Insulin Crystals

III. Determination of the Rhombohedral Zinc-Insulin Unit-Cell by Combined Microscopical and Chemical Examinations

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Cattle insulin is recrystallized by a method yielding perfectly shaped rhombohedral zinc-insulin crystals. During crystallization, samples are taken in which the process of crystallization is arrested by adding zinc ions.

The crystalline fractions in the samples are separated from non-crystalline insulin and determined quantitatively.

The volumes of insulin crystals in the stabilized suspensions as well as the size distributions are determined by a special microscopical counting procedure. Ordinary microscope equipment is used.

From the ratio between the volume of crystals and their content of nitrogen, the dimensions of the unit-cell are calculated to be $a = 50.7 \pm 0.7 \text{ \AA}$ ($P = 0.95$). The volume of crystals containing 1 g of protein (water and salt-free) is calculated to be 1.35 ml. The possible causes of the significant difference between the estimate $a = 50.7 \text{ \AA}$ and the X-ray estimate $a = 49.4 \text{ \AA}$ are discussed.

Crowfoot and Riley¹ in 1939, by means of X-ray analyses, measured the dimensions of the unit-cell of wet zinc-insulin crystals lying in their mother-liquid. The length of the edge of the unit-cell rhombohedron is called a and the obtuse angle of the rhomb is α . They found that $a = 49.4 \text{ \AA}$ (with 0.5 % accuracy) and $\alpha = 114^\circ 16'$.

On a 1.3 mm large perfect zinc-insulin crystal the angle, α , has already been estimated by goniometric measurement² as $\alpha = 114^\circ 22'$. The purpose of the present investigation is to determine the dimension a by a combined chemical and microscopical examination.

THE PRINCIPLE OF INVESTIGATION

It is assumed that the unit-cell contains 6 of the smallest molecular units, the structure of which was established by Sanger and coworkers³. Thus, the insulin content of the unit-cell is $M = 34\,400$ and the nitrogen content is $6 \times 65 = 390$ atoms.

The number of unit-cells, n , present in the rhombohedral crystal is proportional to the volume and hence to the cubes of the edges, l_a (Å), *i. e.*

$$l_a^3 = n \cdot a^3.$$

Thus the nitrogen content, m_N , of a crystal is

$$m_N = \frac{n}{N} \times 390 \times 14.008 \text{ g, where } N = 6.02 \times 10^{23}.$$

Hence,

$$a = 2.086 \times 10^{-7} \left(\frac{l_a^3}{m_N} \right)^{\frac{1}{3}} \text{ Å}.$$

When a drop of an insulin crystal suspension is placed on a slide on the microscope stage, the crystals settle down on their faces. Therefore the greatest diagonal, l , in the crystal projection on the horizontal plane is used as a convenient measure of the crystal size. It follows that

$$l = l_a \times \sec \frac{\alpha}{2}$$

and

$$a = 1.130 \times 10^{-7} \left(\frac{l^3}{m_N} \right)^{\frac{1}{3}} \text{ Å}$$

Consider a suspension of insulin crystals containing c_N g/ml nitrogen belonging to the crystals. The cumulative distribution of crystal sizes, $P(l)$, *i. e.* the number of crystals per ml greater than l , is determined in a counting chamber using a calibrated eye-piece scale for measuring l . Using the volume of a rhombohedron with $l = 1$ Å as volume unit for the crystals, the volume, V , of crystals per ml of suspension is

$$V = \int_0^{l_{\max}} p(l) l^3 \, dl$$

where

$$p(l) = - \frac{dP(l)}{dl}$$

thence,

$$a = 1.130 \times 10^{-7} \left(\frac{V}{c_N} \right)^{\frac{1}{3}} \text{ Å} \quad (1)$$

When V and c_N are known, a can be calculated from eqn. (1). In the following the methods used for the determination of V and c_N are described.

METHODS

The perfect rhombohedral shape which is essential for the investigation is ensured by adding halogen ions to the medium of crystallization². In order to get several estimates of a , samples are taken at different times during the crystallization. The crystallization process in the samples is immediately arrested

by diluting with a zinc salt solution. The crystals in the stabilized suspension are measured and counted, and the amount of nitrogen in the crystals is determined by Kjeldahl analyses on the isolated crystals.

