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A Study of the Binding of Zinc and Cobalt by Insulin

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The extent of binding of zinc and cobalt by amorphous insulin of very low zinc content has been studied by the method of equilibrium dialysis, and the effects of increasing amounts of bound metal ion on the ultracentrifugal and electrophoretic behavior of metal–insulin complexes have been determined. The use of radioactive Zn^{65} and Co^{60} afforded a rapid and simple method for the determination of metal concentrations. At *p*H 7.3 in Veronal–acetate buffer, the amount of zinc bound by insulin (0.1 and 0.25% solutions) is a function of the "free" zinc concentration. Increases in bound zinc lead to increases in sedimentation constants of the resulting zinc-insulin complexes. Electrophoretic studies demonstrated the presence of two components in amorphous insulin. The relative proportion of the two components is a function of protein concentration, and the binding of small amounts of zinc leads to the complete disappearance of the faster moving component. The zinc–insulin interaction appears to be reversible except at very low ratios of bound zinc to insulin. Cobaltous ion was bound to insulin to a much smaller extent than was zinc. The binding of ferric ion by insulin resembled that of cobaltous ion.

The physical behavior of insulin in aqueous solution has been studied by many methods and under varied conditions. In recent years the methods of light scattering,[3] ultracentrifugation-diffusion[4] and osmotic pressure[5] have confirmed the existence of a *p*H and concentration-dependent monomer–polymer equilibrium demonstrated by earlier investigations,[6] and appear to indicate a figure of about 12,000 for the molecular weight of the insulin monomer. This corresponds to an insulin molecule containing two each of the A and B chains of Sanger and coworkers.[7] Some studies[8,9] indicate that a further dissociation into identical molecules of molecular weight 6,000 can occur under special conditions.

It has also been known for many years that interaction occurs between insulin and zinc and between insulin and certain other divalent or trivalent cations. In so far as could be determined, no data were available as to the effect of bound metal ions on the physical state of insulin. Almost all investigations have been carried out with the use of crystalline zinc–insulin preparations.[10] The current study was undertaken to examine the behavior of amorphous insulin in solution at a physiological *p*H and to note the effect of metal ions, particularly zinc, on this system.

Experimental

Equilibrium Dialysis.—The technique of Klotz and his associates[11,12] with minor modifications, was used in the studies of the binding of metal ions by insulin. The volume of protein solution within the "Visking" membranes was routinely 10 ml., while that of the outer solution was 20 ml. Before dialysis all of the metal ion was in the outer solution. The 50-ml. screw cap culture tubes which were used as reaction vessels were placed in specially made racks so that a circular type Warburg apparatus could be used to obtain the agitation needed for rapid attainment of equilibrium at constant temperature. Usually 12–15 hr. were more than sufficient for equilibration in runs carried out at 20 or 25°. Experiments at low temperatures were carried out by placing the dialysis units on a tilted circular motor-driven panel in a cold room. Up to 48 hr. was required for equilibration in this system. Veronal–acetate buffer mixtures were used in most experiments. To obtain solutions of varying *p*H and ionic strength, different quantities of 1 *M* HCl and 1 *M* NaCl were added to a solution of 0.15 *M* Na Veronal and 0.15 *M* Na acetate. Standard zinc solutions were made up by dissolving pure zinc metal in a minimum quantity of HCl and diluting to volume. The accuracy of this procedure was substantiated by colorimetric zinc analyses obtained through the courtesy of Dr. Alfred Staub of Eli Lilly and Company. These solutions were used as sources of known amounts of zinc for the equilibrium dialysis experiments. Standard cobalt solutions were made up from analytical reagent grade cobaltous chloride.

