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The Physical Chemistry of Insulin. II. Hydrogen Ion Titration Curve of Crystalline Zinc Insulin. The Nature of its Combination with Zinc[1-3]

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A complete hydrogen ion titration curve has been obtained for crystalline zinc insulin (one atom of Zn per 11,500 g.) in aqueous solution of ionic strength 0.075, at 25°. Comparison with the corresponding curve for zinc-free insulin indicates that the zinc is combined with imidazole groups, as has previously been found for the combination of zinc with serum albumin. However, two imidazole groups are involved per zinc ion. Polarographic measurements confirm this conclusion. In contrast to the titration curve of zinc-free insulin, the present curve is not reversible. It was observed, however, that identical "reversed" curves are obtained after exposure to acid and to base. The difference between the direct and reversed curves lies largely in the region of incomplete solubility: the direct curve represents equilibrium between hydrogen ion and zinc insulin crystals; the reversed curve, on the other hand, represents equilibrium between hydrogen ion and an amorphous zinc insulin precipitate, which is formed when zinc insulin is freshly precipitated after solution in either acid or base. The *p*H of reversed mixtures was found to drift with time so as to approach the direct titration curve, and, at the same time, zinc insulin crystals were formed.

The original purpose of the present study was to elucidate the nature of the interaction between insulin and zinc ions, both from a comparison of the titration curve of zinc-free insulin (*cf.* preceding paper[4]) with that of zinc insulin, and from polarographic measurements. The solution to this problem proved to be straightforward. As in the case of serum albumin,[5,6] zinc ions appear to combine preferentially with imidazole groups. In contrast to serum albumin, however, each zinc ion combines with two rather than one imidazole group.

A second problem of great interest presented itself, however, as this work got under way. For, not only was the expected difference found between the behavior of zinc insulin and that of zinc-free insulin, but, in addition, entirely unexpected differences were found between the behavior of suspensions of zinc insulin between *p*H 3.5 and 8, and of similar mixtures which had first been dissolved by the addition of acid or alkali, and then returned to this *p*H region. Much of the paper is therefore concerned with this phenomenon.

Experimental

The same procedures and reagents were used as in the preceding paper[4] except for the following.

Insulin.—A sample of five times recrystallized bovine zinc insulin, Lot No. T-2842, was supplied by Eli Lilly and Co. The sample was stated to have an activity of 26.5 u./mg., and a zinc content of 0.429%, *i.e.*, 0.76 zinc ions per insulin monomer.[7] Since the analytical procedure by which this zinc content was measured, U. S. P. XIV, p. 799, is subject to considerable error,[8] the zinc content was redetermined by us, using a polarographic method.[8] A somewhat higher value was found, equivalent to 0.95 ± 0.05 Zn insulin monomer. Within the experimental error, therefore, the zinc content is 1.0 Zn/insulin monomer, and this figure has been used throughout. The ash content (0.7%) indicates the absence of inorganic ions other than zinc. E.m.f. measurements, with silver–silver chloride electrodes, showed that no chloride was present. The *p*H of one sample, suspended in water, was 6.23; in 0.075 *M* KCl the *p*H was 6.005. This corresponds to the presence of a negative charge of 0.5 per insulin monomer (*cf.* Fig. 1), and indicates the presence of an equal amount of some univalent cation. This quantity is too small to require a correction in weighing out samples. The insulin was handled the same way as in the preceding paper.[4] The moisture content was found to be 6.2%.

Polarography.—Polarographic currents were measured on a Sargent Model XXI polarograph. The same drop-time and capillary were used as in a previous study.[5] The cell and technique for oxygen removal are those described by Tanford and Epstein.[9] These measurements were made at 22 ± 1°.

Solubility.—Solubility measurements were performed by the method described by Schwert and Neurath.[10] Each solution made was divided into two parts, one of which was shaken for an hour or more before being measured, the other being processed within a shorter time. Both halves of the solution always agreed within the experimental error, and the differences that did occur were random. The experiments were necessarily crude since a constant temperature centrifuge was not available. The solutions were not exposed, however, to temperatures differing by more than one or two degrees from 25°.

Results and Qualitative Discussion

Whereas the *p*H of all solutions of zinc-free insulin remained unchanged with time and, in addition, the molecular changes with *p*H were reversible, this proved not to be the case in the present study. Brief exposure to either *p*H 2 or *p*H 9 to 11 led to large changes in the titration curve over a considerable region, and resulted also in slow *p*H drifts. These drifts were small (initially about 0.02 *p*H to 0.04 *p*H unit per hour), but well outside the experimental error, the reproducibility normally being consistently ±0.01 *p*H unit. Because the rate of change of *p*H was so slow, initial values were easily obtained by extrapolation to zero time. The titration curves obtained from these are discussed first, the data being given in Tables I to III. The time dependence is discussed subsequently.

Table I shows the experimental data leading to a *direct* titration curve; *i.e.*, each of the mixtures described was prepared by the addition of only the amount of acid or base given in column 2. Over a

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(2) This investigation has received support from a research grant donated by Eli Lilly and Co. The general program, of which this investigation is a part, has been supported by a research grant (RG-2350) from the National Institutes of Health, Public Health Service, and by a grant from the National Science Foundation.

(3) Abstracted, in part, from the dissertation submitted by Jack Epstein in partial fulfillment of the requirements for the Ph.D. degree, State University of Iowa, August, 1953.

(4) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

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

(6) F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952).

(7) As in the preceding paper, the term insulin monomer is used to designate the unit of molecular weight about 11,500.

(8) J. Cholak, D. M. Hubbard and R. E. Burkey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 754 (1943).

(9) C. Tanford and J. Epstein, *Anal. Chem.*, **23**, 802 (1951).

