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The Titration Curve of Insulin in the Presence of Various Bivalent Metal Ions

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1. Titration curves of insulin in the presence and absence of various metal ions are reported. 2. The difference in base consumption with and without the metal ions is compared with calculated curves. 3. These experiments suggest that in dilute solutions Zn^{2+} and Cu^{2+} ions are bound to α-amino groups.

Since the work of Tanford & Epstein (1954*a*,*b*) with titration curves of zinc-free and zinc-containing insulin little work has been done on this aspect of the physical chemistry of insulin. This work represents the most substantial evidence against the theory advanced by Marcker (1960) and Marcker & Graae (1962) that the Zn^{2+} ions form complexes with α-amino groups. It seemed reasonable therefore to reinvestigate the titration curves and discuss them in the light of the information on the insulin molecule that has accumulated since the original work of Tanford & Epstein (1954*a*,*b*). To use the titration curves to calculate binding constants it is important to determine them for a series of known Zn^{2+} ion concentrations. The titrations by Tanford & Epstein (1954*b*) were performed with only one concentration of metal ions. This concentration is estimated by the suppliers (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) to be 0·76 Zn^{2+}/mol. of 12000 Ins [to avoid discussion on the meaning of 'monomer insulin' in connexion with the work of Tanford & Epstein (1954*b*), the molecular weight is given followed by Ins], and by Tanford & Epstein (1954*b*) to be 0·95 Zn^{2+}/mol. of 12000 Ins. For their calculations Tanford & Epstein (1954*b*) use the value 1·0 Zn^{2+}/mol. of 12000 Ins in accordance with the previously accepted view that 3 Zn^{2+}/mol. of 36000 Ins were necessary for crystallization. Even though it now seems reasonable to accept the 0·76 estimate, further experiments with other Zn^{2+} ion concentrations are necessary to gain sufficient information for accurate calculations. I have therefore repeated the experiments of Tanford & Epstein (1954*b*) with Zn^{2+} ion concentrations of 0·0, 0·33 and 0·67 Zn^{2+}/mol. of 12000 Ins.

As Cu^{2+} ion-containing insulin crystals have been used for the determination of the properties of metal-containing insulin crystals (Brill & Venable, 1966), I decided to investigate whether or not Cu^{2+} ions react with insulin in dilute solutions in the same way as Zn^{2+} ions. I have also included experiments with Pb^{2+} ions.

MATERIALS AND METHODS

A pig insulin preparation (lot no. S4765), containing 14·92% N (corresponding to approx. 25·1 i.u./mg.), 97·2% dry matter and less than 0·01% Zn, was kindly provided by Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Determination of biological activity confirmed these values. (The analytical data were supplied by Novo Terapeutisk Laboratorium.)

The metal ions were added in the form of chlorides. All experiments were performed in 0·1 M-KCl. The pH was adjusted to 3·00 with 4 N-HCl and titrations were performed with 0·02 N-NaOH in an automatic apparatus (PHM25+ SBR2; Radiometer, Copenhagen, Denmark). In all experiments insulin was dissolved in 10 ml. of KCl solution, and the final volume of 11 ml. was obtained by adding metal ion solution and KCl. The titrations were usually followed to pH 10·5.

THEORY

The titration of one ionizable group, e.g.:

$$\geqslant NH^+ \rightleftharpoons \geqslant N + H^+ \qquad (1)$$

leads to the well-known titration curve seen as curve *A* in Fig. 1, where the abscissa is the difference pH − pK and the ordinate, x, is the acid fraction, i.e. the mole fraction of the protonated form of the group. If one of the forms of the ionizable group participates in another equilibrium such as:

$$\geqslant N + Me^{2+} \rightleftharpoons \geqslant N\text{–}Me^{2+} \qquad (2)$$

where Me^{2+} is a metal ion, this will cause a shift to the right of equilibrium (1); it is obvious that this shift depends on pH and the equilibrium constant, K_2, for equilibrium (2). Curve *B* in Fig. 1 shows the titration curve as it appears if K_2 is small, whereas curve *C* represents the curve for a very large value of K_2. If we plot the difference $x_1 - x_2$ of the ordinates of the titration curves in the absence and presence of metal ions for varying values of K_2, we obtain the family of curves shown in Fig. 2. It is seen that if K_2 is small the

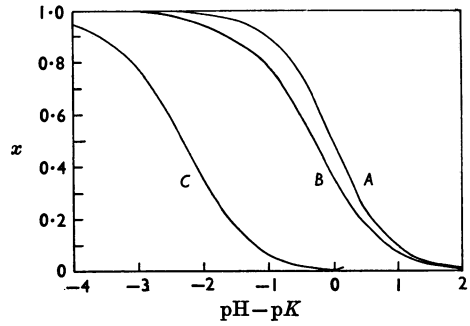

Fig. 1. Theoretical titration curves. *A*, Normal titration curve for a single group (dissociation of 1 H^+). The ordinate is the mole fraction of the protonized form. *B* and *C*, Titration curves as they appear if the base form of this group forms a complex with other ions: *B*, the curve for a small binding constant for this complex; *C*, the curve for a large binding constant.

