

Insulin Crystals

I. The Minimum Mole-Fraction of Metal in Insulin Crystals Prepared With Zn^{++} , Cd^{++} , Co^{++} , Ni^{++} , Cu^{++} , Mn^{++} , or Fe^{++}

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Recrystallized insulin is made zinc-free by a simple salting-out process.

The metal-free insulin is crystallized with Zn^{++} , Cd^{++} , Co^{++} , Ni^{++} , Cu^{++} , Mn^{++} and Fe^{++} . Of these metals, Cu^{++} , Mn^{++} and Fe^{++} have not hitherto been known to form crystals with insulin.

After recrystallization without any further addition of metal ions, the recrystallized insulin contained 2 atoms of metal per crystal unit of insulin. This amount of metal is considered a fundamental part of the crystal structure.

Abel¹ crystallized insulin as the first protein hormone in 1926. The rhombohedral crystals were precipitated in the isoelectric region at pH 5.5—5.6 from an insulin solution buffered with brucine, pyridine and ammonium acetate. The rather poor and uncertain yields were increased in 1929 by Harrington and Scott² who replaced brucine with saponin. The reason for this improvement was discovered in 1934 by Scott³ who found that both the insulin crystals obtained with either method as well as the saponin contained zinc. Scott and Fisher³⁻⁵ showed that the presence of either Zn, Cd, Co or Ni ions is an essential condition for the formation of crystals. Other metals were tried, *e. g.* Mg and Fe, but without success.

It was furthermore demonstrated by Scott and Fisher^{4,5} that the metal content of the insulin crystals is directly proportional to the atomic weight of the metal (0.52 % Zn, 0.77 % Cd, 0.44 % Co and 0.41 % Ni). Cohn *et al.*⁶ observed in 1941 that the zinc content of insulin crystals increases from 0.33 % to 0.65 % when the pH of crystallization is changed from 5.1 to 6.5. Two extremes were reported in the same year by Sahyun⁷ (0.15 %) and Eisenbrand and Wegel⁸ (0.8 %). In 1951 it was shown by the present author and his associates^{9,10} that insulin crystals may combine with zinc to the extent of at least 2.3 % Zn.

Rhombohedral insulin crystals have apparently never been prepared without these metals. In 1949, however, Ellenbogen¹¹ obtained insulin crystals with various other compounds instead of metals, *e. g.* from a solution of insulin and sodium sulphate made slightly acid with sulphuric acid. These crystals were found to be orthorhombic and their unit weight was only one third of that of the rhombohedral crystal.

The presence of anions is also essential for the crystallization of insulin as shown by Hallas-Møller¹². Rhombohedral insulin crystals were not formed with zinc and chloride ions alone; an addition of other anions (*e. g.* citrate, phosphate or carbonate) was found necessary.

From the experimental evidence listed above it can be concluded that the zinc content of insulin crystals is not merely defined by the molecular weight of insulin and the crystal structure; the metal content of the insulin crystals is rather dependent on the chemical composition of the suspension medium. It must nevertheless be assumed that at least a part of the metal found in the crystals is somewhat related to the structure, as the metal ions are necessary for the formation of crystals. The amount of metal which is an essential part of the crystal structure is considered identical with the metal content of crystals prepared with the minimum amount of metal necessary for the crystal formation. This minimum metal content was determined experimentally. The investigation was carried out with Zn^{++} , Cd^{++} , Co^{++} , Ni^{++} , Cu^{++} , Mn^{++} and Fe^{++} .

EXPERIMENTAL

Preparation of zinc-free insulin. Three-times-recrystallized insulin from beef pancreas was salted out 5 times (15 % NaCl, 0.5 % insulin, pH = 2–3) and isolated after precipitation at pH = 5. It contained less than 0.01 % zinc calculated on the dry weight of insulin.

First crystallization. The metal-free insulin was first crystallized from a medium containing a surplus of one of the metal ions. The composition of the mixture was as follows: 0.5 % insulin, 0.05 M sodium citrate, 15 % (v/v) acetone, and a salt of one of the metals, pH = 6.0 (glass electrode). It is preferable to use citrate buffer rather than the phosphate buffer, as the phosphate may precipitate the metal ions so that the crystals will be defiled by metal phosphate¹³. The ammonium acetate buffer which is frequently employed for the crystallization of insulin has the disadvantage that it contaminates the insulin crystals with non-protein nitrogen thus invalidating the subsequent Kjeldahl analyses. The acetone prevents the insulin from precipitating as amorphous particles from the supersaturated solution.

The zinc-free insulin was dissolved in water made slightly acid (pH = 2) with hydrochloric acid. The solution was distributed in glass beakers, acetone and metal salt solutions were added and the pH adjusted to 6.0 by adding a solution of sodium citrate. The mixture was stirred mechanically overnight with a glass propeller at room temperature. The precipitate was collected on a sintered glass filter without washing and dried to a water content of 4–7 %. The yield of a crystallization was expressed in % and calculated on the basis of Kjeldahl analysis.

