Insulin Crystals

VII. The Growth of Insulin Crystals

JØRGEN SCGLICHTKRULL

Novo Terapeutisk Laboratorium, Copenhagen, Denmark

Pig insulin is crystallized from a clear solution containing sodium citrate and acetone. The decreasing concentration of insulin is determined during the process. The final distribution of crystal sizes is similar to that found earlier in the crystallization of beef insulin from a saline suspension of amorphous insulin.

The rate of pig insulin crystallization is

$$\frac{dC}{dt} = 2.8 \times 10^{-7} \times C(95.8 - C)^3$$

where $C$ is the crystalline fraction in per cent of total insulin (1.43 mg insulin nitrogen/ml), and $t$ stands for time in min.

The linear rate, $R_d$, of deposition of insulin on the crystal faces is calculated from the previous and the present data:

- saline, beef: $R_d = 1.63 (c_1 - 0.080)^2 \mu$/min
- citrate, pig: $R_d = 0.38 (c_1 - 0.066)^2 \mu$/min

where $c_1$ is the concentration of dissolved insulin in millimoles per l, each molecule containing 65 atoms of nitrogen ($M = ca. 5700$).

Rhombohedral insulin crystals of perfect shape may be obtained either from pig insulin or by crystallizing beef insulin from a saline solution containing precipitated amorphous insulin. The latter process of crystallization has been analyzed by an experiment using mathematics devised for the purpose. The ratio, $r$, between the total surface, $S$, and volume, $V$, of crystals was found to be constant during crystallization, except for the initial stage. It was assumed that crystals grow with identical rates of linear growth and it was then concluded by mathematical argument that the logarithm of the cumulative size distribution, $P(l)$, is linear, having the slope

$$\frac{d\ln P}{dl} = -3r = -3S/V$$

(1)

This relation was confirmed by the $P(l)$ estimates obtained by counting the crystals according to size. From the constancy of $r$ it was further concluded that the rate of nucleation is proportional to the product of the linear growth.
rate and the total number of crystals present. It was demonstrated that this number is proportional to the total length, \( L \), surface, \( S \), and volume, \( V \), of the crystals during crystallization. A chemical interpretation was suggested to the effect that the crystals are self-reproducing at a rate proportional to their linear growth rate. This rate could be expressed by

\[
\dot{l} = \text{const. } (c_a - c)^2
\]

where \( c_a \) was the actual and \( c \) the final concentration of dissolved insulin, the dot symbol indicating differentiation with respect to time.

The crystallization of pig insulin from a clear solution without addition of sodium chloride has been examined by counting the crystals according to size when crystallization was finished. The cumulative size distribution was found to be very similar to that found for beef insulin, indicating crystal self-reproduction and proportionality during crystallization between the total surface and volume.

It is the purpose of the present investigation to determine the rate of linear growth as a function of the insulin concentration also in this crystallization.

The relation between the rate of growth, \( \dot{l} \), and the rate, \( R_d \), of insulin deposition on the crystal faces has been established in experiments with stained crystals. Thus, the rates of deposition will be calculated from the rates of growth.

**SYMBOLS**

\( l \) = size of crystals, i.e. the largest diagonal in the crystal projection on a plane through one of the faces. As the crystals lie on their faces, the plane is parallel to the slide on the microscope stage. \( l \) is measured in \( \mu \).

\( \dot{l} \) = \( \frac{dl}{dt} \) rate of linear growth of crystals. The time is counted in minutes.

\( P(l,t) \) = cumulative distribution of crystal sizes at the moment \( t \), i.e. the number per ml of suspension of crystals which are greater than \( l \) at the moment \( t \).

\( p(l,t) = - \frac{\delta P(l,t)}{\delta l} \), i.e. distribution of crystal sizes at the moment \( t \).

\( N(t) = \int_{0}^{L_{\text{max}}} p(l,t) dl \) is the number of crystals per ml of suspension.

\( L(t) = \int_{0}^{l_{\text{max}}} tl p(l,t) dl \) is the total length of crystals per ml of suspension

\( S(t) = \int_{0}^{l_{\text{max}}} l^2 p(l,t) dl \) is the total surface of crystals per ml of suspension, the unit area being the area of a rhombohedron with size \( l = 1\mu \).

\[ V(t) = \int_0^{t_{\text{max}}} p(l, t)dl \] is the total volume of crystals per ml of suspension, the unit volume being that of the rhombohedron with size \( l = 1\mu \).

\[ \tau = \frac{S}{V}. \]

\( \lambda = \) distance in \( \mu \) between parallel crystal faces.

\( \alpha = \) obtuse angle of the rhombohedron \( (\alpha = 114^{\circ}22') \).