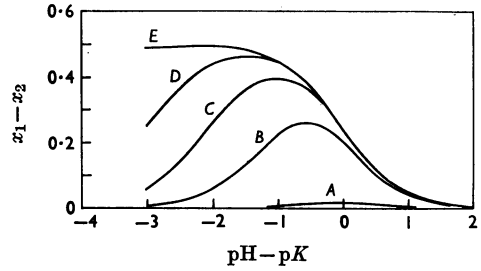

Fig. 2. Calculated titration-curve differences for various binding constants of the metal ion. Insulin concn., 0·15 m-mole/l.; metal concn., 25 μmoles/l. The ordinate (x_1-x_2) is the difference in the values of the acid fraction in the absence and presence of metal ions for given values of pH−pK. K_2 values: *A*, 10^3; *B*, 10^5; *C*, 10^6; *D*, 10^7; *E*, 10^9.

maximum of the difference curve is very near the pK value of the ionizable group (i.e. at pH−pK=0), and the maximum value of the ordinates, $(x_1-x_2)_{max.}$, is much below 0·5, the theoretical value for the concentration used in the calculation. When K_2 is large, the value of $(x_1-x_2)_{max.}$ is close to 0·5, but the maximum is situated far from the pK.

To find the exact expression for the difference curve we can proceed as follows.

For equilibrium (1) we have:

$$K_1 = \frac{[H^+][\geqslant N]}{[\geqslant NH^+]} \qquad (3)$$

If we denote $[\geqslant NH^+]$ by C and $[\geqslant N]$ by C_0-C, where C_0 is the total concentration of the implicated group, and if we calculate H^+ ion concentration relative to the dissociation constant K_1, eqn. (3) becomes:

$$x_1 = h/(h+1) \qquad (4)$$

where $h=[H^+]/K_1$ and x_1 is the acid fraction, C/C_0. In a system where both equilibria, (1) and (2), are established, we have:

$$K_1 = \frac{[H^+][\geqslant N]}{[\geqslant NH^+]} \qquad (3a)$$

and

$$K_2 = \frac{[\geqslant N\text{–}Me^{2+}]}{[Me^{2+}][\geqslant N]} \qquad (5)$$

where $[\geqslant NH^+]$ has a different value from that in eqn. (3). The acid fraction in the presence of metal ions may be expressed in terms of experimental parameters, as shown in the Appendix. The expression is:

$$x_2 = \frac{-K_2MC_0+K_2C_0-h-1\pm\sqrt{(K_2MC_0-K_2C_0+h+1)^2+4h\left(K_2C_0+\frac{K_2C_0}{h}\right)}}{2\left(K_2C_0+\frac{K_2C_0}{h}\right)}$$

where x_2 is the value of x_1 when Me^{2+} is present, M is the ratio of the total number of ionic bonds (3/ion) to binding groups (1/mol. of 6000 Ins) and K_2, C_0 and h are defined as before. Only the positive sign of the square root has a meaning, as the negative leads to negative concentrations.

From the equations for x_1 and x_2 we can now calculate the differences x_1-x_2 as a function of h, or pH−pK_1. This implies estimations of C_0 and M and an assumption for K_2.

In these calculations we have omitted the term $0{\cdot}868jwZ$ (Tanford & Epstein, 1954*a*,*b*), which will influence the calculations in two ways. The shape of the x_2-x_1 curve will not be correct as eqn. (3) should not be (in logarithmic form):

$$pK_1 = \text{pH}-\log\left(\frac{[\geqslant N]}{[\geqslant NH^+]}\right)$$

but:

$$\text{p}K_1' = \text{pH}-\log\left(\frac{[\geqslant N]}{[\geqslant NH^+]}\right)+0{\cdot}868jwZ$$

or:

$$\text{p}K_1' = \text{p}K_1+0{\cdot}868jwZ$$

If, for the jw term, we accept the empirical value 0·1 (Tanford & Epstein, 1954*b*), then the titration of one group will modify the pK by $0{\cdot}868\times0{\cdot}1\times1=0{\cdot}09$, when the p$K$ is near to 7·0. Thus the shape of the difference curves should not be affected significantly by this term.

The error in the determination of the actual pK values will, however, be proportional to the charge of the molecule. The net charge of the insulin monomer (mol.wt. 6000) is changed during the titration of the two histidine groups from +1 to −1 and during the titration of the two α-amino groups from −1 to −3. So the experimental pK values should be corrected by adding 0·00–0·26 as the net charge changes from 0 to 3.