Before dialysis, identical small quantities of radioactive Zn^{65} were added to the outer solution of each dialysis unit of a series. The quantity of zinc represented by a count in each tube could be determined by counting the outer solution of any one of the tubes before dialysis. Then after dialysis equilibrium had been attained, the concentration of zinc in the outer solutions could be obtained rapidly and simply by counting the solutions. All determinations of radioactivity were made on 15-ml. aliquots of the outer solutions, which were pipetted into an annular glass counting cell. A commercial Geiger–Müller tube and scaling unit were employed. The radioactive Zn^{65} (0.362 mc. per mg.) was obtained in the form of a solution of $ZnCl_2$ in 0.033 *N* HCl from the Oak Ridge National Laboratory. The analysis supplied with the sample was 58.3 mg. Zn per ml. Spectral analysis was strong for Zn and indicated traces of Ca, Cd and Pb, and faint traces of Al and Cu. As it was not carrier free, small corrections in the zinc concentrations of the dialysis units were necessitated by its use. In experiments involving cobalt, radioactive Co^{60} was used in an exactly comparable manner except that, while not carrier free, it was utilized in such small amounts (dilution: 1 to 250 or 500) that no corrections in cobalt concentrations were required. Co^{60} in the form of a solution of $CoCl_2$ in dilute HCl was obtained through the courtesy of Dr. B. Connor Johnson of the Department of Animal Nutrition, University of Illinois, who had obtained Co^{60} wire with an activity of 34 mc. per g. from the Oak Ridge National Laboratory.

Careful preliminary studies were made to determine the magnitude of the volume change within the dialysis membrane. This was done by measuring the concentration of insulin at the beginning and at equilibrium by means of its optical density at 277 mμ. In the range of the various experimental variables employed in the measurements reported here, the volume change was within the experimental error and was therefore neglected in calculations. These same data revealed that no loss of insulin occurred due to

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(2) From a thesis submitted by Robert L. Fischer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois, September, 1954.

(3) (a) F. Tietze and H. Neurath, *J. Biol. Chem.*, **194**, 1 (1952); (b) P. Doty, M. Gellert and B. Rabinovitch, THIS JOURNAL, **74**, 2065 (1952).

(4) (a) F. Tietze and H. Neurath, *ibid.*, **75**, 1758 (1953); (b) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

(5) H. Gutfreund, *Biochem. J.*, **50**, 564 (1952).

(6) H. Gutfreund, *ibid.*, **42**, 156, 544 (1948).

(7) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951); F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 366 (1953).

(8) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3087 (1952).

(9) E. Fredericq, *Bull. soc. chim. biol.*, **33**, 420 (1951); E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(10) J. M. Creeth, *Biochem. J.*, **53**, 41 (1953).

(11) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(12) I. M. Klotz and J. M. Urquhart, *J. Phys. Colloid Chem.*, **53**, 100 (1949).

diffusion across the membrane. In all experiments reported here a blank unit which contained no insulin was run at every zinc concentration studied so as to determine the amount of zinc bound to the membrane itself. This correction for non-protein bound zinc was appreciable, but in no case did it exceed 15% of the total bound zinc.

Ultracentrifugation.—Determinations of sedimentation constants were made in the Model E ultracentrifuge of the Specialized Instruments Corporation. All runs were made at a speed setting of 59,780 r.p.m., and the average temperature of the rotor ranged between 20 and 27°. During the course of a single run the rotor temperature rise was between 0.5 and 1.5 degrees. Sedimentation constants were corrected to the medium water as a solvent at 20° ($s_{20,w}$). The values for the partial specific volume of insulin determined by Oncley, *et al.*,[4] were utilized in this calculation. This figure is given as 0.695 near neutral *p*H and 0.707 at more acid *p*H values. A single determination of the sedimentation constant of a given preparation was usually made. Since the highest degree of accuracy was not required for the use of sedimentation constants purely as an index of relative size, only five photographs were taken during the course of a run. With 0.09% insulin solutions the error in $s_{20,w}$ was estimated to be ±0.25 *S*, due chiefly to the difficulty of determining the exact position of the boundary in such dilute solutions. With 0.25% solutions the error was estimated to be about ±0.1 *S*. In solutions where marked polydispersity was observed, the error in $S_{20,w}$ was somewhat higher.

Electrophoresis.—Mobility studies were done in the Tiselius type electrophoresis apparatus constructed by Pearson Associates, New York. All measurements were made at a constant current setting of 10.000 milliamperes. The temperature of the cell was maintained at 0.25 ± 0.03° during all runs. The resistance of the solutions was measured in the Precision Conductivity Bridge designed by Pierce and Roberts.[13]

