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Compounds Formed by Air-dried Insulin Crystals on Uptake of Metal Salts from Ethanolic Solutions

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X-Ray powder diffractometer data from air-dried insulin yield good lattice constants and a surmise of degree of crystallinity. Although the uptake by air-dried insulin of zinc ion and zinc chloride from an ethanolic solution seems to follow an adsorption isotherm, chemical compounds are formed, since lattice constants and reflection intensities in the products vary systematically with increasing zinc content. From X-ray data the uptake is seen to fall in three distinct stages: I, until 2 zinc atoms per Sanger unit are taken up; II, till 4 zinc atoms are taken up; III, when more zinc is taken up. As shown earlier step I is an exchange of zinc ions for hydrogen ions. In steps II and III zinc chloride is taken up. Lattice constants for a few compounds with other metal salts are given.

The shrinking of insulin crystals on drying is not reversed by uptake of water vapour. Air-dried insulin took up 18 % of water, but the lattice constants remained unchanged.

Marcker [1] recently described some new compounds of insulin with metals (metal ions) and metal salts, formed by treating air-dried insulin crystals with ethanolic solutions of metal salts. An X-ray crystallographic study of the compounds was begun at an early stage of this work. It has given a more detailed picture of the compounds than unaided chemical methods. A few experiments of the influence of air-drying and subsequent moistening of insulin crystals on their lattice constants will also be described.

X-RAY TECHNIQUE

Insulin crystals under mother liquor are well ordered, and give X-ray reflections up to glancing angles corresponding to interplanar spacings to about 1 Å. Air-dried insulin crystals, on the other hand, have lost the finer details of their internal order. X-Ray reflections corresponding to interplanar spacings shorter than 5—7 Å have vanished on drying. Since Marckers com-

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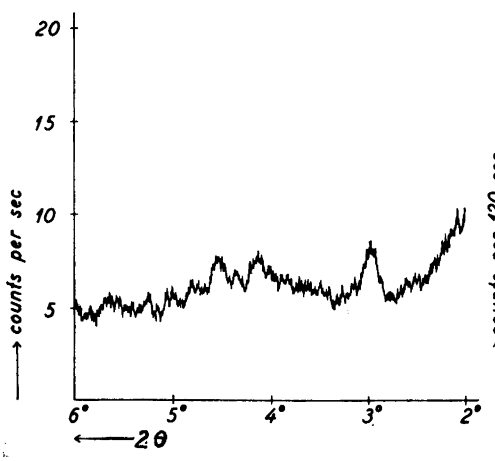

Fig. 1. Powder diagram of air dried insulin crystals. Specimen 1. 6.8 atoms of zinc per Sanger unit. Cu*Kα* radiation, automatic recording. 2Θ from 2 to 6°.

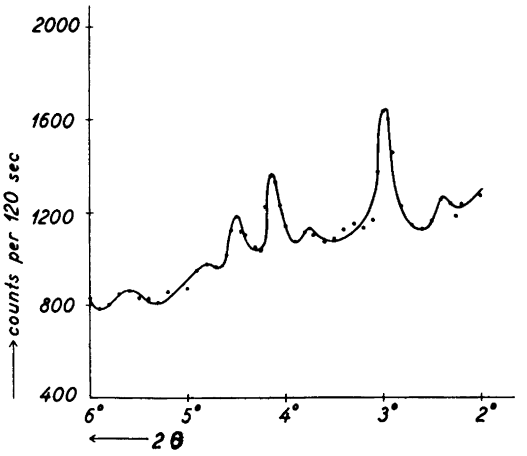

Fig. 2. Powder diagram of air dried insulin crystals. 2Θ from 2° to 6°. Specimen as in Fig. 1. Cu*Kα* radiation. Stationary 120 secs. counting at intervals of 0.05° to 0.10° in 2Θ.

pounds were prepared from air-dried insulin, and because the crystals were small, no attempt was made to apply single crystals methods to them.

The powder method, unpromising at first, was found quite useful. In the early days of protein crystallography, powder methods were used a good deal. However poor the diagrams, they contained lines and gave evidence of internal order, and of the very long interplanar spacings present. But the lines of early powder diagrams of protein crystals were in general »clashings» between several reflections. Only two cases are known to us where the indexing of four or five lines from a protein crystal powder diagram has been claimed: ferritin and apoferritin (Fankuchen [2]).

