerning the possible relationships between the intermolecular packing in the air-dried forms of the two structures (Low, 1952).

Two crystalline forms, type *A* and type *B*, have been found in some preparations of beef insulin sulphate. Both type *A* and type *B* crystals are orthorhombic, space group $P2_12_12_1$, and both usually give the same air-dried form, here described as type *P*. There are two molecules (of molecular weight 5733) in the asymmetric unit. This approximately 12,000 molecular weight dimer is, within this pH range, the solution monomer. The two-dimensional Patterson projections and sections through the three-dimensional vector structure of the *P* form have been described (Low, 1952). This paper presents crystallographic data for the two 'wet' forms, type *A* and type *B*, of insulin sulphate, and describes the Patterson projections of these two forms.

Experimental

One procedure used in growing beef insulin sulphate crystals has been described elsewhere (Low & Richards, 1954). Several slightly modified procedures have also been employed. The method described gives large, well-developed crystals of length approximately 1–2 mm. and diameter 0·5–1 mm. Type *A* crystals predominate in all preparations; type *B* crystals have been found in some, but not in all preparations. The procedure necessary for obtaining type *B* crystals at will has not yet been established. They have not been found at all in recent preparations.

The optical examination and X-ray studies were carried out in a cold room at a temperature of 0 ± 2 °C.

For X-ray studies, the 'wet' crystals were mounted and sealed in thin-walled glass capillaries. Precession photographs of the three principal zones of wet crystals of both type *A* and type *B* were taken using the Buerger precession camera, Cu $K\alpha$ radiation, and a 30° precession angle. A modified backstop with a small cup was used. Special layer-line screens with narrow annuli (2·5 mm.) were used in order to prevent the recording of non-zero layers of the reciprocal lattice.

The intensities were estimated visually with the aid of intensity strips. Timed exposures and duplicate film techniques were used. Lorentz polarization factor corrections were taken from a chart published by Waser (1951). Corrections were not made for absorption either by the crystal or by the capillary walls.

Morphology and optics

Type *A*. These crystals show two predominating habits: (1) Diamond-shaped tables lying on (010) and bounded by {101}. (2) Tables elongated along [010], lying on (001), and bounded by {010} and {100}.

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The crystals are biaxial positive with $\alpha \parallel b$, $\beta \parallel a$, and $\gamma \parallel c$. $2V$ is small.

The refractive indices have not been measured accurately but limits were obtained using the Becke line test. The refractive indices all lie within the range $n = 1{\cdot}50 - 1{\cdot}53$.*

When a crystal is removed from its mother liquor and carefully freed from adhering droplets of liquid, changes in optical properties may be observed as the crystal dries, either in air or in xylene. In several crystals observed in this way, the drying process appeared to proceed by stepwise changes. Correlation of these changes with stepwise changes in the crystal lattice on drying has not been established. When the (010) face is observed as the crystal dries, the birefringence of the section increases. When the (001) face is observed, the birefringence initially decreases until it disappears; later it increases until the whole face exhibits higher birefringence $(\beta - \alpha)$ than before. The orientation of the optical indicatrix has now changed to $\alpha \parallel a$, $\beta \parallel b$, and $\gamma \parallel c$.

Refractive indices for the dry crystals determined as above lie broadly within the limits $n = 1{\cdot}53 - 1{\cdot}56$.*

Type *B*. Optical and morphological measurements of type *B* crystals have not been made.

Crystallographic data

The unit-cell dimensions and minimum observed spacings are given in Table 1 for type *A*, type *B*, and, for purposes of comparison, type *P* crystals.

The cell dimensions of 'wet' crystals of both type *A* and type *B* crystals show a range of values dependent both on the detailed preparative procedure employed and on the age of the preparation. The maximum observed range of variation for each axial length is 1 – 1·5 Å. These limited variations are accompanied by a very few minor changes in the intensity distribution of the diffraction maxima.

Table 1. *Crystallographic data**

	Type *A*	Type *B*	Type *P*
a	58·0 Å	46·7 Å	44 Å
b	51·5	51·9	51·4
c	38·5	44·6	30·4
Minimum spacing	2·1	1·7	5·5
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Z†	4	4	4

* There is an error in the tabulation of the data (Low, 1953) for type *A* 'wet' insulin sulphate crystals. The values for the lengths of the axes *a* and *b* have been transposed.

† The protein component of the asymmetric unit in all three crystal structures is two (5733 molecular weight) molecules.