Insulin Preparations.—The insulin used in all experiments was an amorphous product of high biological activity but very low zinc content, furnished through the kindness of Eli Lilly and Company. Two lots were employed, W 1302 (0.0075% zinc, equivalent to 0.014 mole Zn per 12,000 g. insulin), and W 1282 (0.011% zinc, equivalent to 0.020 mole Zn per 12,000 g. insulin). Emission spectrographic semi-quantitative analyses carried out through the kindness of Dr. Otto K. Behrens, Eli Lilly and Company, showed the following results, expressed as parts per million: *W 1282*, Cu 11, Pb 52, Sn 3, Fe 20, Zn 10, Si 80, Mg 2, Ag 3; *W 1302*, Cu 17, Pb 87, Sn 24, Fe 100, Zn 18, Si,1600. In the case of W 1302, 100 p.p.m. of Fe are equivalent to 0.021 mole Fe per 12,000 g. insulin and 18 p.p.m. of Zn are equivalent to 0.003 mole Zn per mole of insulin. All figures presented for percentage insulin concentration and all calculations of the quantity of zinc bound per mole of insulin are based on the dry weight obtained by heating the insulin in a vacuum oven at 110° to constant weight and on the assumption of a molecular weight of 12,000 for the hormone. The insulin concentration in all electrophoresis runs and in certain of the ultracentrifugal and equilibrium dialysis studies was checked by measuring the optical density at 277 mμ.

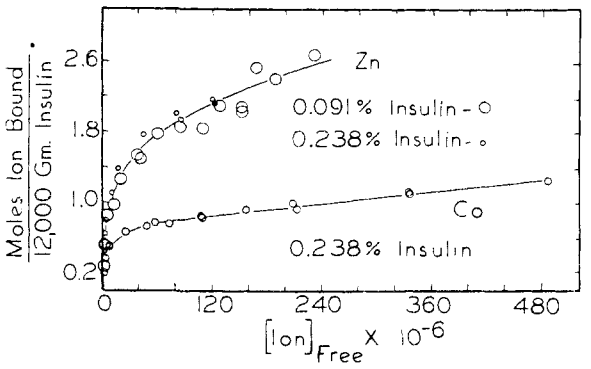

Fig. 1.—Binding of metal ions by amorphous insulin; Veronal-acetate-NaCl, Γ/2 = 0.1, *p*H 7.3, 20°.

(13) G. R. Pierce and H. C. Roberts, *Proc. Instru. Soc. Amer.*, **8**, 55 (1953).

Results

The results obtained from equilibrium dialysis studies of the zinc–insulin interaction at two protein concentrations and at *p*H 7.3 are included in Fig. 1. It is apparent that there is no leveling off of the binding curves, which might indicate the maximum number of metal ions which could be bound by a mole of insulin. When the reciprocal of the moles of zinc bound per mole of insulin is plotted *versus* the reciprocal of the free zinc concentration, a straight line is not obtained. This indicates that this ion–protein interaction cannot be attributed to purely statistical effects.[14]

Since data have been obtained[15] which show that the zinc in crystalline zinc–insulin cannot be removed by dialysis at neutral *p*H, the reversibility of formation of the zinc–insulin complexes in these and similar experiments was studied by the technique suggested by Klotz. The membranes containing the inner solutions after the first equilibration were placed in 20 ml. of fresh buffer and allowed to re-establish equilibrium. The results of such an experiment are shown in Fig. 2. It is

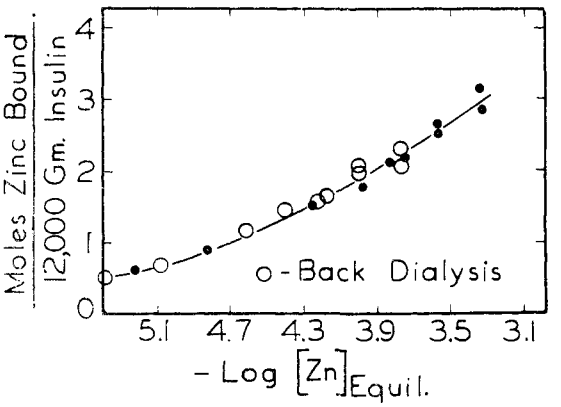

Fig. 2.—Reversibility of zinc binding; Veronal–acetate–NaCl, Γ/2 = 0.18, *p*H 7.6, 25°, 0.046% insulin.

apparent that the zinc–insulin interaction is entirely reversible throughout the range shown. Another experiment, which was done at a higher insulin concentration thus improving the relative accuracy of the calculation, is presented in Table I. The data indicate clearly that no net removal of bound zinc occurs in the the region from 0.2 to 0.6 mole of zinc per mole of insulin under the conditions of back-dialysis described. These data, then, agree with the findings on the stability of the zinc–

TABLE I

ZINC–INSULIN INTERACTION

Insulin concentration, 0.238% W 1282; buffer, 0.026 *M* Na Veronal, 0.026 *M* Na Acetate, 0.048 *M* NaCl, adjusted to *p*H 7.3 with HCl; temperature, 20°.