(10) M. H. Schwert and H. Neurath, THIS JOURNAL, **72**, 2785 (1950).

TABLE I

DIRECT TITRATION CURVE, $T = 25°$, $\mu = 0.075$

Moles insulin monomer/ kg. H_2O $\times 10^4$	H^+ added moles/ kg. H_2O $\times 10^4$	pH	H^+ free moles/ kg. H_2O $\times 10^4$	H^{+a} bound moles/ insulin monomer	H^{+b} moles dissociated/ insulin monomer
11.75	464.61	1.595	295.8	13.9	0.1
10.60	384.94	1.70	233.3	13.8	0.2
6.50	221.47	1.94	135.2	12.8	1.2
5.77	168.63	2.097	94.84	12.3	1.7
5.90	148.2	2.215	72.28	12.4	1.6
7.27	150.68	2.27	63.97	11.4	2.6
5.33	130.17	2.275	63.24	12.1	1.9
5.31	103.29	2.483	39.45	11.5	2.5
7.28	112.58	2.625	28.58	11.0	3.0
4.05	61.68	2.815	18.58	10.1	3.9
7.00	78.89	3.13	9.10	9.5	4.5
7.87	78.50	3.29	6.34	8.7	5.3
4.19	43.60	3.33	5.78	8.5	5.5
5.89	47.76	3.66	2.73	7.2	6.8
5.00	30.03	3.985	1.30	5.2	8.8
7.35	40.19	4.005	1.25	4.8	9.2
5.93	26.47	4.078	1.05	3.8	10.2
3.97	12.26	4.40	0.50	2.5	11.5
4.23	10.59	4.53	0.37	1.9	12.1
7.30	15.35	4.695	0.25	1.6	12.4
5.93	5.96	5.21	0.08	0.5	13.5
	OH^- added moles/ kg. H_2O $\times 10^4$		OH^- free moles/ kg. H_2O $\times 10^4$	OH^{-a} bound moles/ insulin monomer	
2.83	0.00	6.005	0.00	0.5	14.5
4.68	2.46	6.41	.00	1.0	15.0
4.61	4.69	6.715	.00	1.5	15.5
4.19	7.22	7.133	.00	2.2	16.2
4.29	8.90	7.27	.00	2.6	16.6
3.64	8.65	7.40	.00	2.9	16.9
4.86	14.79	7.53	.00	3.5	17.5
3.89	13.90	7.68	.00	4.1	18.1
4.52	18.69	7.865	.01	4.6	18.6
4.44	21.62	8.18	.02	5.4	19.4
4.02	20.23	8.205	.02	5.5	19.5
4.63	28.75	8.71	.07	6.7	20.7
4.28	30.80	9.09	.18	7.6	21.6
4.88	37.59	9.32	.30	8.1	22.1
4.58	37.31	9.475	.42	8.5	22.5
4.51	39.96	9.70	.71	9.2	23.2
4.28	44.70	10.04	1.56	10.6	24.6
5.02	56.93	10.27	2.65	11.3	25.3
4.82	60.31	10.425	3.78	12.2	26.2
4.77	66.59	10.615	5.86	13.2	27.2
4.31	73.57	10.87	10.54	15.1	29.1
7.65	127.57	11.005	14.39	15.3	29.3
5.54	110.97	11.185	21.78	16.6	30.6
4.60	110.99	11.37	33.34	17.4	31.4
4.99	142.85	11.60	56.62	17.8	31.8
5.02	144.9	11.615	58.61	17.7	31.7
6.58	238.78	11.925	119.7	18.6	32.6
4.51	207.59	11.938	123.3	19.2	33.2
5.02	226.65	11.965	131.2	19.5	33.5
4.41	230.98	12.013	146.6	19.6	33.6
9.69	424.64	12.223	237.7	19.8	33.8

[a] Referred to the isoelectric point, which, in this case, is the pH where two H^+ ions are dissociated from isoionic protein. [b] Referred to the pH of maximum acid binding.

TABLE II

REVERSIBILITY FROM ACID END, $T = 25°$, $\mu = 0.075$

Moles insulin monomer/ kg. H_2O $\times 10^4$	Net H^+ added moles/ kg. H_2O $\times 10^4$	pH[c]	H^+ free moles/ kg. H_2O $\times 10^4$	H^{+a} bound moles/ insulin monomer	H^{+b} moles dissociated/ insulin monomer
4.63	49.02	3.32	5.92	8.8	5.2
3.88	19.89	4.255	0.70	4.4	9.6
4.08	20.06	4.295	.64	4.3	9.7
4.58	6.12	5.429	.05	0.8	13.2
3.86	3.38	5.601	.03	0.4	13.6
	Net OH^- added moles/ kg. H_2O $\times 10^4$		OH^- free moles/ kg. H_2O $\times 10^4$	OH^{-a} bound moles/ insulin monomer	
4.30	9.30	6.805	0.00	2.7	16.7
4.21	21.66	8.087	.02	5.6	19.6
4.08	29.24	9.013	.15	7.6	21.6

[a,b] *Cf.* Table I. [c] Where appreciable pH drift occurred (0.02 pH unit per hour or more), these pH values are extrapolated to zero time.