\( R_d = \dot{\lambda} = \) the linear rate of deposition of insulin on the faces of the growing crystal.

\( c_* = \) the concentration of dissolved insulin in per cent of the initial concentration which is 1 % or 1.43 mg insulin nitrogen per ml.

\( C = \) the crystallized fraction in per cent of the initial insulin concentration \( (C = 100 - c_*) \).

\( c_1 = \) insulin concentration in millimoles per l. An amount of insulin containing 65 atoms of nitrogen is considered equivalent to one molecule \( (M = 5778) \).

**METHOD**

**Crystallization.** The method of crystallization has been described recently. The crystallization mixture contains: 1 % insulin, 0.04 % Zn++, 0.05 M sodium citrate, 15 % (v/v) acetone, pH = 6.4. Two solutions are made, one of them contains insulin, the other one is a buffer solution.

(A) 10 g recrystallized insulin from pig pancreas is dissolved in 500 ml water containing 9 ml 1 N HCl. The solution is sterilized by filtering through an asbestos filter previously rinsed with diluted HCl. 125 ml acetone and water are added to the filtrated solution to make a total of 875 ml.

(B) 0.05 mole sodium citrate, 0.80 g ZnCl₂, 25 ml acetone + water to make 125 ml. When (A) and (B) are mixed in the proportion 7:1, pH = 6.4. It may be necessary to add NaOH or HCl to solution (B) in order to produce this pH.

The solutions (A) and (B) are mixed at the time \( t = 0 \) in a glass beaker. The surface of the liquid is covered with a layer of paraffin oil to prevent evaporation and the beaker is covered with a glass lid. The mixture is stirred slowly for 24 h at 21° -- 22°C.

**Determination of the insulin concentration, \( c_* \).** At intervals, 20—50 ml samples are filtered on sintered glass. The moments, \( t_1 \), at the beginning of filtration and those at the end, \( t_2 \), are noted. The averages of \( t_1 \) and \( t_2 \) are used as the \( t \)-estimates in the calculations. The concentrations of nitrogen in the filtrates are determined by semi-micro Kjeldahl analyses. Determinations of total nitrogen are also made to check that evaporation is insignificant.

**Determination of the cumulative distribution of crystal sizes, \( P_*(l) \).** \( P_*(l) \), the number per ml of suspension of crystals greater than \( l \), is determined when crystallization is finished, using the technique described earlier.
THEORY

The linear growth rate, \( \dot{i} \). It follows from the definition of \( S \) and \( V \) (see list of symbols) that

\[
dV = 3Sdl
\]  
(3)

and

\[
\dot{V} = 3Sl.
\]  
(4)

Assuming a constant ratio, \( r \), between surface and volume, it follows that

\[
(lnV) = 3r\dot{i}
\]  
(5)

and, as \( V \) and \( C \) are proportional,

\[
i = \frac{(lnC)}{3r}
\]  
(6)

from which \( \dot{i} \) can be calculated by the \( t, C \) data when \( r \) is known. The ratio \( r \) is calculated from eqn. (1)

\[
r = -\frac{dlnP_s(l)}{3dl}
\]  
(7)

Hence

\[
i = -\frac{(log C)}{dlogP_s(l)/dl}
\]  
(8)

The linear rate, \( R_d \), of insulin deposition on the crystal faces. During crystallization insulin is deposited on the plane crystal faces which thereby move outwards in directions perpendicular to the faces. The relation between the linear rate of deposition, \( R_d \), and the linear growth rate, \( \dot{i} \), is found by geometrical calculus. The distance between the parallel faces of the obtuse rhombohedron is called \( \lambda \). It can be demonstrated that

\[
\lambda = \sin\left(\frac{\alpha}{2}\right) \cdot \sqrt{1 + 2\cos\alpha \cdot l}
\]  
(9)

where \( \alpha \) is the obtuse angle of the rhomb and \( l \) is the crystal size, i.e. the greatest diagonal in the crystal projection on a plane through a face. The angle, \( \alpha \), has been estimated by goniometric measurement \(^1\) as 114°22'. Thus,

\[
\dot{\lambda} = 0.351 \dot{i}
\]  
(10)

It has been shown by experiment that the insulin is not deposited on the three crystal faces meeting in one of the obtuse vertices, but that it is deposited on the three remaining faces, with identical rates of deposition \(^3\). Hence,

\[
R_d = 0.351 \dot{i}
\]  
(11)