First equilibrium		Second equilibrium	
$[Zn^{++}]_{free}$, $M \times 10^{-6}$	Moles Zn / Mole insulin	$[Zn^{++}]_{free}$, $M \times 10^{-6}$	Moles Zn / Mole insulin
0.69	0.24	0.08	0.24
3.4	0.62	1.1	0.61
18.4	1.38	10.9	1.30
85.2	1.93	39.8	1.75

(14) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

(15) E. J. Cohn, J. D. Ferry, J. J. Livingood and H. M. Blanchard, THIS JOURNAL, **63**, 17 (1941).

insulin bond in crystalline zinc–insulin, since the zinc content of the crystals probably ranges from 0.28 to 0.55 mole of zinc per mole of insulin.[16]

In all equilibrium dialysis studies, progressively larger amounts of zinc–insulin complex precipitated as more zinc was bound. The point at which precipitation first occurred varied with the *p*H and ionic strength of the solution. The reversibility of this interaction was most striking when such a precipitate redissolved during the second equilibration. These precipitates were examined microscopically and no evidence of crystallinity was observed. Eisenbrand and his associates had previously shown[17,18] that similar insulin preparations of high zinc content were not crystalline.

The binding of zinc by insulin was also investigated in Veronal–acetate buffer at *p*H 3.75. From Table II it may be seen that the ability of insulin to bind zinc is lost completely at this low *p*H. This is in agreement with earlier data[15] which showed that the zinc of crystalline insulin could be removed by dialysis at low *p*H.

TABLE II

LACK OF ZINC-BINDING BY INSULIN AT *p*H 3.75

Veronal-acetate buffer, $\Gamma/2 = 0.052$; 0.09% insulin 20°.

$[Zn^{++}]_{free}$, $M \times 10^{-6}$	Moles Zn / 12,000 g. insulin	$s_{20,w}$
16.3	0.02	2.7
29.3	.01	2.7
67.8	− .004	2.7
127	.17	2.5
207	.03	2.9
414	− .13	2.3

In order to determine the effect of bound zinc on the size of the insulin molecule, the sedimentation constants of the zinc–insulin complexes formed in the "Visking" membranes during equilibrium dialysis were determined. The results of such studies on the complexes formed in the experiments shown in Fig. 1 are given in Fig. 3. Relatively small amounts of bound zinc increase the sedimentation constant from that of amorphous insulin to about 3.5 *S*. Further increases in bound zinc cause no further increase in $s_{20,w}$ until about 0.6 to 0.8 mole of zinc is bound per mole of insulin. Above this point further increases in the amount of bound zinc lead to a linear rise in $s_{20,w}$. Although sedimenting boundaries of 0.09% insulin solutions are relatively difficult to study, several definite qualitative features were apparent. The boundary of 0.09% amorphous insulin showed considerable polydispersity in the form of trailing toward the meniscus. The peak sharpened markedly when small quantities of zinc were bound. This was not as noticeable in 0.24% solutions, however, since this concentration of amorphous insulin does not exhibit nearly so much trailing as lower concentrations. In both cases, however, when the zinc content exceeded 0.6 to 0.8 mole of zinc per mole of insulin, marked polydispersity reappeared in the form of rapidly spreading, fairly symmetric boundaries of higher sedimentation constants. It is also of interest to note that in the region of highest zinc content studied, a boundary of considerably higher $s_{20,w}$ than any reported here for zinc–insulin complexes was observed in addition to the main boundary. It was estimated to comprise 10–15% of the total protein concentration. This boundary spread very rapidly indicating that it represented a group of zinc–protein complexes of similar form.