TABLE III

REVERSIBILITY FROM ALKALINE END, $T = 25°$, $\mu = 0.075$

Moles insulin monomer/ kg. H_2O $\times 10^4$	Net H^+ added moles/ kg. H_2O $\times 10^4$	pH[c]	H^+ free moles/ kg. H_2O $\times 10^4$	H^{+a} bound moles/ insulin monomer	H^{+b} moles dissociated/ insulin monomer
4.86	35.31	3.835	1.84	6.4	7.6
4.46	16.47	4.655	0.28	3.1	10.9
4.67	12.59	4.885	.16	2.2	11.8
4.73	10.37	5.069	.11	1.7	12.3
4.38	6.40	5.378	.05	1.0	13.0
4.68	4.67	5.531	.04	0.5	13.5
	Net OH^- added moles/ kg. H_2O $\times 10^4$		OH^- free moles/ kg. H_2O $\times 10^4$	OH^{-a} bound moles/ insulin monomer	
4.46	3.23	6.326	0.00	1.2	15.2
5.42	11.30	6.74	.00	2.6	16.6
4.49	15.89	7.475	.00	4.0	18.0
4.51	42.11	9.787	.87	9.6	23.6

[a,b] *Cf.* Table I. [c] *Cf.* Table II.

wide range of pH, from pH 3.5 to 8, the insulin in these mixtures (*ca.* 5 g./liter) was incompletely soluble, and measurements of pH were made on the resulting suspensions. Outside this range of pH, solution was complete, except that between pH 8 and 9 a few coarse grains of precipitated material were generally present. Microscopic examination of the suspensions between pH 3.5 and 8 showed that all or most of the insulin precipitate was present in crystalline form. The pH of all mixtures of this table, both those completely soluble and those incompletely soluble, did not change perceptibly with time over a period of many hours. The data of Table I are therefore taken to represent the equilibrium between insulin and hydrogen ion. In the region of partial insolubility, they represent equilibrium with crystalline zinc insulin. Because the solubility range of this precipitate is different from that of amorphous insulin, and because this particular crystalline form cannot be obtained except in the presence of zinc or certain other

metals[11] it can be concluded that in this crystalline precipitate there are intermolecular bonds in which the zinc is intimately concerned. The nature of these bonds will be discussed below.

Table II shows the experimental data leading to an *acid-reversed* titration curve. All of the mixtures described were prepared by the addition of sufficient acid to dissolve the insulin and to lower the *p*H to about *p*H 2. Potassium hydroxide was then added to bring the *p*H to its final value. The amount of acid or base added, given in column 2, is the *net* amount, *i.e.*, the difference between the added acid and base. The mixtures described in the table formed suspensions over a narrower region than those described in Table I; in fact, the region of partial insolubility proved to be the same as for zinc-free insulin, *p*H 4 to 7. Microscopic examination of fresh suspensions showed that the precipitated insulin was amorphous; no crystals at all were present.

Table III shows the experimental data leading to a *base-reversed* titration curve. All of the mixtures described were prepared by the addition of sufficient base to dissolve the insulin and to raise the *p*H to about *p*H 11. (In a few solutions, a maximum *p*H of 9 instead of 11 was used. This made no noticeable difference.) HCl was then added to bring the *p*H to its final value. As in Table II, column 2 of Table III represents the *net* amount of acid or base added. The region of partial insolubility proved to be the same as for acid-reversed points. The precipitated insulin was again amorphous.

The three sets of data described are plotted in Fig. 1, and the experimental data from *p*H 4 to 7 are shown in detail in Fig. 3. The acid- and base-reversed curves are seen to differ considerably from the direct titration curve in the region of partial insolubility. It is apparent, however, that the acid- and base-reversed curves are themselves indistinguishable. There is apparently only one "reversed" curve, and it must be concluded that it represents a reversible equilibrium between hydrogen ion and the amorphous zinc insulin precipitate described above. The nature of this precipitate must be independent of whether it is obtained from an acid or basic solution of insulin. The fact that the region of insolubility is identical with that of zinc-free insulin, suggests that this precipitate is very similar to that which is obtained for insulin in the absence of zinc; *i.e.*, in this case the zinc does not participate in the intermolecular bonds leading to precipitation.

The difference between the precipitates of the direct and reversed titration, and the fact that most of the reversed mixtures showing *p*H drift in the region of incomplete solubility changed in *p*H so as to approach the direct titration values (see below), suggested that the *p*H instability might be due to a change in the nature of the precipitate. This proved to be the case. Several mixtures in the region of partial insolubility were examined microscopically, and it was found that typical zinc insulin crystals were formed whenever *p*H changes took place. No crystals were found when no *p*H change occurred.

These observations provide strong support for the contention that the direct titration curve represents true equilibrium between hydrogen ion and crystalline zinc insulin; the equilibrium between hydrogen ion and the amorphous precipitate is apparently a metastable one.

One other interesting feature of the titration curves of Fig. 1 is that the points near *p*H 12 are less scattered than the corresponding points on the curve for zinc-free insulin,[4] and show quite clearly the titration of the two guanidine groups known to be present per insulin monomer.

Comparison with Zinc-free Insulin. The Site of Combination with Zinc.—In addition to the titration curves of zinc insulin, Figs. 1 and 3 show (dotted lines) the hydrogen ion titration curve of zinc-free insulin, reported in the preceding paper.[4] It is seen at once that the over-all number of groups titrated in zinc insulin is greater than the number titrated in zinc-free insulin. The difference is two groups per insulin monomer, and presumably corresponds to the dissociation of two hydrogen ions from the hydration sphere of the zinc.[12] This difference between the direct titration curve of zinc insulin and that of zinc-free insulin develops, as is expected, above *p*H 7; for up to *p*H 7 the over-all number of groups titrated is the same. In the reversed curve the titration of the zinc appears to begin at a somewhat lower *p*H.