Fig. 1 shows an automatic recording of the powder diagram of crystalline air-dried insulin from $2\Theta = 2°$ to $2\Theta = 6°$. The diagram was taken with a General Electric X-Ray Diffractometer (XRD-3) fitted with a special linear amplifier, radiation filtered Cu*Kα*. The slit system was very narrow as seen from the low intensity. The diagram shows 3 dubious lines. Fig. 2 shows the improvement obtained when the same diagram is constructed from 120 sec. stationary countings at points 0.05° to 0.10° apart. Seven lines are seen now. The same technique was adopted in all further work, and similar diagrams obtained, except of course for samples whose structure had deteriorated as a result of chemical treatment of the sample. The lines could always be indexed by an adaption of Crowfoots lattice constants for air-dried insulin. An example is given in Table 1, where the results from the diagram, Fig. 2, are given. All lattice constants have been referred to the hexagonal cell whose volume is three times that of the true rhombohedral cell, and contains 18 Sanger units of molecular weight 5 800 plus water *etc.* Since the innermost part of the powder diagram is so easily indexed it is tempting to proceed further out, *inter alia* in

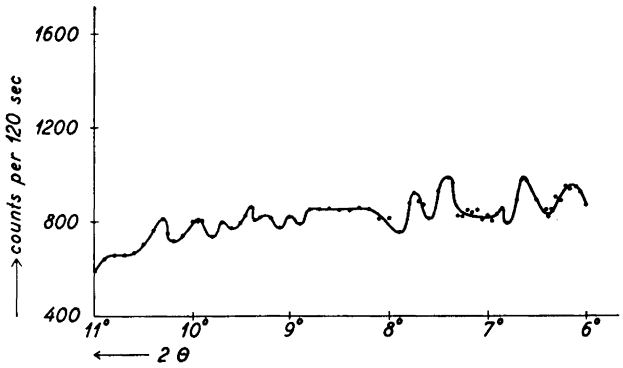

Fig. 3. Powder diagram of air dried insulin crystals. 2Θ from 6° to 11°. Same technique as in Fig. 2.

order to obtain more accurate lattice constants. A glance at Fig. 3 which gives the diagram from $2\,\Theta = 6°$ to $2\,\Theta = 11°$ will show why such attempts at indexing failed. A considerably refined technique or independent knowledge of relative intensities would be necessary, since calculation shows that there are some 30 possible reflections in this part of the diagram.

INSULIN-ZINC AND INSULIN-ZINC-$ZnCl_2$ COMPOUNDS

The following work was done using twice recrystallized pig-insulin *. Table 2 gives lattice constants for 4 chemically untreated samples of insulin crystals, all data obtained as described. The classical data by Crowfoot-Hodgkin [3] and Crowfoot and Riley [4] for dry and wet insulin are also tabulated. In column 4 of Table 2 are given the lattice constants for a specimen of insulin under its mother liquor **. In column 5 is given the lattice constants for the same speci-

Table 1. X-Ray diffractometer data for insulin, 6.8 Zn atoms per Sanger unit (specimen 1). Comparison of observed and calculated $\sin^2\Theta$-values. Cu-$K\alpha$ radiation.

$2\,\Theta$	Indices $h\,k\,i\,l$	$10^4 \times \sin^2\Theta_{obs.}$	$10^2 \times \sin^2\Theta_{calc.}$	Relative intensity obs.
2.36°	$11\bar{2}0$	4.24	4.26	11
2.97°	$10\bar{1}1$	6.72	6.79	100
3.76°	$20\bar{2}1$	10.76	11.05	5
4.13°	$30\bar{3}0$	12.98	12.78	46
4.49°	$21\bar{3}1$	15.34	15.31	23
4.70°	$22\bar{4}0$	16.81	17.04	1
5.60°	$10\bar{1}2$ $13\bar{4}1$	23.86	22.90 23.82	15

Unit cell $a = b = 74.7 \pm 0.4$ Å
$c = 33.3 \pm 0.2$ Å

* kindly placed at our disposal by Roskilde Medical Company, Roskilde.
** a phosphate buffer with pH = 6.7, total phosphate molarity approximately 0.035.

Table 2. Lattice constants for untreated insulin crystals.