ANALYSIS OF THE DATA

The ratio between surface and volume \((r = S/V)\). In the crystallization of beef insulin from a saline suspension of amorphous insulin, perfectly shaped rhombohedral single crystals were developed. However, in the pig insulin crystallization from clear solution, crystal agglomeration occurs to such an extent as to weaken the significance of the notions. The crystals are counted, irrespective of whether they are single or attached to other crystals, and as the shape of attached crystals varies, it follows that the relations between size, surface and volume of a crystal also vary. Thus, the equations set up on the assumption of geometrical similarity do not strictly hold true in this crystallization. It is nevertheless assumed that the deviations from similarity do not significantly alter the significance of the relations leading to eqn. (1). It is assumed that

\[
S/V = r = - \frac{\ln P_s(l)}{3d l}
\]  

(12)

in the main part of crystallization, i.e. from the moment when a few per cent of insulin have crystallized onwards. This assumption is based upon the similarity between the observed distributions of sizes in this experiment and in the former experiment on cattle insulin crystallization, but it may be incorrect.

The \(P_s(l)\) estimates from three countings are shown in Fig. 1.

The slope, \(-3rM = -0.029\) was calculated by using the method of least squares on the section \(l < 75 \mu\). Thus, \(r = 0.022\) which is identical with the ratio found earlier in beef-insulin crystallizations \(^2\).

---

**Fig. 1.** The cumulative distribution of crystal sizes.

**Fig. 2.** \((\log C)\) as a function of \(c_s\).

Fig. 3. The third root of \((\log C)\) as a function of \(c_s\).

The growth rate, \(\dot{t} = (\ln C)/3t\). The \(t\) and \(C\) estimates are listed in Table 1. Estimates of \((\dot{\log} C)\) are obtained by the approximation

\[
(\log C_i) \approx \frac{\log C_{i+1} - \log C_{i-1}}{t_{i+1} - t_{i-1}} \tag{13}
\]

where \(i\) is the sample number. The estimates are plotted in Fig. 2 against the concentration of insulin, \(c_s\).

It appears that \(\dot{t}\) is a power function of \(c_s\). The curve drawn into the figure is

\[
\frac{\Delta \log C}{\Delta t} = 1.206 \times 10^{-7} (c_s - 4.2)^3 \tag{14}
\]

The constants in this equation were determined by trial and error using the chronometric integral below and aiming at the best agreement between observed and calculated \(C\)-values.

The third root of \(\Delta \log C/\Delta t\) is plotted in Fig. 3 against the third root of \(1.206 \times 10^{-7} (c_s - 4.2)^3\).

Thus, using eqns. (8) and (14)

\[
\dot{t} = \frac{1.206}{0.029} \times 10^{-7} (c_s - 4.2)^3 = 4.2 \times 10^{-6} (c_s - 4.2)^3 \tag{15}
\]

The chronometric integral, \(t(C)\), is calculated from eqn. (14) which (since \(C + c_s = 100\)) is written as follows:

\[
dt = \frac{10^7 \cdot M \cdot dC}{1.206 \cdot C(95.8 - C)^3} \quad (M = 0.4343) \tag{16}
\]

Fig. 4. The chronometric integral as calculated for each of the observed $C$-values.

It follows by integration that

$$t(C) - t(C_1) = 3.60 \times 10^6 \int_{C_1}^{C} \frac{dC}{C \cdot (95.8 - C)^3}$$

(17)

Estimates of $t(C) - t(C_1)$ have been calculated for $C_1 = 0.7$ using the formula

$$\int \frac{dx}{x(a-x)^3} = \frac{(3a-2x)}{2a^2(a-x)^2} + \frac{a^{-3} \cdot \ln[x/(a-x)]}{a^3}$$

(18)

By subtracting these estimates from the experimental $t(C)$ values, $t(C_1)$ was found to be 27.0 min (in the average). Thus,

$$t(C) = 3.60 \times 10^6 \int_{0.7}^{C} \frac{dC}{C \cdot (95.8 - C)^3} + 27.0$$

(19)

The $t$-values calculated from eqn. (19) are tabulated (Table 1) and plotted (Fig. 4) against the experimental $t$-values. The discrepancies are within the range of the experimental error.

The analytical estimates, $C(t)$, of insulin which has crystallized at the time $t$ are plotted against $t$ in Fig. 5. The curve in the figure represents eqn. (19), the chronometric integral. The difference between the observations and the curve is insignificant. Hence, it is possible to account for the course of crystallization by a simple mathematical expression.

Fig. 5. The percentage of crystalline insulin, \( C \), as observed — the dots — and as calculated from eqn. (19) — the curve.