In addition to the over-all difference of two titratable groups, one other prominent feature differentiates the titration curves of zinc insulin and zinc-free insulin. Two groups which in zinc-free insulin are titrated between *p*H 6.5 and 7, and which are thus immediately identifiable as imidazole groups, appear in the direct titration curve of zinc insulin near *p*H 4 instead, while in the reversed titration curve they are titrated between *p*H 4 and 6. This type of decrease of the *p*H at which hydrogen ions are dissociated is a typical result of complex formation between metals and weak bases,[13] or between metals and basic groups of proteins.[6] It appears certain, therefore, that the zinc in zinc insulin is complexed to imidazole groups, since it is the titration range of these groups which is shifted to lower *p*H values. This finding is not unexpected, for it has already been shown that the principal binding sites for zinc in both human and bovine serum albumin[5,6] are imidazole groups, and that this is to be expected from the properties of imidazole itself.[6] A similar conclusion has been

(11) D. A. Scott, *Biochem. J.*, **28**, 1592 (1934). Crystals of salts of insulin have also been obtained, but these are quite different from the isoelectric crystals here discussed.

(12) Potentiometric studies by H. Diehl (personal communication) show that the *pK* for the dissociation of the first hydrogen ion from $Zn(H_2O)_4^{++}$ is 8.0. In insulin the zinc is complexed, but this need not necessarily alter the value for this dissociation constant since, as is shown in this section, only two of the coördination positions of the zinc are concerned in the bonding to insulin; at the other two positions, water molecules are probably still attached. The second dissociation constant of $Zn(H_2O)_4^{++}$ is, in all likelihood, of the same order of magnitude as the first. The dissociation of the last two hydrogen ions to form $ZnO_2^{=}$, however, does not take place until the *p*H is greater than 12, even in the absence of other complexing. In the present case, where $ZnO_2^{=}$ could not be formed without rupture of the insulin–zinc bonds, its formation would not be expected to occur within the *p*H region of the titration curve.

(13) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, Denmark, 1941.

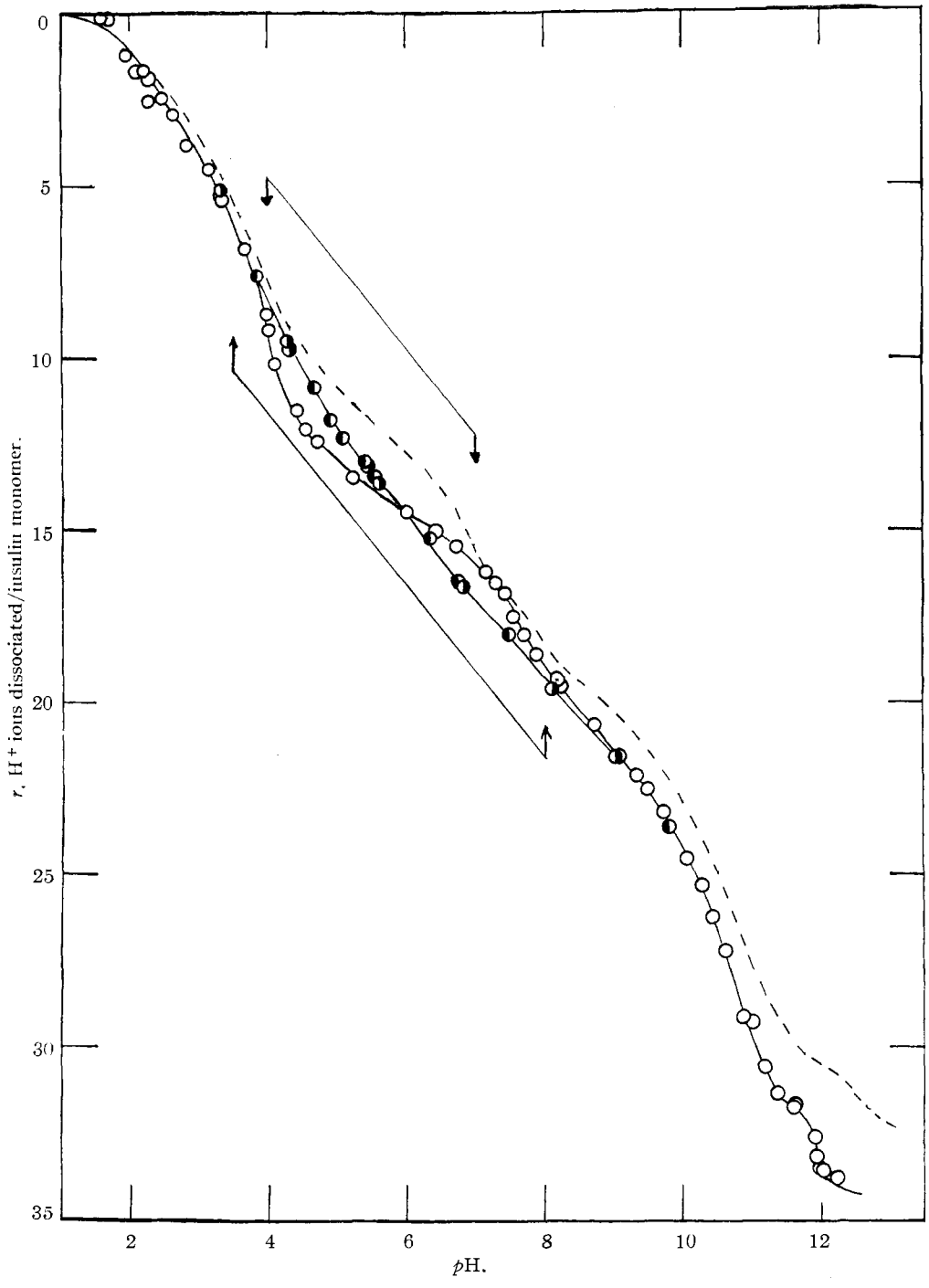

Fig. 1.—Hydrogen ion titration curves of zinc insulin at 25°, μ = 0.075: O, direct curve; ◑, acid-reversed; ◐, base-reversed. The dotted line is the curve for zinc-free insulin, taken from the preceding paper. The regions of partial insolubility of the direct and reversed curves are indicated, respectively, by the lower and upper arrows.

reached for the binding of cadmium.[14] Sulfhydryl groups probably are also involved in the combination of zinc with serum albumin,[15] but these groups are absent, of course, in insulin.