The unit-cell volume of type *B* crystals is less than that of type *A*. It is pertinent therefore to inquire whether it represents an inadvertant drying stage of type *A* crystals, perhaps the result of careless handling of the crystals during mounting. Several distinct drying stages have been observed and characterized during the complete transition wet → dry for type *A* crystals by Einstein (1958). At one drying stage of type *A* beef insulin sulphate a crystal form appears which is closely similar to the type *B* form both in unit-cell dimensions and in intensity distributions. The minimum spacing observed for this drying stage is, however, 3·5 Å, which is greater than that for type *A* crystals (Table 1), and much greater than that for type *B* crystals. Moreover repeated efforts to produce type *B* crystals by drying have not proved successful. We may therefore conclude tentatively that the type *B* form is a true wet form in equilibrium with the mother liquor.

Intensity distributions and Patterson projections

The corrected relative intensities for the three principal zones* of both type *A* and *B* crystals were used to compute Patterson projections at intervals of *a*/60, *b*/60 and *c*/60. The computations were carried out on I.B.M. machines by Mr J. R. Steinberg at the Statistical Services Laboratory, Massachusetts Institute of Technology using the *M*-card system (V. F. H. Schomaker). The projections are shown in Fig. 1(*a*), (*b*).

The intensity distributions for the three principal zones of reflections of type *B* crystals are markedly pseudo-centered. (Further, for all reflections where $h+k+l = 2n+1$ the type *B* diffraction maxima are in general either weak or absent.) The most prominent non-origin peak in all three projections is at $\frac{1}{2}, \frac{1}{2}$. The peak distributions in *b* and *c* plane projections of type *B* crystals are rather similar to each other.

There is a close similarity between the type *B* and type *P* crystals which is most clearly evident when a direct comparison is made for both forms of the weighted reciprocal lattices. This is particularly striking when the *hk*0 zones of reflection are compared. There is also a marked similarity between the two 0*kl* zones of reflection. Some similarity does appear in a comparison of the *a* axis projection of both forms (Low, 1952). The Patterson projection along the *c* axis of type *B* crystals may not immediately appear closely similar to the corresponding projection of the air-dried type *P* form. However, when the *P* form *c* plane projection is compared to the *c* plane projection of the *B* form calculated using only those terms corresponding to spacings of > 7 Å the relationship becomes much more obvious.

The diffraction maxima from type *A* crystals in general show little evidence of pseudo-centering. Pseudo-centering is remarked in the 0*kl* intensity

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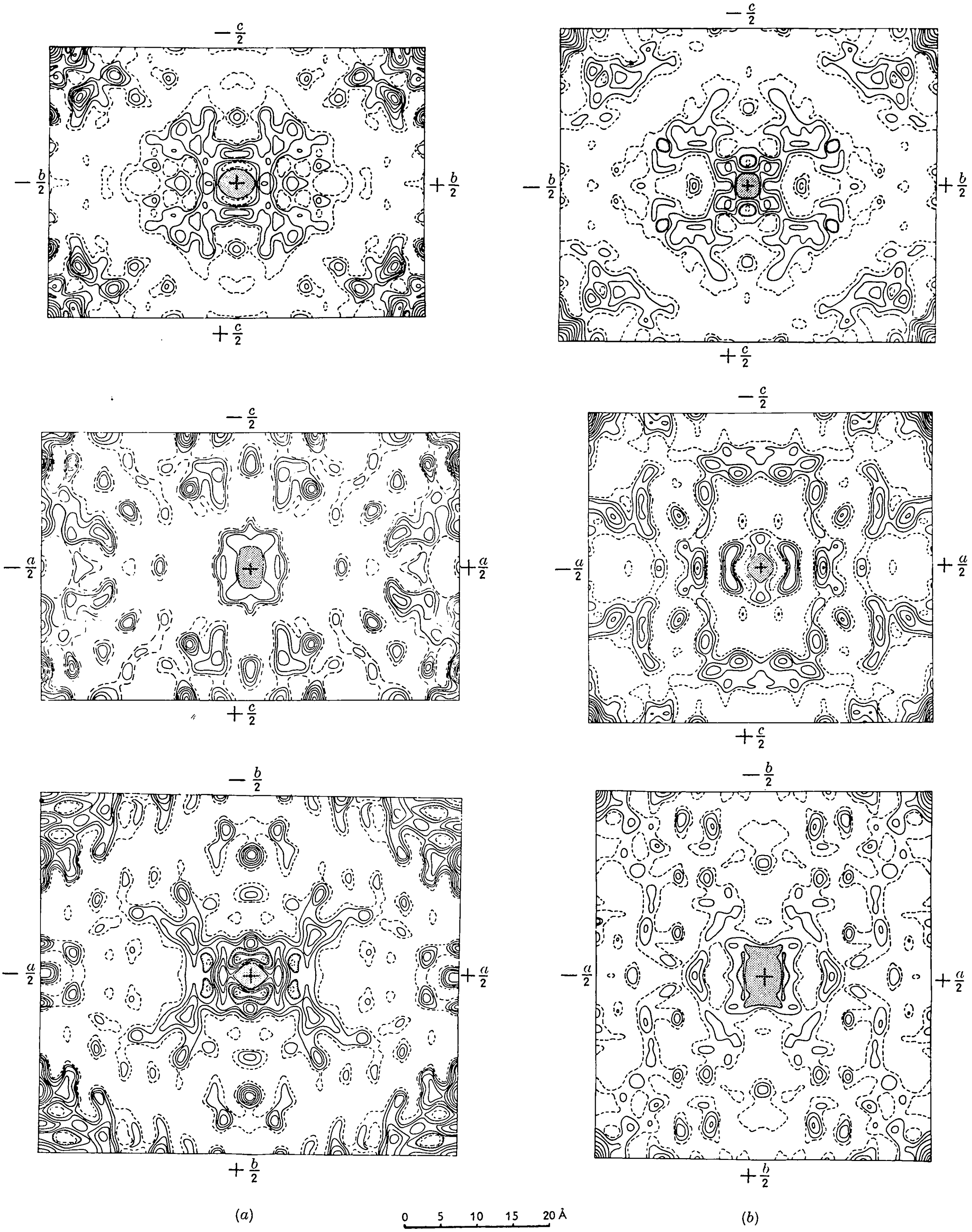
* Values of *dn*/*dt* for the immersion liquids used have been assumed equal to – 0·0005. The refractive indices given in the text are computed from the literature values for 20 °C.