Determination of V

About 0.1 ml of the stabilized suspension is drawn up into a narrow pipette and let out into the chamber (Fuchs-Rosenthal) which is 0.200 mm deep. (Verified by check-up). The 2 great squares (4×4 mm each) are both divided into 256 small squares each corresponding to c ml of the crystallization mixture, $c = 1.25 \times 10^{-6}$ ml. The crystals are measured and classified. Class limits $l_1 = 0, l_2, l_3, \text{etc.}$ are devised, $l_i - l_{i-1} = 3-6 \mu$. The inspection may begin in one corner and proceed as when reading a page. In A_i small squares, n_i crystals are found to be greater than l_i . A_i and n_i are counted simultaneously. The areas inspected within the large squares are not chosen at random as this process is very time consuming. As no significant differences have been observed when changing the system of inspection, it is believed that systematic errors are unimportant. About 30 crystals are counted per class limit, but not more than one large square is generally inspected per class. When the area is insufficient, the chamber is refilled. Two determinations of V are made for each suspension.

The number of crystals per square unit, n_i' , belonging to the class $l_i < l < l_{i+1}$ is

$$n_i' = \frac{n_i}{A_i} - \frac{n_{i+1}}{A_{i+1}}$$

The volume V_i of these crystals is calculated from the assumption that $p(l_i)$ is constant in the intervals.

It follows that

$$V_i = \int_{l_i}^{l_{i+1}} p(l) l^3 dl = p(l_i) (l_{i+1} - l_i) \frac{l_{i+1}^4 - l_i^4}{4(l_{i+1} - l_i)} \quad (2)$$

Since

$$p(l_i) (l_{i+1} - l_i) = n_i'/c$$

then, estimates of V_i may be obtained from

$$V_i = f_i n_i'; f_i = \frac{m_i^3 + m_i d_i^2}{8c} \quad (3)$$

where

$$m_i = l_{i+1} + l_i; d_i = l_{i+1} - l_i.$$

The distribution of particles according to weight may be obtained from eqn. (3). The volume per ml of all the crystals is then obtained by summation of V_i ,

$$V = \sum_{i=1}^{i=i_{\max}} V_i$$

The limit $i_{\max}$ is the smallest limit above which no crystals are found.

Error: As $p(l)$ is not strictly constant within the classes, a systematical error is introduced by eqn. (2). The error is estimated from an examination of the size distributions. It can be shown that

$$P(l) = c_1 \cdot \exp. (-c_2 l); c_1, c_2 > 0; l_1 < l < l_{1+1}$$

or

$$p(l) = c_1 \cdot c_2 \cdot \exp. (-c_2 l).$$

This relation is observed when the logarithms of the numbers in column (4) are plotted against the class limits (1) in Table 1. Deviations which are insignificant in this connection occur in the outer regions of the distributions ⁵.

Hence, the actual volume of crystals within the size interval (l_1, l_{1+1}) is

$$V_1 = c_1 \cdot c_2 \int_{l_1}^{l_{1+1}} l^3 \exp. (-c_2 l) dl = c_1 [F(l_1) - F(l_{1+1})] \quad (4)$$

where

$$F(l) = (l^3 + 3l^2 c_2^{-1} + 6l c_2^{-2} + 6c_2^{-3}) \exp. (-c_2 l)$$

Table 1. Determination of crystal volume (V) by the counting procedure.