There is, however, an important difference between the binding of zinc by serum albumin and by insulin. Whereas in the former one imidazole group is involved per zinc ion, two such groups are clearly involved in the present case.[16]

It remains to account for the difference between

(14) C. Tanford and M. L. Wagner, THIS JOURNAL, **75**, 434 (1953).

(15) I. M. Klotz, J. M. Urquart and H. A. Fiess, *ibid.*, **74**, 5537 (1952).

(16) For the reversed curve this is perhaps not completely obvious from Fig. 1, because of the relatively low *p*H at which the water of hydration of the complexed zinc ions begins to be titrated. It should be noted, however, that there is a difference of two groups between the reversed curve and the curve for zinc-free insulin at *p*H 6, where dissociation of the zinc hydration sphere can hardly be appreciable. That approximately two groups are involved is, in any event, virtually proved by subsequent calculations (see text).

the direct and reversed curves of crystalline zinc insulin. In the latter, as indicated above, the zinc is not involved in the intermolecular bonds leading to precipitation; the precipitate is loose and highly hydrated, as in zinc-free insulin, and ions can pass in and out freely. Chelation probably occurs in a random fashion: any two imidazole groups from the same or from adjacent insulin monomers may be involved. In zinc insulin crystals, however, the association between monomer units is organized and much more intimate, free passage of ions is likely to be hindered, and the zinc is most probably chelated to two particular imidazole groups from adjacent monomer units (otherwise it would be difficult to account for the fact that the zinc bonding is the critical factor leading to crystallization).[17] Reaction of this inter-monomer bridge with hydrogen ion is likely to be difficult or impossible unless the crystalline structure is broken; *i.e.*, the reactions $-\mathrm{Im}-\mathrm{Zn}^{++}-\mathrm{Im}- + 2\mathrm{H}^{+} \rightarrow 2\ (-\mathrm{ImH}^{+}) + \mathrm{Zn}(\mathrm{H_2O})_4^{++}$, and $-\mathrm{Im}-\mathrm{Zn}^{++}-\mathrm{Im}- + 2\mathrm{H_2O} \rightarrow 2\mathrm{H}^{+} + (-\mathrm{Im})_2\ \mathrm{Zn}(\mathrm{OH})_2$ are likely to be pushed to lower and higher *p*H values, respectively, than in the amorphous precipitate, thus accounting for the difference between the direct and reversed titration curves of Figs. 1 and 3.

Rate of *p*H Changes.—The rate of change of *p*H of reversed mixtures is most rapid near the isoelectric point (*ca.* *p*H 5.6). The slow decrease in *p*H continues for several hours, and amounts to close to 0.2 *p*H unit at the end of 24 hours. The *p*H reached in this time is never as low as that corresponding to the direct curve (the separation is about 0.3 *p*H unit). However, conversion to the crystalline form was not judged to be complete, either.

Below *p*H 5 no appreciable *p*H change occurs. At this *p*H the protein has a charge of +2 per insulin monomer, and repulsive forces presumably prevent the more intimate alignment to the crystalline form.

At *p*H 6 the direct and reversed titration curves cross, and, above that *p*H, the *p*H drift, if it is to be explained as due to transformation to the crystalline state, should be toward higher *p*H values. Changes in *p*H in this direction were observed, though they appeared to be slower than those occurring acid to the isoelectric point, perhaps because the binding of zinc is almost complete above *p*H 6 (*cf.* Fig. 2) and dissociation must occur before realignment can take place.[18]

Finally, it should be noted that the reversed solutions at *p*H 9.0 and 9.8 (but not those of the direct curve in this *p*H range) showed a downward drift in *p*H. The cause of this is unknown.

Polarographic Measurements.—The polarographic current of a protein solution containing a metal ion, above the characteristic reduction potential of that ion, is made up of two portions.[5] The first is due to the reduction of free metal ion, the second, having a greatly reduced current per unit concentration, due to the reduction of protein-complexed metal ion. In the present case, the very tight binding of the zinc, coupled with the incomplete solubility of the insulin over the *p*H range of greatest interest, should reduce the contribution of protein-bound zinc virtually to zero. As a first approximation, therefore, the polarographic current can be taken as a measure of the free zinc concentration only.

If the conclusions reached above are correct, the *p*H-dependence of the polarographic current should thus show an irreversibility of the same kind as is found in the titration curve. This proved, indeed, to be the case, as shown by the data of Fig. 2.

Solubility Measurements.—In connection with the calculations reported below, some crude solubility studies were made. Temperature could not be adequately controlled in these measurements, as indicated in the experimental section. However, acid to the isoelectric *p*H, the results for the direct solubility curve agree tolerably well with a more precise determination (in 0.1 *M* NaCl) by Fredericq and Neurath,[19] whose data are also plotted in Fig. 2. Above the isoelectric point, however, the solubility observed by us (both direct and reversed) is much lower than found by Fredericq and Neurath, who, however, used a different lot of insulin from that studied in this paper. (It is quite possible that different lots, especially if of different zinc content, may behave differently.)

