Insulin Crystals

IV. The Preparation of Nuclei, Seeds and Monodisperse Insulin Crystal Suspensions

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Nuclei were formed by freeze-drying a crystallization mixture containing dissolved insulin. The freeze-dried insulin was suspended in a buffered insulin solution whereupon the nuclei grew up to $1-2\ \mu$ crystals which were used as seeds for the preparation of monodisperse insulin crystal suspensions with different crystal sizes (5, 25 and $40\ \mu$)

It is the purpose of the present investigation to demonstrate the effect of seeding with a large number of small seed crystals on insulin crystallization.

The size of a rhombohedral insulin crystal has been defined¹ as the greatest diagonal in the projection of the crystal on a plane through a face. An amount of seed-crystals weighing w_s g contains n seeds of almost uniform size, $l_s\ \mu$. Hence,

$$w_s = knl_s^3$$

where k is a constant. The total weight of insulin gained as crystals after seeding is w_i . Assuming that the crystals grow with identical rates of linear growth, it follows that

$$\alpha w_i = knl^3$$

where $(1-\alpha)$ is the fraction of spontaneously formed crystals, and l is the size of the grown up seeds. Hence,

$$l = l_s \sqrt[3]{\frac{\alpha}{1-\alpha}} \quad (1)$$

or

$$\alpha = r \left(\frac{l}{l_s} \right)^3 \quad (2)$$

where r is the ratio w_s/w_i between weight of seeds and total insulin gained as crystals. The fraction, α , of uniform crystals depends on the conditions of crystallization as well as on l_s and r . When the conditions and the size of seeds are fixed, a decrease in r causes α to decrease.

When seeding is employed immediately after mixing the components for crystallization, it follows from the assumption and eqn. (1) that the upper limit of crystal size is

$$l_u = l_s \sqrt[3]{\frac{1}{r}}$$

PREPARATION OF SEED-CRYSTALS

Solutions: (1). 5 g beef insulin, recrystallized from a citrate buffer ² + 42.5 ml 0.1 N HCl + water to make 500 ml; (2). 500 mg citric acid + 20 mg Zn⁺⁺ (as chloride) + 800 mg methyl-*p*-hydroxybenzoate + 11.2 ml 1 N NaOH + water to make 500 ml.

Freeze-drying: To 100 ml of sol. (1) were added 100 ml (2) and 1 N NaOH to pH = 6.7. The solution was frozen at once and freeze-dried.

Crystallization: The freeze-dried insulin was suspended in 400 ml of the alkaline (pH 8.5) solution (2) and immediately thereafter 400 ml of sol. (1) were added. The mixture (pH = 6.2–6.5) was shaken over night at room temperature. The crystals which developed were 1 μ large.

CRYSTALLIZATION WITH AND WITHOUT SEEDS

The first experiment is a comparison between crystallizations with and without seeding.

The crystallization mixture had the following composition ³:

1.7 % beef insulin, 155 μ g/ml Zn⁺⁺ (as chloride, including the zinc from the insulin), 7 % NaCl, 0.1 M CH₃COONa + HCl to pH = 5.45 to 5.55.

One ml of the 1 μ -seed crystal suspension containing 6 mg insulin was diluted with water to 20 ml and added to 4 litres of crystallization mixture containing 68 g insulin. The final distribution of crystal sizes was determined by the technique described earlier ¹. The cumulative distribution of insulin units was calculated and presented in a graph, Fig. 1, in which can be seen that 90 % of the weight was concentrated in the size range 20–30 μ .

In the control, which was not seeded, 90 % of the insulin was found in the much greater interval 25–100 μ . It is concluded that seed crystals grow with identical rates of linear growth in accordance with the assumption for eqn. (1).

VARIATION OF THE AMOUNT OF SEED-CRYSTALS

The dependence of α on r is illustrated by an experiment in which the conditions of crystallization remain as in the previous experiment while r is varied. The size of seeds was 2–3 μ . The resulting cumulative distributions of units (or weight) on crystal size are shown in Fig. 2. The median size is taken as

Fig. 1. Cumulative distribution of insulin units.

Fig. 2. Cumulative distribution of insulin units. The suspensions were prepared with different amounts of seeds (see Table 1).

representative of l , the size of grown up seeds. The results from 3 crystallizations are presented in Table 1. It is assumed that spontaneous crystallization is negligible ($\alpha = 1$) in the first case where r is relatively large. Hence, by inserting l_{50} , r and $\alpha = 1$ in eqn. (1) it is calculated that $l_s = 3.0 \mu$. When eqn. (2) is employed in case 2 and case 3, α is found to be 0.9 and 0.7, respectively. These values of α are confirmed by Fig. 2 as they are found to be the ordinates corresponding to deflections of the curves. These points presumably divide the distributions in the two parts containing, respectively, spontaneously formed crystals and grown up seeds.

Table 1. Variation of the amount of seed-crystals.

No.	r	l_{50}	$\alpha = r \left(\frac{l}{3} \right)^3$
(1)	12×10^{-2}	6μ	1.0
(2)	21×10^{-4}	23μ	0.9
(3)	30×10^{-5}	39μ	0.7

Legend: r , The ratio between weight of seed-insulin and total insulin.
 l_{50} , The median size in the distribution of insulin units.
 α , The weight of spontaneously formed crystals is $(1-\alpha)w_i$, where w_i is the total weight of insulin gained as crystals.

DISCUSSION

The freeze-drying method originated from the incidental observation that crystallization yielded smaller crystals when using glass apparatus which had not been cleansed after a previous crystallization process. The small amounts of dried-in insulin on the propeller rod and in the beaker were apparently responsible. The number of crystals was further increased hundredfold when freeze-dried insulin was added.

When suspended in the alkaline ($\text{pH} = 8.5$) solution (2), the freeze-dried insulin is partially dissolved. The pH may even be increased to 11 (glass-electrode). In this case the insulin disappears into solution, apart from a great number of particles which are visible in the dark field microscope. The seeding properties of the colloidal solution vanish within one hour together with the small particles.

The faces of the seed-crystals do not grow with equal growth rate upon seeding. Thus the seed-bodies from $3\text{--}5\ \mu$ seeds are finally found in the obtuse vertices of the crystals. Smaller seeds are barely or not at all visible in the final crystal suspension.

REFERENCES

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