* These data have been omitted from the paper in order to save space; they are available from the authors on request.



(*a*) (*b*)

Fig. 1. (*a*) Insulin sulphate Type *A* crystals: *a*, *b* and *c* axis Patterson projections. (*b*) Insulin sulphate Type *B* crystals: *a*, *b* and *c* axis Patterson projections. Contours at equal arbitrary intervals. The broken line is the zero contour. The marked lines outline troughs lying within peak regions.

distribution and the highest non-origin peak in the a plane projection is at $b/2$, $c/2$ (Fig. 1(a)). Further, the constellations of peaks within a radius of ~ 12 Å about 0, 0 and $b/2, c/2$ show certain similarities to each other and to the corresponding peaks in the related type B projection.

In the b plane projection of the A form the highest non-origin peak is at $x=\frac{1}{2}$, $z=\frac{24}{60}$ rather than at $x=\frac{1}{2}$, $z=\frac{1}{2}$. In spite of the absence of this one feature of pseudo-centering, the type A and type B plane projections do show certain related similarities in peak distribution about 0, 0; $x=\frac{1}{2}$, $z=\frac{1}{2}$ (type B) and 0, 0; $x=\frac{1}{2}$, $z=\frac{24}{60}$ (type A). There are perhaps less pronounced similarities between the c plane projections of the A and B forms.

Discussion

The relationships between the projections of the A, B and P forms outlined above do suggest that the molecular orientation in type B crystals may be simply related to that in type A crystals. The molecular shifts which would be involved in the (hypothetical) transition type $A \rightarrow$ type B are clearly not only simple translations, with linear components along the three axial directions, although such translations are probably the major components of the overall implied molecular shifts. Further, the molecular shifts which would be required for the transition type $A \rightarrow$ type B appear similar to those actually involved in the type $A \rightarrow$ type P transition. A detailed study of this transition is in progress.

A tentative packing model for the insulin structure was proposed earlier (Low, 1953), after a study of the two and three-dimensional Patterson series of air-dried insulin sulphate crystals (Low, 1952). The structure may simply be described as made up of rod-like regions of high electron density (perhaps coiled or folded peptide chain) in close-packed array parallel to the a axis. It is pertinent to inquire whether the vector projections described here provide any evidence concerning the overall validity of this model. The a plane projections certainly do not provide straight-forward evidence for the existence of simple rods viewed end-on as does the a plane projection of type P. On the other hand, the similarities between the b and c plane projections of type B crystals are not incompatible with a packing structure made up of some rod-like regions approximately parallel to the a axis.

A study of these projections, of oblique projections for the type B crystals, and optical diffractometer studies of model structures have recently been interpreted in terms of a new molecular model with many features of the old (Low, McGavin & Einstein, 1958).

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