Insulin Crystals

II. Shape of Rhombohedral Zinc-Insulin Crystals in Relation to Species and Crystallization Media

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The shape of insulin crystals depends on the species of insulin. When crystallized by ordinary methods (citrate, phosphate, ammonium acetate buffer), recrystallized pig insulin precipitates as single, sharp and perfect rhombohedral bodies. The shape of cattle and sheep insulin crystals often shows a twinned appearance (e.g. star shape). The faces are curved.

Pig and cattle insulins are found to have identical biological insulin activities (units per molecule). The confidence limits for the ratio between pig and cattle insulin activity were 0.99 and 1.07 ($P = 0.95$).

Recrystallized insulin from all tree species yields perfectly shaped rhombohedral single crystals when urea or a halogenide are present in the mother liquid during crystallization.

The angle of a large rhombohedron is measured with an eye-piece goniometer. The estimate, $114^{\circ}22'$, is in agreement with the X-ray analysis, $114^{\circ}16'$.

In 1929 Abel¹ crystallized insulin and observed that the crystals were rhombohedral, and in 1935 this structure was proved by X-ray diffraction analysis by Crowfoot². In the papers by Abel¹, Harrington and Scott³, Scott⁴, Netter⁵ (Ni-insulin crystals), Romans *et al.*⁶ and Sahyun⁷ the rhombohedral shape is apparent from the photomicrographs. Scott⁴ showed by means of a movie strip, however, that the crystals were actually twins, and he writes in this connection: "This twinned appearance of insulin crystals is the rule rather than the exception, but of course, is not observed unless the crystal is moved to expose various faces to view". When crystallizing at pH 5.2 instead of at the usual pH 6, Scott^{4,8} obtained dumb-bell shaped crystals (wedge-shaped, no plane faces). Crowfoot⁹ reviewed the literature dealing with insulin crystals of various shapes and studied some of these crystals by X-ray.

The primary purpose of the present paper is to define, within limits of particular interest, the dependence of the crystal shape on insulin and the conditions of crystallization. A new crystallization medium is introduced contain-

ing saline and sodium acetate. This medium causes the development of rhombohedral shape regardless of insulin species.

The second part of the present paper comprises a goniometric confirmation of the X-ray estimate of the obtuse angle of the rhombohedron, for which purpose large, perfectly shaped rhombohedral single crystals were prepared.

EXPERIMENTS WITH CRYSTAL SHAPES

In order to establish the sources of shape variation, the conditions of crystallization are initially fixed and the quality of insulin varied, with respect to species (pig, cattle and sheep). The dependence of shape on purity within the species cannot be properly studied as the impurities are not well defined.

1. Crystallization from a buffered solution

The crystallization mixture contains 0.5% insulin; 0.04% Zn^{++} (as chloride); 0.05 M sodium citrate; 15 % (v/v) acetone; and the pH = 6.0—6.3. The insulin is dissolved in water containing HCl (pH = approximately 2.5), whereupon acetone and buffer solution are added. The crystallization is carried out at room temperature without stirring.

Only a few observations were made with sodium phosphate or ammonium acetate buffers. The results were identical with those obtained with the citrate buffer.

A. Insulin from pig pancreas. Fig. 1 shows pig insulin crystals obtained by recrystallization. The crystals are beautiful single rhombohedrons. All attempts, without any exception, to obtain this shape by recrystallizing various lots of pig insulin proved successful. The pig insulin crystals (citrate buffer, recrystallized insulin) are thus defined as perfectly shaped, rhombohedral single crystals.

B. Insulin from cattle pancreas. Cattle insulin crystals from several times recrystallized insulin are shown in Fig. 2. They have the star-shaped appearance described by Crowfoot⁹. When agitation is avoided the crystals may grow even larger and develop the needle shape⁹. The gradual perfection of shape seen when recrystallizing pig insulin has not been observed in this laboratory in the case of cattle insulin even after as many as ten recrystallizations.

The shape of cattle insulin crystals is correlated with the size of crystals. When agitating, a great number of smaller crystals (5—10 μ) are obtained. These crystals, apparently twins, resemble those shown in the publications listed above. They have rounded edges and faces and their vertices are not well defined. The curved surface of cattle insulin crystals may be explained by the presence of impurities which are not removed by recrystallization. To test this question, cattle insulin was fractionated by salting out with NaCl at pH 3 or by extracting the crystals with neutral citrate or phosphate buffers. No perfect rhombohedral shape could be observed in crystals obtained by crystallization of these fractions.

Fig. 1. Rhombohedral zinc-insulin crystals from pig pancreas. $\times 250$.

Fig. 2. Twinned zinc-insulin crystals from beef pancreas. $\times 250$.

C. *Insulin from sheep pancreas.* Only one lot of sheep insulin crystals has been examined. After recrystallization the crystals developed a shape similar to that of cattle insulin crystals.