No.	1	2	3	4	5	6
Specimen	Crowfoot [3]	R M C	Novo	R M C	No. 4 dried	Crowfoot and Riley [4]
Zinc content	?	0.8 %	2.2 %	0.6 %	0.6 %	?
Humidity	air dried	air dried	air dried	wet	air dried	wet
a, Å	74.8	74.1	75.0	90.4	77.0	83.0
c, Å	30.9	32.8	31.8	32.8	31.7	34.0

men after air drying. The figures in columns 4 and 5 are not as accurate as the other figures in Table 2, but they prove beyond doubt that our wet specimen has been in a higher state of hydration than that of Crowfoot and Riley (column 6). In order to see whether the lattice constants of dry and shrunk insulin crystals varied when the crystals took up humidity from the air — in which case we would have had to work in a controlled atmosphere — we left a specimen overnight in an atmosphere of 95 % relative humidity at room temperature. The weight of the sample increased by 18 %. We registered its powder diagram in the humid state, dried it again and registered the powder diagram a third time. The same glancing angles and reflection intensities were found in the three experiments within experimental error. So the shrinking was not reversed by the uptake of humidity from the air, and controlled atmospheric humidity is unnecessary when working with air-dried crystals. Whether the molecules of the 18 % of water taken up were placed in holes in the insulin crystals proper, or in an amorphous fraction undoubtedly present, is not clear to us.

In Figs. 4 and 5 the lattice constants of one untreated specimen of air-dried insulin crystals and of 7 specimens treated with ethanolic solutions of zinc chloride are plotted against the zinc content of the specimens. For details

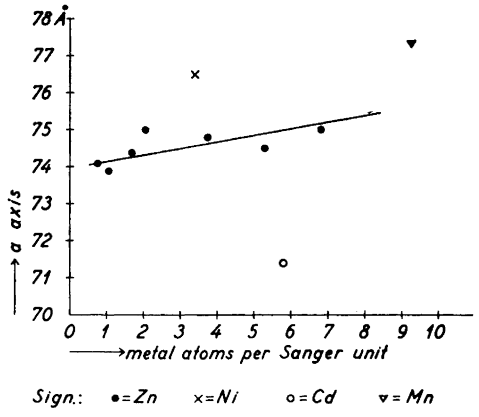

Fig. 4. Variation in *a*-axis length of dry insulin with increasing zinc content.

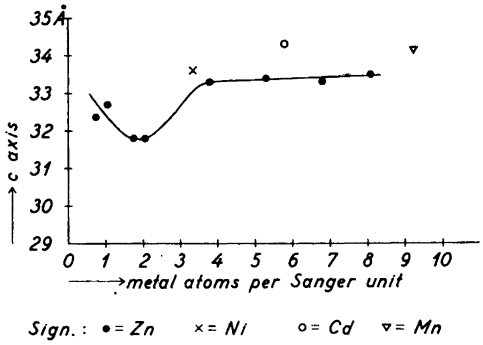

Fig. 5. Variation in *c*-axis length of dry insulin with increasing zinc content.