Chloride Binding.—A determination of the effect of *p*H on the binding of chloride by zinc insulin in 0.075 *M* KCl was reported, together with data for zinc-free insulin, in the preceding paper.[4]

Theory and Calculations

It would be desirable to apply to the direct and reversed titration curves of zinc insulin a theoretical analysis similar to that used for zinc-free insulin. Such an analysis would be especially interesting in the region of *p*H 4 to 8, where the most significant differences between the three titration curves of Fig. 1 occur. However, as reported in the preceding paper, it is not possible to arrive at an unequivocal analysis in this *p*H region even for zinc-free insulin, because there are too many variable parameters whose values are unknown. This difficulty is even more formidable in the case of zinc insulin. Not only are the same parameters present (and they do not necessarily have the same values as in the absence of zinc), but, in addition, it is necessary to introduce values for the association constant of zinc with imidazole and for the dissociation constants of the hydrated zinc–imidazole complex. Furthermore, account must be taken of the fact that above *p*H 7 or 8 the amino groups are able to compete with the imidazole groups for the zinc, as a result of which several new unknown constants appear.

These difficulties are counterbalanced to some extent by the existence of additional data for zinc insulin: the theoretical parameters used in fitting the titration curve must also satisfy the polarographic data of Fig. 2. Accordingly, the calculations reported below have been made to see whether reasonable theoretical assumptions can approximately account for the experimental data. The principal conclusion is that they can do so only if each zinc ion is bound to two imidazole groups of the insulin molecule, in confirmation of the same conclusion reached above from a qualitative examination of the titration curves.

Reversed Curve.—Evidence presented above indicates that the amorphous precipitate, the hydrogen ion dissociation of which is represented by the reversed titration curve, is very similar to the insulin precipitate obtained in the absence of zinc. Accordingly, the simplest possible assumption which can be made is that the intrinsic dissociation constants of all groups are the same as for zinc-free insulin,

(17) It is probable that two carboxyl groups are involved also, so that the inter-monomer bridge becomes electrically neutral. If a model is constructed, based on Sanger's order of amino acids and on Pauling's 3.7 residue helix, two carboxyl groups are found to be located in correct positions for this to occur.

(18) Two solutions between *p*H 6 and 6.4 behaved anomalously. There was a rapid initial *p*H drop of several hundredths of a *p*H unit, which preceded a subsequent *p*H rise. No explanation of this *p*H drop can be given. Only a slow *p*H rise was found for three solutions examined between *p*H 6.4 and 6.8.

(19) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

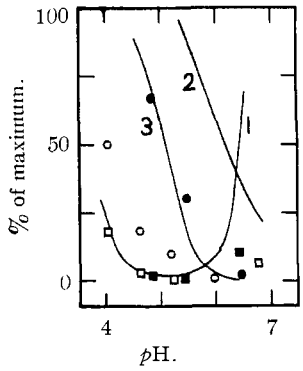

Fig. 2.—Polarographic diffusion current (circles) and solubility (squares) expressed as per cent. of maximum in 4.2 $\times 10^{-4}$ molal insulin. Open circles and squares represent direct titration, black circles and squares represent acid-reversed titration. Curve 1 is the solubility curve of Fredericq and Neurath. Curves 2 and 3 are computed reversed curves for the polarographic current, based on the binding of zinc to one and to two imidazole groups, respectively.

except that now some of the imidazole groups are complexed by zinc. The values of jw are also assumed the same as for zinc-free insulin at the same value of Z'. (The value of Z' at a given pH differs somewhat from that for zinc-free insulin because of the presence of bound Zn^{++}.)[20]

We may assume first of all that each Zn^{++} ion is bound to a single imidazole group only (as in serum albumin). At a given pH, let $\bar{\nu}_{Zn}$ indicate the average number of zinc ions so bound per insulin monomer. The degree of dissociation, x_i, of each type of acidic group is given by

$$\log \frac{x_i}{1 - x_i} = pH - (pK'_{int})_i + 0.868\, jwZ' \quad (1)$$

where the intrinsic dissociation constants, $(pK'_{int})_i$, are those given in Table II of the preceding paper,[4] and the alues of jw at a given value of Z' the same as those of Fig. 4 of the preceding paper[4] at the corresponding value of Z'. Equation 1 applies to all acidic groups other than to imidazole groups, and to $(4 - \bar{\nu}_{Zn})$ of the imidazole groups, *i.e.*, to all those not complexed.

For equilibrium between zinc and imidazole groups, we have

$$\log \bar{\nu}_{Zn}/\bar{\nu}_{IM} = \log C_{Zn^{++}} + \log K_{Zn} - 1.736 jwZ' \quad (2)$$

Here $\bar{\nu}_{IM}$, the average number of free non-protonated imidazole groups per insulin monomer, is equal to $x_{IM}(4 - \bar{\nu}_{Zn})$; $C_{Zn^{++}}$, the concentration of free zinc in solution, is equal to $(1 - \bar{\nu}_{Zn})P$, P being the total protein concentration[21]; K_{Zn} is the intrinsic association constant between Zn^{++} and free imidazole groups at the ionic strength used, the probable value being approximately, $\log K_{Zn} = 3.0$[22]; and the last term, representing electrostatic interaction, is twice as great as the corresponding term in equation 1 because a bivalent ion is involved, and opposite in sign because association, and not dissociation is involved.

With these substitutions, equation 2 becomes

$$\log \frac{\bar{\nu}_{Zn}}{(1 - \bar{\nu}_{Zn})(4 - \bar{\nu}_{Zn})} = \log x_{IM} + \log P + \log K_{Zn} - 1.736 jwZ' \quad (3)$$

In the present experiments, the value of P for all points in the neutral range is close to 4.2 $\times 10^{-4}$ molal.