i:	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	0.0	53	2	27	12	0.003	0.0	0.0
2	2.5	30	2	15	0	0.046	0.0	0.0
3	5.0	30	2	15	2	0.203	0.4	0.0
4	7.5	26	2	13	3	0.547	1.6	2.2
5	10.0	31	3	10	0	1.15	0.0	5.6
6	12.5	29	3	10	2.5	2.10	5.3	18.6
7	15.0	30	4	7.5	4.1	3.45	14.1	30.6
8	17.5	37	11	3.4	0.7	5.30	3.7	12.2
9	20.0	32	12	2.7	0.4	7.70	3.1	19.5
10	22.5	27	12	2.3	1.41	10.7	15.1	32.7
11	25.0	25	28	0.89	0.22	14.4	3.2	10.3
12	27.4	32	48	0.67	0.38	20.3	7.7	8.6
13	31.3	28	96	0.29	0.12	29.5	3.5	3.9
14	35.3	27	160	0.17	0.10	41.4	4.1	2.7
15	39.2	27	384	0.070	0.035	57	2.0	0.8
16	43.6	9	256	0.035	0.023	81	1.9	1.4
17	49.4	3	256	0.012	0.012	116	1.4	0.9
18	55.6	0	256	0.000	0.000	162	0.0	0.0
						Total: 67	150	

(1) $10^{-4} \cdot l_i$ (l_i in Å)

(2) n_i (number of crystals greater than l_i)

(3) A_i (number of squares in which n_i are counted)

(4) n_i/A_i

(5) $n_i' = n_i/A_i - n_{i+1}/A_{i+1}$

(6) $10^{-21} \times f_i$; $f_i = (m_i^2 + m_i d_i^2) / 8c$; $c = 1.25 \times 10^{-6}$ ml

(7) $10^{-21} \times f_i n_i'$

(8) $10^{-22} \times n_i (f_i^2 + f_{i-1}^2) / A_i^2$

$$V = 67 \times 10^{21}; \sigma_V^2 = 150 \times 10^{22}$$

The volume calculated from eqn. (3) is

$$V_i = \frac{m_i^3 + m_i d_i^2}{8c} [P(l_i) - P(l_{i+1})] \cdot c$$

or

$$V_i = \frac{m_i^3 + m_i d_i^2}{8} \cdot c_i [\exp. (-c_2 l_i) - \exp. (-c_2 l_{i+1})]$$

Hence, the ratio between this volume and that from eqn. (4) is

$$r = \frac{[\exp. (-c_2 l_i) - \exp. (-c_2 l_{i+1})] (m_i^3 + m_i d_i^2)}{8 [F(l_i) - F(l_{i+1})]} \quad (5)$$

It is found that $r = 1.00$ — 1.02 so that this error can be neglected.

The variance of V is the sum of the variances of V_i .

$$\sigma_V^2 = \sum_{i=1}^{i=i_{\max}} \sigma_{V_i}^2$$

where

$$\sigma_{V_i}^2 = f_i^2 \sigma_{n_i}^2$$

The error of A_i is considered negligible, then

$$\sigma_{n_i}^2 \approx \frac{\sigma_{n_i}^2}{A_i^2} + \frac{\sigma_{n_{i+1}}^2}{A_{i+1}^2}$$

If it is further assumed that the crystals are classified without error and that they are distributed in the chamber according to the Poisson distribution, it follows that

$$\sigma_{n_i}^2 \approx n_i$$

and

$$\sigma_V^2 \approx \sum_{i=1}^{i=i_{\max}} n_i \cdot \frac{f_{i-1}^2 + f_i^2}{A_i^2}, \quad (f_0 = 0) \quad (6)$$

In order to keep σ_V^2 within reasonable limits, the terms in the last equation must not be allowed to increase too much with l_i . Aiming at constant terms it follows from eqn. (6) that the area of inspection shall be

$$A_i = \text{const.} \cdot P(l_i) \cdot l_i^6$$

The specific feature of this counting method is that both the particles and the squares are counted; the latter increase with the particle size.

Determination of c_N

The isolation of insulin crystals from the stabilized suspensions is achieved by extracting the amorphous particles.

Solvent:

8.15 g CH_3COONa , $3\text{H}_2\text{O}$

42.0 g NaCl

150 ml acetone

+ water to make 500 ml.