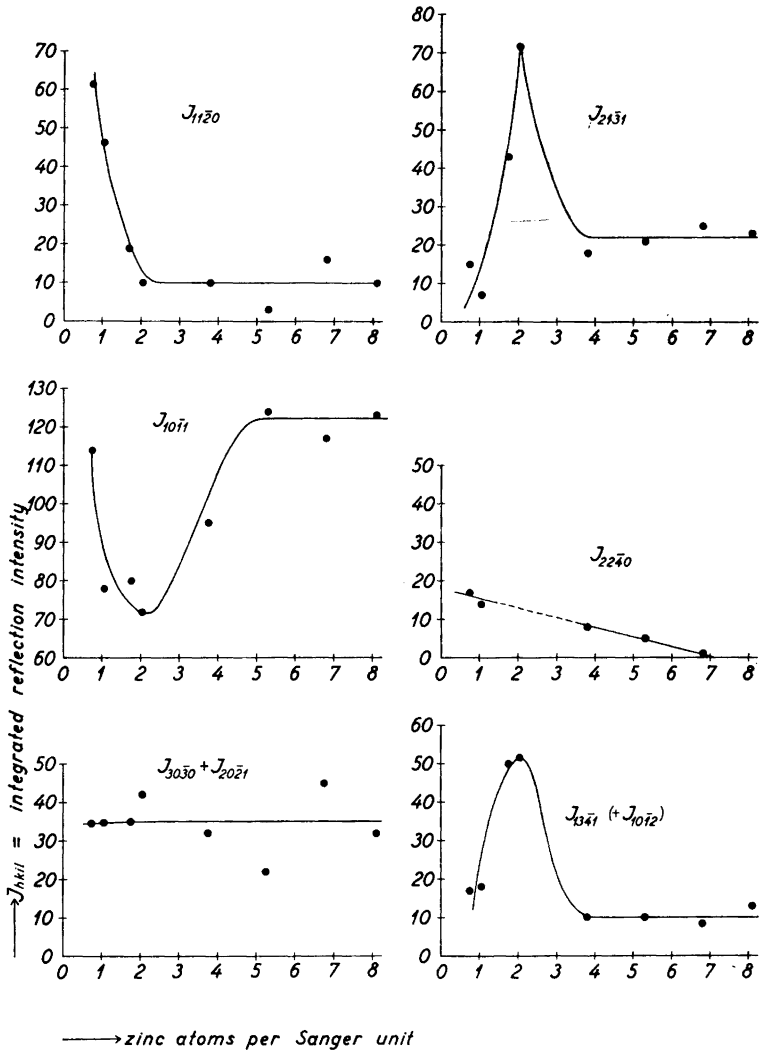

Fig. 6. Variation in integrated reflection intensity with increasing zinc content for the innermost lines in the powder diagram of dry insulin.

of preparation see Ref.[1] When the accuracy of the a-axis values ($\pm$ 0.4 Å) is borne in mind it is difficult to conclude anything from Fig. 4, except that the uptake of zinc brings about a gradual increase in a. On the other hand, it seems legitimate to conclude from Fig. 5 that the c-axis (accuracy $\pm$ 0.2 Å) shrinks when the zinc content increases to 2 atoms per Sanger unit, and expands again as two more atoms are taken up. If the zinc content increases from 4 to the maximal 8 atoms per unit the c-axis remains constant. This indicates

that the reaction is a 3-stage process. Fig. 4 from which nothing definite could be concluded is perhaps not incompatible with a 3-stage process.

Considerably more evidence for a 3-stage process is provided by the variation in reflection intensity with zinc content for 6 lines, as seen from Fig. 6. The intensities are given as integrated intensities measured as peak area over a smoothed background. Four of the curves indicate a break at about 2 zinc atoms per Sanger unit, three curves another break at about 4 atoms of zinc per unit. No significant variation occurs at higher values. The diagram for reflection $30\bar{3}0$ plus $20\bar{2}1$ presents a confused picture. The shape of the combined peaks made a graphical separation impossible. In addition the combined peak area contained in 2 out of 3 diagrams a third peak of an unknown impurity. This peak was the only one found in any diagram which could not be accounted for by the "quadratic form" for insulin. Its area could not be measured and subtracted, and since it varied between zero and a finite value its presence adds to the confusion in reflection intensity.

The break at 2 atoms of zinc was explained later when it was found by chemical analysis that at this point the reaction between insulin crystals and an ethanolic zinc chloride solution changes from an exchange of hydrogen ions in the crystal for zinc ions from the solution to an uptake of zinc and chloride ions [1]. The break at 4 zinc per Sanger unit is so far unexplained chemically. From chemical evidence, especially the remarkable work by Schlichtkrull [5], it seems beyond doubt that the zinc atom per 3 Sanger units which is necessary to obtain insulin in the crystalline state, is placed in a fixed position in the crystal. From our work we think it may be safely concluded that the zinc ions subsequently taken up by ion exchange until two atoms are present, *and* the first two moles of zinc chloride taken up when the ion-exchange capacity is exhausted, are likewise placed in fixed positions. Were they placed at random it would be difficult to understand the gradual but drastic changes in reflection intensity caused by their gradual introduction. So we must conclude that these products are chemical compounds. On the other hand, there seems to be no evidence for a fixed position of the last four molecules of zinc chloride, since they cause no systematic change in reflection intensities.

It is somewhat unexpected that the reaction between insulin crystals and an ethanolic solution of zinc chloride is a three-stage process where first and second stage lead to chemical compounds, since the entire reaction seems to follow an adsorption isotherm; see Fig. 1 in Ref.[1] But there is in fact nothing contradictory in the findings. Stage 3 *is* most probably an adsorption process, stage 2 is the formation of a kind of interstitial compound, and stage 1 an ion exchange process, all of which follow a function similar to an adsorption isotherm. The experiments, the results of which are given in Fig. 1 in Ref. [1] could hardly be expected to distinguish between one adsorption isotherm and three adsorption isotherms pieced together: one from ordinates 0 to 2, another between ordinates 2 and 4 and a third at ordinate values higher than 4.

COMPOUNDS OF ZINC-INSULIN WITH OTHER METAL CHLORIDES

The reaction between dry insulin crystals and ethanolic solutions of $NiCl_2$, $CdCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$ and $CoCl_2$, briefly mentioned in Ref.[1] was

Table 3. Chemical composition and X-ray characterisation of compounds formed when crystalline zinc-insulin was suspended in an ethanolic solution of another heavy metal chloride.