Simultaneous solution of equation 3 and the several equations of the form of equation 1, by a method of successive approximation, yields a value for r, the total number of hydrogen ions dissociated per insulin monomer, as a function of pH

$$r = \bar{\nu}_{Zn} + (4 - \bar{\nu}_{Zn})x_{IM} + \Sigma n_i x_i \quad (4)$$

where n_i is the total number of groups of the i^{th} kind per insulin monomer, and the summation extends over all groups except imidazole groups. The charge, Z', per insulin monomer at any pH is given by

$$Z' = 12 - r + 2\bar{\nu}_{Zn} \quad (5)$$

Equation 4 is the equation of the titration curve (Fig. 1). The polarographic current curve of Fig. 2 should be equal to the ratio of free zinc to total zinc present

$$\frac{i}{i_{max}} = \frac{(1 - \bar{\nu}_{Zn})}{1} = 1 - \bar{\nu}_{Zn} \quad (6)$$

where i_{max} represents the maximum current, obtained when all of the zinc present, *i.e.*, 1 ion per insulin monomer, is free in solution.

The titration curve computed in this way is shown by curve 2 of Fig. 3. The computed polarographic curve is shown by curve 2 of Fig. 2. It is clear that neither curve agrees with the experimental data: the pH shift predicted is much less than that observed, the calculated polarographic currents are higher. The binding of zinc, in other words, is much stronger than has been assumed in this calculation.[23]

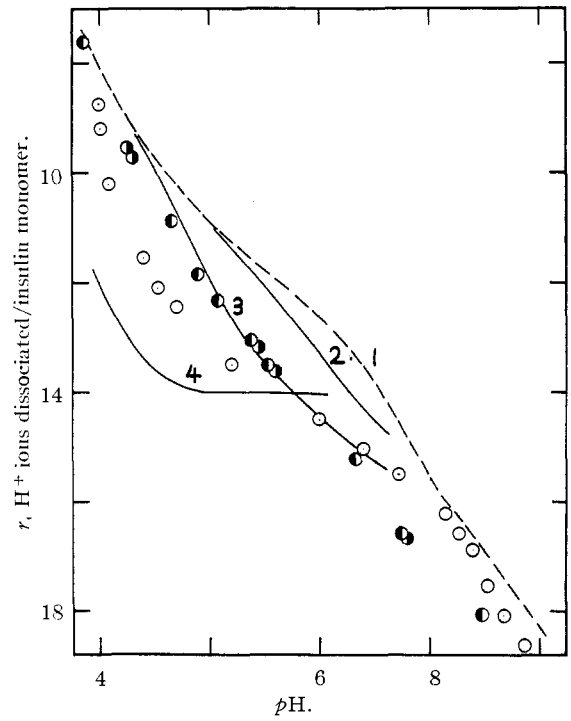

Fig. 3.—Computed titration curves for the region of pH 4 to 7. Curve 1 is the curve for zinc-free insulin, taken from the preceding paper. Curves 2 and 3 are computed reversed curves for zinc insulin, based on the binding of zinc to one and to two imidazole groups, respectively. Curve 4 is a computed direct titration curve, based on inaccessibility of the crystalline precipitate to hydrogen and hydroxyl ion. Experimental points: ○, direct curve; ◑, acid-reversed; ◐, base-reversed.

(20) In the preceding paper[4] it was shown that two sets of parameters could fit the titration of the imidazole and α-amino groups of zinc-free insulin. Only one of these, however, assigning intrinsic pK values of 6.4 and 7.45, respectively, to these groups, could be used in the region of precipitation. Since we are here concerned primarily with this region, it is these values which have been used.

(21) This relation arises from the fact that all the zinc present in solution must come from dissociation of the zinc initially present on the insulin. No additional zinc was added.

(22) The somewhat lower values reported for serum albumin, $\log K_{Zn} = 2.76$ (ref. 6) and $\log K_{Zn} = 2.9$ (ref. 5), were based on the value of 6.1 for the intrinsic hydrogen ion dissociation pK of the imidazole groups of that protein. A somewhat higher value should probably have been used (*cf.* ref. 24 of preceding paper[4]), which would have the effect of increasing the values of $\log K_{Zn}$ calculated in the references cited.

(23) The reason for the small pH shifts predicted for 1:1 binding, as compared to the much larger shifts observed elsewhere (*e.g.*, ref. 6), is that the amount of zinc in solution is extremely low. At the point of 50% binding it is only about 2 $\times 10^{-4}$ M, *i.e.*, one-half the zinc present originally in the insulin, compared to concentrations between 10^{-3} and 10^{-2} M, in the experiments of Gurd and Goodman.[6]

Thus one is led to conclude that most of the zinc is bound to two rather than to one imidazole group. One simple model is to suppose that all of the imidazole groups may be divided into binding sites, two per insulin monomer, each site consisting of two groups. These two groups may be on the same monomer unit, or on adjacent ones.[24,25]

As before, equation 1 represents the hydrogen ion equilibria of the dissociable groups. However, for each zinc ion bound, two imidazole groups (rather than one) are now removed from participation in this equilibrium, *i.e.*, it applies to $(4 - 2\bar{\nu}_{Zn})$ of the imidazole groups. In place of equation 2 we now have

$$\log \frac{\bar{\nu}_{Zn}}{\nu_{F.S.}} = \log C_{Zn^{++}} + \log K_{Zn} - 1.736jwZ' \quad (7)$$

where $\nu_{F.S.}$ represents the number of free sites per insulin monomer, *i.e.*, the number of sites with both imidazole groups free of both H^+ and Zn^{++}. The number of sites without zinc is $2 - \bar{\nu}_{Zn}$; the fraction of these entirely free from H^+ is x^2_{1M}, *i.e.*, $\nu_{F.S.}$ is equal to $x^2_{1M}(2 - \bar{\nu}_{Zn})$. All the other parameters have the same meaning as before, but the value of log K_{Zn} should now have about twice its previous value, *i.e.*, it is equal to 6.0.[26]

Substituting for $\nu_{F.S.}$ and, as before, for $C_{Zn^{++}}$, equation 7 becomes

$$\log \frac{\bar{\nu}_{Zn}}{(2 - \bar{\nu}_{Zn})(1 - \quad_{Zn})} = 2 \log x_{1M} + \log P + \log K_{Zn} - 1.736jwZ' \quad (8)$$

The value of r is now

$$r = 2\bar{\nu}_{Zn} + (4 - 2\bar{\nu}_{Zn})x_{1M} + \Sigma n_i x_i \quad (9)$$

the summation again extending over all groups other than imidazole groups. Equations 5 and 6 are unchanged.