2. Crystallization from a buffered solution of urea or a halogenide

If it is assumed that the impurities of both pig and cattle insulin are removed by the recrystallizations from citrate buffer it follows that the peculiar shape of cattle insulin crystals is not due to impurities. In order to investigate the question of equal purity, the biological activities of recrystallized pig and cattle insulin were estimated by biological assays on rabbits. The activity is expressed in units per molecule of insulin. The ratio between the activities of insulin from the two species was estimated as:

$$\frac{\text{activity of pig insulin}}{\text{activity of cattle insulin}} = 1.03 \pm 0.04 \text{ (confidence limits: } P = 0.95\text{)}.$$

The activities are found identical. This finding also supports the hypothesis of equal purity.

The crystallization of pig insulin was then compared with the crystallization of cattle insulin in order to find an explanation of the shape difference. Under apparently identical conditions (citrate, pH, acetone, etc.) cattle insulin crystals are formed more rapidly than pig insulin crystals, and this is more pronounced when the pH is increased above 6. The solubility of the two species of insulin crystals also presents a difference. By increasing the pH from 6 to a value below 7, the solubility of pig insulin is increased more than

that of cattle insulin. This difference in solubility and rate of crystallization may or may not have any relation to the shape of the crystals. In order to investigate this question, these differences were tentatively eliminated by using solubilizing agents for the crystallization of cattle insulin. Urea, which is known to have solubilizing properties, was added to the citrate medium for the crystallization of cattle insulin. The solubility of the cattle insulin was thereby increased and the rate of crystallization decreased. Sodium chloride which also increases the solubility of the crystals in the citrate buffer was likewise tested.

A. Addition of urea, citrate buffer, cattle insulin. Acetone was tentatively substituted by 20 % urea, which caused the insulin to stay in solution. 10 % urea was tried with the result that perfect rhombohedrons developed which could not be distinguished from pig insulin crystals. After recrystallization without urea, the beautiful sharp rhombohedrons were transformed into the usual shape typical of cattle insulin. Thus the shape seems to be related to solubility and the rate of crystallization rather than to the presence of impurities.

B. Addition of halogenides. The solubilizing effect of sodium chloride is less pronounced, but when acetone is replaced by 7–8 % sodium chloride, rhombohedrons are obtained which are just as perfect as those obtained with 10 % urea. Sodium acetate, nitrate, sulphate, citrate or phosphate were found ineffective, whereas KCl, NH_4Cl , CaCl_2 and $(\text{HOCH}_2\text{CH}_2)_3\text{NHCl}$ produced the effect. Thus the effect seems to be due to the chloride ion.

Sodium fluoride was ineffective, but the following halogenides, KCl, KBr, KI, were all effective, especially KI as judged from the dependence of shape upon molar concentration of the halogenide.

In order to demonstrate that the effect of the halogenides is not contingent on the presence of citrate buffer, the following composition may be employed:

0.5–4.0 % insulin
0.8 % Zn^{++} (as chloride) calculated on the weight
of insulin
0.1 M sodium acetate
7.0 % sodium chloride
HCl to pH 5.45–5.55.

The insulin is dissolved in water containing HCl and ZnCl_2 , whereupon a solution of sodium acetate and sodium chloride is added. The crystallization is carried out at room temperature under mechanical stirring. The process is completed within one or two days. The advantages of this method are the following:

- 1) The shape of crystals is perfectly rhombohedral irrespective of the species of insulin and, to a certain extent, the purity.
- 2) Non-protein nitrogen is not introduced (as with ammonium acetate).

When the acid insulin solution is mixed with the alkaline buffer solution to produce pH 5.5, the insulin is precipitated as amorphous particles and gradually converted into crystals. When impure insulin is used, an amorphous

Fig. 3. 1.3 mm large insulin crystal from pig pancreas.

fraction is left which may be removed by acetone (30 % (v/v)). The acetone dissolves the amorphous fraction so that the crystals can be isolated by filtration or centrifugation. When pure, recrystallized insulin is crystallized by this method, only a small fraction, approximately 3 % of the protein, is left as amorphous particles, and the amount of insulin left in solution is of the same magnitude.

The halogenide ions do not alter the shape of pig insulin crystals.

MEASUREMENT OF THE ANGLE BETWEEN THE EDGES OF THE RHOMBOHEDRON

Several times recrystallized insulin from pig pancreas was crystallized from the acetone-citrate buffer at pH 6.2 (1 litre). Agitation was avoided. Within a few days, large (1 mm), beautiful, extremely sharp and colourless rhombohedrons could be observed on the glass walls, at the bottom of the beaker and hanging on the surface. The crystals shrink, harden and become crackled when taken up and left in the air. Therefore, for the microscopical examination, a crystal was placed in its mother liquid on a slide and covered with a cover glass, a ring of vaseline between the glasses providing space for the crystal. The crystal was lying on a face which was therefore in level (see Fig. 3). The horizontal position was checked by adjusting the microscope to the edges of the face. A $10\times$ objective in connection with a $8\times$ eye-piece goniometer was used for the measurements. Ten goniometer readings of the obtuse angle between the edges were made with the following result: $\alpha = 114^{\circ} 22' \pm 4'$ (S.D.). This estimate agrees with the X-ray estimate for wet insulin crystals, viz.: $\alpha = 114^{\circ} 16'$, as determined by Crowfoot and Riley¹⁰.

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