Metal chloride	Me atoms/unit	Zn atoms/unit	*a*, Å	*c*, Å	powder diagram
$NiCl_2$	2.8	0.5	76.5	33.6	good
$MnCl_2$	8.8	0.4	77.3	34.1	good
$CdCl_2$	5.4	0.3	71	34	bad
$CoCl_2$	7.8	<0.1			no diagram
[untreated	0	0.7	74.1	32.4	good]

not followed up by a systematic X-ray study as in the case of the reaction with a $ZnCl_2$ solution. Lattice constants and compositions for three compounds of insulin and manganese, nickel and cadmium chlorides are given in Table 3. The compounds are also entered in Figs. 4 and 5. The lattice constants are seen to differ widely from those of compounds in the $ZnCl_2$ series. The partial replacement of zinc ions with other heavy metal ions, and the uptake of additional metal ion and metal chloride apparently cause more drastic changes than the reaction with zinc chloride.

This phenomenon deserves a closer study. Chemical composition of the products and the character of their powder diagrams are also given in Table 3. It is seen that as long as 0.4 atoms of zinc per Sanger unit are left in the crystals their X-ray diagram is good. When only 0.3 atoms are left their structure has deteriorated, and when less than 0.1 atom is left the structure is so deranged that no powder diagram lines are visible, although the appearance of the crystals under the microscope is unchanged. The deterioration sets in between the limits of 0.4 and 0.3 atoms of zinc per Sanger unit. It will be remembered that one metal atom per 3 Sanger units is the minimum number of metal atoms that can produce crystalline insulin. It is tempting to identify the minimum number of zinc atoms (roughly 0.35) that must be left in air-dried insulin if the ordered internal structure shall not break down, with the number of atoms (*i.e.* 0.33) that must be built in, if insulin shall crystallize from an aqueous solution. It is true — as known from Schlichtkrulls tracer experiments [6] with ^{65}Zn — that any zinc atom in wet insulin crystals can be replaced rapidly by any other zinc atom without deterioration of structure. But it is not improbable that the replacement of a zinc atom with another zinc atom be a milder process than the replacement of a zinc atom with a cobalt atom. The cobalt atom might cause a change of coordination, or it might occupy another site than the zinc atom and hereby cause a breakdown of the order still left in insulin crystals after drying.

CONCLUSION

The study of powder diagrams — or as here diffractograms — is not a general method for the investigation of crystalline proteins. Insulin with its small and highly symmetric unit cell is uniquely favourable. There is hardly any other proteins with which experiments similar to those described above could be made. But the method might be of interest in other studies of

that particularly important protein. It is a routine method, it is "non-destructive", and it yields well defined physical constants, *viz.* degree of crystallinity, lattice constants and reflection intensities from known sets of lattice planes, properties whose variations under different circumstances are of some interest.

ADDENDUM

The number (N) of metal atoms per Sanger unit has been calculated from

$$N = \frac{\%\ \text{Me}}{\text{atomic weight of Me}} \Big/ \frac{100 - \%\ \text{Me} - \%\ \text{Cl}}{6\,000} \qquad \text{(A)}$$

In this equation the weight of a Sanger unit of air-dry pig-insulin including 6 % of water has been set equal to 6 000, and it has been assumed that no water is expelled when metal and salt are taken up. In a previous paper [1] the metal contents of the same specimens were calculated from

$$N = \frac{\%\ \text{Me}}{\text{atomic weight of Me}} \Big/ \frac{100}{6\,000} \qquad \text{(B)}$$

which gives the number of metal atoms per Sanger unit, provided the amount of water expelled equals that of the metal plus metal salt taken up. We think (A) is less arbitrary than (B). However, only complete analysis of a specimen can decide which assumption be the better. For specimens containing less than four metal atoms per Sanger unit there will be no significant differences. For the specimens with higher metal contents discussed in Ref.[1] we think the following stoichiometric formulae calculated from (A) are more probable than those given by Marcker (shown in brackets)

Insulin, 2 Zn, 6 $ZnCl_2$	(Insulin, 2 Zn, 5 $ZnCl_2$)
Insulin, $\frac{1}{3}$ Zn, $1\frac{2}{3}$Mn, 7 $MnCl_2$	(Insulin, $\frac{1}{3}$ Zn, $1\frac{2}{3}$ Mn, 6 $MnCl_2$)
Insulin, $\frac{1}{3}$ Zn, $1\frac{2}{3}$ Cd, 4$CdCl_2$	(Insulin, $\frac{2}{3}$Zn, $1\frac{2}{3}$ Cd, 3 $CdCl_2$)
Insulin, 2 Co, 6 $CoCl_2$	(Insulin, 2 Co, 5 $CoCl_2$)

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