15 ml of the stabilized suspension are centrifuged and the supernatant liquid is discarded. The precipitate is resuspended in 5 ml of distilled water, 10 ml of the solvent are added and mixed with the suspension. The suspension is centrifuged and the supernatant liquid containing the dissolved amorphous fraction is discarded. The crystals are suspended in 10 ml of 60 % (v/v) acetone, centrifuged and the acetone is discarded. The nitrogen content of the remaining crystals is determined by semi-micro Kjeldahl analysis. The result is expressed in g of nitrogen per ml of the original crystallization medium. The test has been included in the British Pharmacopoeia ⁶.

Error: Mixed suspensions containing amorphous and crystalline insulin in different proportions have been analyzed. The results of analyses were in agreement with the values of c_N predicted from analyses of the original suspensions used for the mixing. A small systematical error is caused by the fact that a part of the amorphous particles is denatured and heavily soluble, which causes overestimation of the crystal fraction. This error which amounts to 2—3 % of the total insulin-nitrogen, is corrected by making analyses of the crystallization mixture before crystals are formed.

The standard deviation of a single determination has been estimated from double analyses as 1.0×10^{-5} g N/ml.

EXPERIMENTAL

Composition of crystallization mixture: 2.42 mg/ml insulin-nitrogen (equiv. to 1.7 % insulin)

155 $\mu\text{g/ml}$ Zn^{++} , as chloride (including the Zn^{++} from the insulin)

7 % NaCl

0.1 M CH_3COONa

+ HCl to pH = 5.45—5.55.

Solutions: (I). 68 g recrystallized insulin from beef pancreas, containing 9.68 g insulin-nitrogen were dissolved in water containing 80 ml 1 N HCl and so much ZnCl_2 that the final solution contained a total of 0.62 g Zn^{++} . The solution was sterilized by filtering through an asbestos filter previously rinsed with diluted HCl . The filtered solution was diluted with water to 3 000 ml.

(II). 54.4 g CH_3COONa , $3\text{H}_2\text{O}$ + 280 g NaCl + 44 ml 1 N NaOH + water to make 1 000 ml.

The crystallization: The solutions (I) and (II) were poured simultaneously into an 8-litre glass beaker placed in a water thermostat at 25° C. The beaker was covered with a glass lid. Slow, continuous stirring was provided with a glass propeller through a hole in the lid.

Initially about 95 % of the insulin was precipitated as amorphous particles. During the first 30 min between one fourth and one third of the amorphous insulin was dissolved but only a few crystals were formed. After a further 30 min the crystallization became

rapid, but soon slowed down again and was practically finished within 12 h. At that time approximately 92 % of the insulin was crystalline, 3 % was still in solution and the rest was in the amorphous state.

The stabilized samples: The process of crystallization was arrested by combining the insulin with zinc ions at neutral reaction ⁴. A 10 ml sample was added to 90 ml of a neutral solution of zinc chloride containing 7.2 mg Zn^{++} , 100 mg methyl-*p*-hydroxybenzoate and 2.75 ml 0.1 N NaOH. The total volume was adjusted to 100 ml. The insulin thereby immediately combined with some of the zinc ions. The crystals and the amorphous particles were not visibly altered, whereas the dissolved insulin from the samples was precipitated as stable amorphous particles.

RESULTS

Two crystallizations were made and analyzed with an interval of 19 months. Fourteen samples were taken during the first crystallization and seventeen in the second experiment. Each sample was subjected to counts twice. The results of the 62 countings are too numerous to be compiled in a table, but for the purpose of illustration the result from one of them is presented in Table 1. The volume V is estimated from this counting to be 67×10^{21} . The systematical error is calculated by means of eqn. (5) using $c_2 = 0.18$. When $l_i = 50$ and $l_{i+1} = 55$, it is found that $r = 1.021$ and likewise for $l_i = 22$, $l_{i+1} = 25$, $r = 1.017$. This error is considered insignificant. The standard deviation of the V estimate is calculated by means of eqn. (6) and found to be 12×10^{21} .