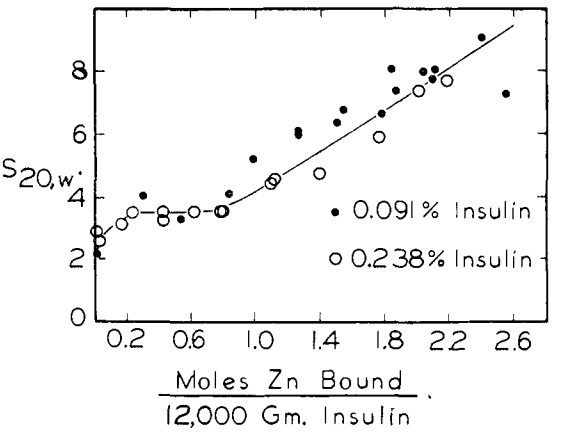

Fig. 3.—Sedimentation constants of zinc–insulin; Veronal–acetate–NaCl, $\Gamma/2 = 0.1$, *p*H 7.3, 20°.

When the sedimentation constants of the contents of the cellophane tubes of the equilibrium dialysis experiment at *p*H 3.75 were determined, no progressive effect of increasing zinc concentration could be detected. This confirms the results from equilibrium dialysis which indicated that no zinc is bound under these conditions. These data are presented in Table II.

Since most physico-chemical studies of insulin have been made utilizing phosphate buffers, it was decided to attempt binding studies in this medium. As expected, the precipitation of zinc phosphates prevented the obtaining of data of high reliability from such an investigation. It was fairly well established, however, that a relatively low order of zinc binding occurred, up to 0.78 mole of zinc per mole of insulin in one case but usually less than 0.2 mole.

This was confirmed by the ultracentrifugal study of the resulting complexes, as shown in Table III. As in the case of Veronal–acetate buffer, small amounts of zinc led to a rise in $s_{20,w}$ from that of amorphous insulin to about 3.5 *S*. Marked sharpening of the boundary also was observed. However, no indication of an increase above that figure could be observed at the highest levels of total zinc studied.

TABLE III

SEDIMENTATION CONSTANTS OF ZINC–INSULIN IN PHOSPHATE (0.001 *M*)–NaCl (0.1 *M*) CONTAINING VARYING AMOUNTS OF ADDED ZINC

*p*H 7.3, 0.09% insulin, 20°.

$[Zn^{++}]_{est.}$, $M \times 10^{-6}$	$s_{20,w}$	$[Zn^{++}]_{est.}$, $M \times 10^{-6}$	$s_{20,w}$
9.4	3.5	22.9	3.3
10.2	3.3	28.5	3.6
10.2	2.9	54.0	3.5
15.8	3.5	79.4	3.4
16.8	3.1	105	3.5
22.9	3.3		

(16) E. J. Cohn, J. D. Ferry, J. J. Livingood and H. M. Blanchard, *Science*, **90**, 183 (1939).

(17) J. Eisenbrand and F. Wegel, *Z. physiol. Chem.*, **268**, 26 (1941).

(18) J. Eisenbrand, *Med. Chem.*, **4**, 259 (1942).

Inasmuch as cobalt ions had also been shown to interact with insulin to form cobalt–insulin crystals, a comparison of the binding curve of the zinc–insulin interaction with that of the cobalt–insulin interaction was made in the hope that any difference might yield information as to the nature of the metal–protein linkage. The data are included in Fig. 1. It is apparent that there is a very considerable difference in the behavior of these two similar ions toward the hormone. Under identical conditions, cobalt is not bound to nearly the same extent as zinc. This difference is borne out by ultracentrifugal studies on the cobalt–insulin complexes. Fig. 4 shows that increasing amounts of bound cobalt cause a rise in $s_{20,w}$ from that of amorphous insulin to values between 3.5 and 4.0 *S*. As was the case with zinc, considerable precipitation of the complexes of higher metal content caused the sedimenting boundary of the complex left in solution to be smaller in area, thus leading to an increased error in the determination of the sedimentation constants. This presumably accounts for the scattering of points in this curve. This was also the reason why higher cobalt concentrations were not studied. In the cobalt studies no evidence was obtained for the higher molecular weight component mentioned above.