**THE RATE OF DEPOSITION ON THE CRYSTAL FACES**

*Saline.* When beef insulin was recrystallized from a saline medium it was found\(^2\) that

\[
\dot{l} = 4.64 \ (c_i - 0.080)^2 \ \mu/\text{min}
\]

(20)

where \(c_i\) is the concentration of insulin in millimoles per l. An amount of insulin containing 65 atoms of nitrogen is considered equivalent to one molecule, \(M = 5734\). It follows from eqns. (11) and (20) that

\[
R_a = 1.63 \ (c_i - 0.080)^2 \ \mu/\text{min}
\]

(21)

**Table 1.** The experimental data.

<table>
<thead>
<tr>
<th>(i)</th>
<th>(t_1)</th>
<th>(t_2)</th>
<th>(t)</th>
<th>(C)</th>
<th>(10^3\Delta \log C/\Delta t)</th>
<th>(t(C))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1'30&quot;</td>
<td>2'30&quot;</td>
<td>1.8</td>
<td>0.0</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29'45&quot;</td>
<td>30'00&quot;</td>
<td>29.9</td>
<td>0.7</td>
<td>35.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>34'45&quot;</td>
<td>35'15&quot;</td>
<td>35.0</td>
<td>4.9</td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39'35&quot;</td>
<td>40'10&quot;</td>
<td>39.9</td>
<td>11.2</td>
<td>6.73</td>
<td>45.2</td>
</tr>
<tr>
<td>5</td>
<td>44'45&quot;</td>
<td>45'15&quot;</td>
<td>45.0</td>
<td>23.1</td>
<td>4.81</td>
<td>45.2</td>
</tr>
<tr>
<td>6</td>
<td>49'50&quot;</td>
<td>49'55&quot;</td>
<td>49.9</td>
<td>33.9</td>
<td>2.75</td>
<td>49.7</td>
</tr>
<tr>
<td>7</td>
<td>54'55&quot;</td>
<td>55'00&quot;</td>
<td>55.0</td>
<td>43.5</td>
<td>1.83</td>
<td>54.6</td>
</tr>
<tr>
<td>8</td>
<td>59'45&quot;</td>
<td>60'05&quot;</td>
<td>59.9</td>
<td>51.7</td>
<td>0.98</td>
<td>60.2</td>
</tr>
<tr>
<td>9</td>
<td>70'00&quot;</td>
<td>70'15&quot;</td>
<td>70.1</td>
<td>61.1</td>
<td>0.45</td>
<td>70.2</td>
</tr>
<tr>
<td>10</td>
<td>89'30&quot;</td>
<td>90'05&quot;</td>
<td>89.8</td>
<td>70.6</td>
<td>0.20</td>
<td>90.3</td>
</tr>
<tr>
<td>11</td>
<td>119'45&quot;</td>
<td>120'20&quot;</td>
<td>120.1</td>
<td>77.1</td>
<td>0.078</td>
<td>121.5</td>
</tr>
<tr>
<td>12</td>
<td>170'35&quot;</td>
<td>171'00&quot;</td>
<td>170.8</td>
<td>81.7</td>
<td>0.034</td>
<td>170.6</td>
</tr>
<tr>
<td>13</td>
<td>259'45&quot;</td>
<td>240'15&quot;</td>
<td>240.0</td>
<td>84.7</td>
<td>0.014</td>
<td>237</td>
</tr>
<tr>
<td>14</td>
<td>399'50&quot;</td>
<td>400'20&quot;</td>
<td>400.1</td>
<td>87.8</td>
<td>0.003</td>
<td>394</td>
</tr>
<tr>
<td>15</td>
<td>1400&quot;</td>
<td>1400&quot;</td>
<td>1400</td>
<td>91.7</td>
<td>1268</td>
<td></td>
</tr>
</tbody>
</table>

The maximum $R_d = 0.7 \mu/min$ occurred at $c_1 = 0.73$.

*Citrate buffer.* Provided that eqn. (12) is valid in this crystallization just as in the former, it follows from eqn. (15) that

$$i = 1.1 (c_1-0.066)^3 \mu/min$$

from which

$$R_d = 0.38 (c_1-0.066)^3 \mu/min$$

The maximum $R_d = 1.3 \mu/min$ occurred at $c_a = 100 \%, i.e. at c_1 = 1.57$ mmole/l.

*Acknowledgements.* My thanks are due to Dr. Hallas-Møller for his critical advice which has been of considerable benefit to the work. In the mathematical part of the study I have received encouragement, advice and technical assistance from Dr. A. Hald, Professor of Statistics at the University of Copenhagen. It is with pleasure I express my sincere gratitude for this kind help.

I also want to thank K. Petersen, with whom I have had many discussions, my colleagues I. Nørgaard and F. Sundby who contributed to the experimental work.

REFERENCES


Received April 15, 1957.