Recrystallizations. The insulin crystals were recrystallized from a medium of the same composition as used for the first crystallization except that no metal salts were added. The crystals thus obtained contained less metal than the original crystals. This procedure was repeated until the crystal shape deteriorated severely (spherical shape) and amorphous particles appeared. The yield of a recrystallization was expressed in % and calculated on the basis of Kjeldahl analysis.

RESULTS

The precipitates are characterized by their microscopical appearance. Normal twinned rhombohedrons are indicated as NC (normal crystals) in Table 1. A tendency towards spherical shape is denoted by DC (defective crystals). Furthermore, if amorphous insulin particles are present in considerable amounts, an A is added.

From the paper by Sanger and co-workers¹⁴ it is known that the smallest molecule of insulin contains 65 atoms of nitrogen. As it is assumed that one crystal unit contains 6 of the small molecular units, it contains 390 atoms of nitrogen. Thus the metal content per crystal unit is calculated as atoms of metal per 390 atoms of nitrogen.

Table 1. Crystallization of insulin with Zn^{++} , Cd^{++} , Co^{++} , Ni^{++} , Cu^{++} , Mn^{++} , and Fe^{++} .

(1)	Zn^{++} $ZnCl_2$	Cd^{++} $3CdSO_4 \cdot 8H_2O$	Co^{++} $CoCl_2 \cdot 6H_2O$	Ni^{++} $NiCl_2 \cdot 6H_2O$	Cu^{++} $CuCl_2 \cdot 2H_2O$	Mn^{++} $MnCl_2 \cdot 4H_2O$	Fe^{++} $FeCl_2 \cdot 4H_2O$
(2)	94	95	94	96	95	89	93
(3)	94	96	87	95	93	68	61
(4)	85	91	88	93	94	—	—
(5)	46	45	48	48	30	274	135
(6)	2.12	3.28	2.23	2.16	2.07	3.43	2.38
(7)	1.92	1.99	1.90	1.96	1.92	—	—
(8)	1.92	1.91	1.81	1.96	1.90	—	—
(9)	NC	NC	NC	NC	NC	NCA	NC
(10)	NC	NC	DCA	DCA	NC	(DC)A	A
(11)	DC	NC	DCA	DCA	DC	—	—

(1) Metal salt employed.

(2), (3) and (4) Yields from 1st, 2nd and 3rd crystallization, respectively, expressed in per cent nitrogen recovered.

(5) Total amount of metal present during the first crystallization, expressed as atoms of metal per 390 atoms of nitrogen.

(6), (7) and (8) Metal content of crystals from 1st, 2nd and 3rd crystallization, respectively, expressed as atoms of metal per 390 atoms of nitrogen.

(9), (10) and (11) Character of precipitate from 1st, 2nd and 3rd crystallization, respectively. NC = normal appearance of crystals; DC = defective crystals, *i. e.* tendency towards spherical shape; A = amorphous insulin particles present in considerable amounts.

DISCUSSION

It has been a common practice to remove the zinc from insulin by electro-dialysis. Preference was, however, given to a more easy method with no detrimental effect on the insulin. As already shown by Netter¹⁵ in 1939, hydrogen ions may displace metal ions bound to insulin. The ordinary salting-out procedure for insulin, using an acid insulin solution and salt, is a similar process, and the zinc actually remains in the solution while the insulin is precipitated. The zinc content of insulin crystals can also be simply extracted by acid saline which does not dissolve the crystals.

It may be inferred from Scott and Fisher's¹⁶ findings that the metal ions are chemically bound to the protein in a stoichiometric relation. The unit weight of the rhombohedral insulin crystal has been found to be 37 000 by Crowfoot¹⁷ in 1935. Thus, the zinc content determined by Scott and Fisher (0.52 %) corresponds to 3 atoms of zinc per unit weight of insulin. The interpretation of the metal content is nevertheless complicated by the later findings of diverging zinc contents. In view of the present investigation it seems that 2 rather than 3 atoms of metal per crystal unit form the structural basis of the insulin crystal.

The metal content of crystals prepared in the presence of a surplus of metal ions (first crystallization) varies considerably, 2.1—3.4 atoms per crystal unit. This variation is explainable, as the metal content of the crystals is known to depend on the concentration of metal ions in the medium.

In the recrystallization process Mn^{++} and Fe^{++} crystals failed to reappear. The Mn^{++} and Fe^{++} ions must be present in a relatively high concentration to bring about crystallization.

The change in metal ion concentration in the medium from the first to the second crystallization did not greatly influence the Zn, Co, Ni and Cu contents of the crystals. This invariability is quite compatible with the concept of a fundamental metal insulin compound.

The minimum metal content of insulin crystals prepared with Zn^{++} , Cd^{++} , Co^{++} , Ni^{++} or Cu^{++} was found to be 2—5% less than 2 atoms per crystal unit. A closer proximity to the integer cannot be claimed when it is considered that the crystal structure becomes visibly imperfect through repeated recrystallization. The structure may already be somewhat defective after the second crystallization.

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