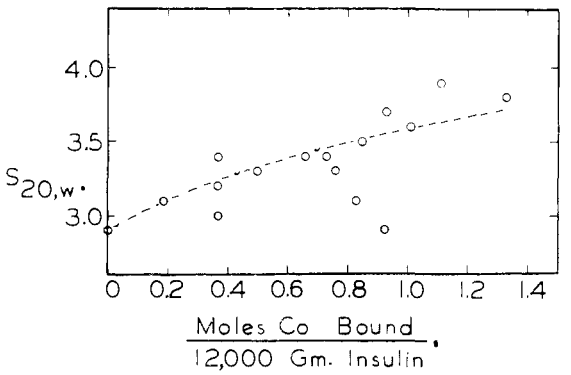

Fig. 4.—Sedimentation constants of cobalt–insulin; Veronal–acetate–NaCl, $\Gamma/2 = 0.1$, *p*H 7.3, 20°, 0.238% insulin.

Preliminary studies on the binding of iron, Fe^{+++}, by insulin and of the sedimentation constants of iron–insulin complexes indicated a behavior similar to that of cobalt rather than to zinc. The Fe^{55} used was a solution of $FeCl_3$ in dilute HCl as supplied by the Oak Ridge National Laboratory.

Several runs were also made to determine the concentration dependence of the sedimentation constant of amorphous insulin in Veronal–acetate, in tris-hydroxymethylaminomethane (TRIS) and in phosphate buffer. The results of these experiments are shown in Fig. 5. There is a definite rise in the $s_{20,w}$ of amorphous insulin with increasing protein concentration. This shows that aggregation of amorphous insulin can occur in the presence of only trace amounts of zinc although it seems certain that it is effected through a mechanism different from that of the "zinc type" of aggregation.

As a means of obtaining further information on the various types of aggregation observed in the preceding experiments, electrophoretic studies were

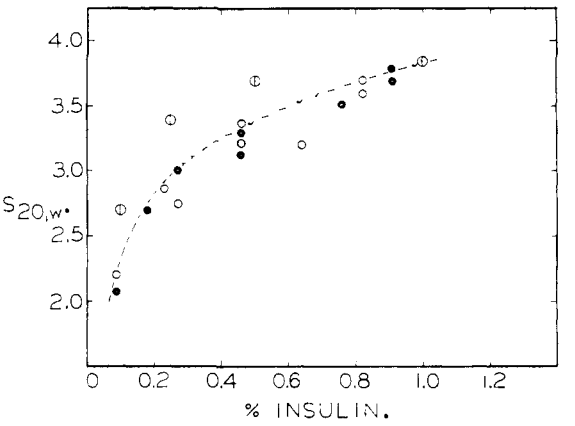

Fig. 5.—Concentration dependence of the sedimentation constants of amorphous insulin: ⊕, TRIS–NaCl, $\Gamma/2 = 0.15$, *p*H, 7.0, 20°; ○, Veronal–acetate–NaCl, $\Gamma/2 = 0.1$, *p*H 7.3, 20°; ●, phosphate, 0.001 *M*–NaCl, 0.1 *M*, *p*H 7.3, 20°.

carried out. Since no data were available in the literature on the behavior of zinc-free insulin preparation in electrophoresis, a study of this type was initiated. The results of representative experiments are given in Table IV. The most striking feature is the appearance of two distinct peaks in the insulin preparations. The faster moving peak is designated, α, and the slower, β. The mobility of both components increases with increasing protein concentration, but there appears to be an equilibrium between the two such that the more slowly moving component is predominant at higher protein concentrations. The use of Veronal–acetate and of TRIS buffers showed that the nature of the buffer ion was not involved in these effects.

TABLE IV

ELECTROPHORETIC BEHAVIOR OF METAL-FREE INSULIN

		Component α		Component β	
	Insulin concn., %	Mobility, cm.² v.⁻¹ sec.⁻¹ × 10⁻⁵	% of total area	Mobility, cm.² v.⁻¹ sec.⁻¹ × 10⁻⁵	% of total area
A.[a]	0.1	5.78	54	4.42	46
	0.25	6.18	15	5.00	82
	0.5	6.40	10	5.22	90
	1.0	6.72	13	5.84	87
B.[b]	0.15	4.03	100	..	0
	0.3	4.47	40	3.27	60
	0.5	5.01	38	4.03	62
	1.0	5.22	20	4.46	78

[a] Veronal–acetate buffer, *p*H 7.35, $\Gamma/2$ 0.1. [b] TRIS buffer, *p*H 7.0, $\Gamma/2$ 0.15.