Table 2 contains all the condensed data. The two rectangular parts correspond to the two crystallizations and each row represents a sample. As crystallization proceeds, both V and c_N increase, while a remains constant except for casual variations. The systematical error of V has been calculated for the samples in Table 2. In the first crystallization it is found to be 2 % or less and in the second where $c_2 = 0.07$ it is less than 1 %. Hence, this error is neglected. The standard deviation of the V estimate from a counting has been calculated from eqn. (6) to be 12×10^{21} (Table 1). As two countings are made on each of the samples it is also possible to calculate s_V from the double determinations. However, as V increases, s_V increases also as seen from the ranges in column (3), but in spite of this a pooled estimate of the standard deviation has been calculated in the usual way. It is found that $s_V = 13 \times 10^{21}$, with 14 and 17 d.f. from each of the two experiments, in close agreement with the result, 12×10^{21} , from Table 1.

The standard deviation of a is calculated from the variation of a between the 14 resp. 17 samples taken during each of the two crystallizations.

The estimates of a from I and II are pooled as their difference is insignificant. $a = 50.7 \text{ \AA}$ with confidence limits $\pm 0.7 \text{ \AA}$ ($P = 0.95$). The volume of the unit-cell is calculated from the values $a = 50.7 \text{ \AA}$ and $\alpha = 114^\circ 22'$ using the formula

$$\text{Volume} = 2 \cdot \sin^2\left(\frac{\alpha}{2}\right) \cdot \sqrt{1 + 2\cos\alpha} \cdot a^3$$

The volume is $0.591 \times 50.7^3 = 7.70 \times 10^4 \text{ \AA}^3$. The weight of insulin within the unit-cell is $6 \times 5734 / 6,02 \times 10^{23} = 5.71 \times 10^{-20} \text{ g}$. Hence the volume of crystals containing 1 g of insulin is 1.35 ml.

Table 2. Results of analyses of two crystallizations.

I. (March 1954)						II. (November 1955)					
(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)	(3)	(4)	(5)	(6)
27	35	8	31	37	49.5	21	16	5	10	23	49.3
67	66	1	66	70	51.4	33	34	1	34	35	52.0
78	78	0	78	85	51.0	25	45	10	35	49	46.9
95	96	1	96	97	52.3	48	45	3	47	64	47.3
111	86	25	99	114	50.1	83	74	9	79	81	52.0
152	145	7	149	149	52.4	71	74	3	73	97	47.7
156	141	15	148	175	49.6	101	86	5	94	108	50.1
167	227	60	197	189	53.2	99	118	19	109	121	50.6
187	149	38	168	197	49.7	104	115	11	110	142	48.1
204	189	15	197	202	52.0	179	130	49	155	147	53.3
188	215	27	202	204	52.3	165	162	3	164	165	52.3
184	178	6	181	209	49.9	164	178	13	171	172	52.3
197	183	14	190	214	50.4	179	190	11	185	190	52.0
167	151	16	159	216	47.3	179	175	4	177	220	48.8
						239	239	0	239	221	53.8
						221	208	13	215	227	51.5
						242	203	39	223	226	52.2

(1) $10^{-21} \times V$ from first counting;(2) $10^{-21} \times V$ from a second counting;

(3) The range between (1) and (2);

(4) The average of (1) and (2);

(5) $10^6 \times c_N$;(6) $a = 1.130 \times 10^{-7} \left(\frac{V}{c_N} \right)^{\frac{1}{3}}$ Å.I. $s_V = 13 \times 10^{21}$ with 14 d.f. $a = 50.8 \pm 0.4$ Å (S.E.)II. $s_V = 13 \times 10^{21}$ with 17 d.f. $a = 50.6 \pm 0.5$ Å (S.E.) $a_{I,II} = 50.7 \pm 0.7$ Å (confidence limits, $P = 0.95$)

DISCUSSION

The estimate $a = 50.7$ Å differs significantly from the X-ray value $a = 49.4$ Å and it may be due to an overestimate of V by 8 %. In that case a systematic error is present in the counting method, but this possibility cannot be verified from the present data. However, the crystals examined by X-ray were lying in their mother-liquid of crystallization while in the present investigation the crystals were actually insulin-zinc crystals containing approximately 2.3 % zinc calculated on dry weight⁴. It appears possible that the stabilization with zinc causes the lattice to expand, just as drying causes it to shrink. Thus experimental conditions may be responsible for the difference observed in a .

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