The titration curve computed in this way is shown by curve 3 of Fig. 3; the computed polarographic curve by curve 3 of Fig. 2. Both curves are in reasonable agreement with the experimental data. This does not, of course, indicate that the simple model on which the calculation is based is correct, but it leaves little doubt as to the conclusion that most of the zinc ions, even on the reversed curve, are chelated to *two* imidazole groups.

The above calculations have not taken into account the dissociation of hydrogen ions from the hydration sphere of the zinc ion. If they are extended to higher *p*H values a curve is obtained which becomes identical with the curve for zinc-free insulin above *p*H 7.

In actual fact, the reversed curve of Fig. 1 differs from the curve for zinc-free insulin by nearly 2 groups at *p*H 6.75. This difference is far too large to be accounted for by dissociation of hydrogen ions from the hydration sphere of the zinc ion, if the first intrinsic dissociation constant of $(-1m)_2Zn(H_2O)_2^{++}$ is taken to be equal to Diehl's value[12] for the first dissociation constant of $Zn(H_2O)_4^{++}$, *i.e.*, log $K = -8.0$. A much larger value, log $K = -6.5$, must be used instead.[27] If this value is used, a value of *ca.* 0.16 must be assigned to jw to fit the titration curve above *p*H 7 to *p*H 9. For zinc-free insulin, for the most reasonable pK values, a constant value of jw of 0.10, corresponding to an actual molecular weight of 12,000, had to be used in this *p*H range. The value of 0.16, corresponds to the existence of insulin as a trimer in this *p*H range, in agreement with observed values of the molecular weight.[28,29] This suggests the possibility that the molecular weight of insulin on the alkaline side may depend on whether or not zinc is present.

Direct Curve.—The crystalline precipitate of zinc-insulin probably consists of a closely-knit array of monomer units, in contrast to the loosely-connected structure of the amorphous precipitate. The passage of ions in and out of the crystals is likely to be difficult. It is this which causes the flattening of the titration curve in the isoelectric region. Passage of ions is, however, not completely inhibited, for, if it were, the direct titration curve would fall along curve 4 of Fig. 3, and, since only dissolved insulin could contribute to the polarographic current, the direct polarographic data of Fig. 2 would fall along the solubility curve below *p*H 5.6, instead of above it.

(24) The model used represents, of course, a tremendous oversimplification. The number of possible arrangements in a randomly oriented precipitate is virtually infinite. Even on insulin in solution (in the *p*H range under consideration it exists largely as tetramer) it is most unlikely that all of the imidazole groups can be conveniently paired off, as we have assumed. The assumption that Zn^{++} is bound only to two imidazole groups, is, of course, also oversimplified. Undoubtedly some zinc ions are bound to only one imidazole group; others may be bound to as many as three or four.

(25) Structural considerations suggest that the two groups must be on adjacent monomer units. However, the simple equilibrium constants written are independent of the location of the groups.

(26) In the case of zinc, in contrast to cadmium and most other metals, the standard free energy change accompanying bonding to a second complexing group is approximately equal to that accompanying bonding to the first such group. *Cf.* ref. 13.

(27) The second dissociation constant has been assigned a value one fourth that of the first, allowing for the usual statistical factor. Electrostatic interaction with the charged protein molecule has been taken into account in making the calculations.

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

(29) J. L. Oncley and E. Ellenbogen, *J. Phys. Chem.*, **56**, 85 (1952).

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NOTES

Reactions of *p*-Dimethylaminochalcones with Acetic Anhydride

BY GERALD BRANCH AND JEROME F. THOMAS

RECEIVED DECEMBER 31, 1953

Katzenellenbogen and Branch[1] measured the spectra of the isomeric *p*-dimethylaminochalcones, *p*-$C_6H_5COCH{=}CHC_6H_4N(CH_3)_2$ and *p*-$(CH_3)_2NC_6H_4COCH{=}CHC_6H_5$, in methanol, in methanol 0.1 N with respect to hydrochloric acid, in acetic anhydride with 0.4% by volume of sulfuric acid and in concentrated sulfuric acid. Intense bands were found at 419 and 387 mμ in methanol, 294 and 315 mμ in acidified methanol, 483 and 490 mμ in acidified acetic anhydride and 425 and 396 mμ in sulfuric acid. The first of each pair of wave lengths is for the first-mentioned dimethylaminochalcone.

From each of these solutions the unchanged base was recovered by simple means. Assuming that the easy recoveries of the chalcones were simple formations of bases from salts, it was concluded that the four spectra from each chalcone were those of the free base and its ions with one, two and three charges. This would mean that the bands at 483 and 490 mμ were due to $C_6H_5CO^+HCH{=}CHC_6H_4N^+H(CH_3)_2$ and $(CH_3)_2\overset{+}{N}HC_6H_4C\overset{+}{O}HCH{=}CHC_6H_5$.

However, the peak of the principal band of C_6H_5-

(1) E. Katzenellenbogen and G. Branch, THIS JOURNAL, **69**, 1615 (1947).