Electrophoretic studies were next performed on the zinc–insulin complexes obtained from dialysis–equilibrium experiments. Again, as recorded for representative experiments in Table V, a rise in mobility was found, due in this case to an increase in the amount of zinc bound to the insulin. This rise in mobility continues until approximately one mole of zinc is bound per mole of insulin. At this point a leveling off or an actual decrease in mobility occurs as more zinc is bound. The most pronounced effect of bound zinc, however, is shown in Fig. 6. The binding of amounts of zinc as low as

0.2 mole per mole of insulin leads to the disappearance of the α-component of amorphous insulin, leaving a single symmetrical peak.

TABLE V

EFFECT OF ZINC BINDING ON THE ELECTROPHORETIC MOBILITY OF AMORPHOUS INSULIN, $\Gamma/2 = 0.1$

Buffer	pH	Mobility, cm.^{2} v.$^{-1}$ sec.$^{-1}$ $\times 10^{-5}$	Moles zinc/mole insulin
Veronal–acetate	7.5	6.00	0
		6.06	0.05
		6.18	0.44
		6.30	0.71
		6.42	1.07
TRIS	7.35	4.84	0
		5.32	0.24
		5.75	0.77
		5.75	1.30

Similar studies were made on solutions of cobalt and iron complexes of insulin. The binding of iron led to an initial increase in mobility, followed by a decrease. It failed to cause the disappearance of the faster moving component. The behavior of cobalt was very similar to that of zinc in the region studied.

Discussion

All the data presented appear to be consistent with the hypothesis that there exist at least two types of zinc–insulin linkage. The one which occurs in the presence of relatively small amounts of zinc accounts for several observations; (1) a rapid rise in $s_{20,w}$ from that of amorphous insulin to about 3.5 S, (2) the loss of the α peak in electrophoretic studies, (3) the irreversibility of the linkage under the conditions of dialysis employed, (4) the occurrence of this binding in phosphate buffer where there exists a strong competition between phosphate and insulin for zinc ions. The second type of binding is characterized by the linear rise in $s_{20,w}$ with increasing amounts of bound zinc, its free reversibility to dialysis, and by the fact that it does not occur in phosphate buffer.

The interaction of cobalt with insulin gives rise to results very similar to those obtained in the first type of zinc linkage. Preliminary investigations, however, indicate that the cobalt–insulin interaction is reversible. The iron–insulin complexes studied did not clearly fall into either class. The relationship between zinc binding and the imidazole

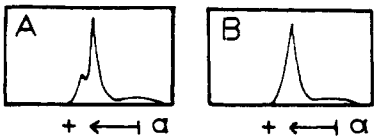

Fig. 6.—Effect of zinc binding on the electrophoretic pattern of amorphous insulin; TRIS, $\Gamma/2 = 0.1$, pH 7.0, field strength = 2.72 v. cm.$^{-1}$: A, 0.5% amorphous insulin, 178 min.; B, 0.5% insulin, 0.2 mole zinc/12,000 g. insulin, 177 min.

groups of histidine recently shown by Gurd and Goodman,[19] and by Tanford,[20] and Tanford and Epstein,[21] is interesting in relation to the data reported here. On the basis of their observations it seems reasonable to ascribe the type I or "irreversible" binding to an imidazole–zinc type of linkage. There are four histidine residues per molecule of insulin of molecular weight 12,000. If two histidine residues are involved per mole,[21] a maximum value of two moles of bound zinc per 12,000 g. insulin should be approached. In no case did it appear that the binding curve approached two as a maximal number. The changes in the physical state of insulin in the presence of zinc suggest cross linking, but no exact mechanism for such polymerization may be inferred from these data.

To point up the effect of very small amounts of zinc on the physical size of insulin it should be mentioned that a considerable variation in apparently duplicate runs on the low-zinc amorphous insulin preparations was finally ascribed to the fact that traces of zinc were left behind in the centrifuge cell when it was cleaned following a run on a zinc–insulin complex of high zinc content. While extra care eliminated this, it does show rather clearly the very marked effects of small amounts of zinc on the sedimentation constant of insulin.

In addition to the effect of metal ions on the physical state of insulin, it has been shown by both ultracentrifugal and electrophoretic experiments that interaction occurs between insulin sub-molecules of very low zinc content and that increasing protein concentration leads to an aggregation of these particles.

URBANA, ILLINOIS

(19) F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(20) C. Tanford, *ibid.*, **74**, 211 (1952).

(21) C. Tanford and J. Epstein, *ibid.*, **76**, 2170 (1955).