Since the charge of the molecule and the nature of the groups binding the metal ions are not known, the right correction cannot be introduced from the start. However, the purpose of this investigation is only to distinguish between binding by histidine groups and α-amino groups, whose pK values differ by 1·05 according to Tanford & Epstein (1954*a*); thus the correction is not really necessary and can be neglected.

The calculations were made by using an ALGOL programme and model curves were calculated on a GIER digital computer with different values assumed for K_2, M and C_0. An example of these curves for $C_0 = 0{\cdot}15$ m-mole/l., $M = 0{\cdot}5$ and different values for K_2 is given in Fig. 2. What could be expected from the qualitative considerations previously described is clearly seen. If K_2 is small (approx. 10^3) the difference represents only about 5% of the theoretical maximum and the actual maximum is located at the pK, and if K_2 is large (approx. 10^9) the maximum approaches the theoretical value (0·5), but is situated 2–3 pH units below pK_2.

RESULTS AND DISCUSSION

In Fig. 3 are recorded the results from a typical experiment with different concentrations of Zn^{2+} ions, representing 1, 2, 3 and 4 Zn^{2+}/mol. of 36000 Ins. If we compare curve *1* in Fig. 3 with the calculated curves in Fig. 2 it is seen that the experimental curve is situated between the curves representing $K_2 = 10^5$ and $K_2 = 10^6$.

Table 1 allows us to determine more accurately the pK of the group involved. The Table records the values of $(x_1 - x_2)_{max.}$ and $(pH - pK)_{max.}$, the co-ordinates of the maxima of the curves in Fig. 2, together with the experimental values of $(x_1 - x_2)$ and the corresponding pH values from Fig. 3. It is seen that the curve representing 1 Zn^{2+}/mol. of 36000 Ins has a value for $(x_1 - x_2)_{max.}$ corresponding to $K_2 = 5 \times 10^5$ and that representing

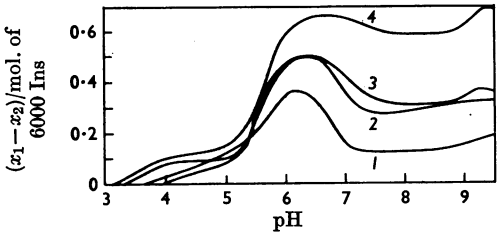

Fig. 3. Difference in base consumption on titration of insulin with and without Zn^{2+} ions. Curves *1*, *2*, *3* and *4* represent 1, 2, 3 and 4 Zn^{2+}/mol. of 36000 Ins respectively.

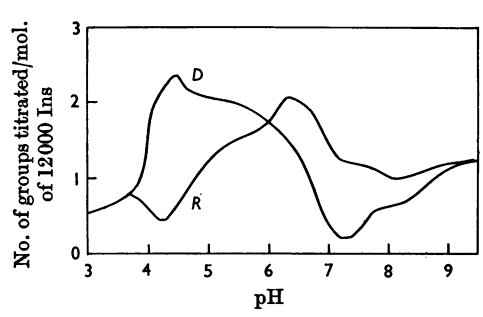

Fig. 4. Curves similar to Fig. 3 calculated from the direct (*D*) and reversed (*R*) titration curves of Tanford & Epstein (1954*b*, Fig. 1).

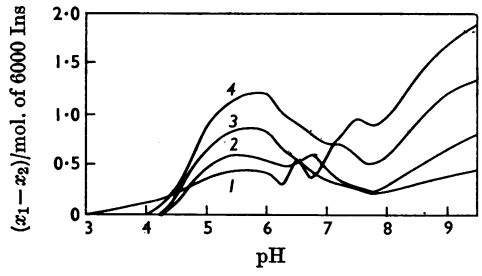

Fig. 5. Curves similar to those of Fig. 3, for Cu^{2+} ions.

Table 1. *Values of* $(x_1 - x_2)_{max.}$ *and* $(pH - pK)_{max.}$ *from insulin titration curve*

The theoretical values for $10^{-5}K_2$ 1–6 are taken from Fig. 2, and the experimental data from Fig. 3. For definition of the symbols see the text.

Zn^{2+}/mol. of 36000 Ins	1		2	
$10^{-5}K_2$	$(x_1 - x_2)_{max.}$	$(pH - pK)_{max.}$	$(x_1 - x_2)_{max.}$	$(pH - pK)_{max.}$
1	0·27	0·56	0·45	0·56
2	0·32	0·68	0·54	0·68
3	0·34	0·76	0·58	0·92
4	0·36	0·80	0·61	0·76
5	0·37	0·85	0·63	0·80
6	0·38	0·87	0·65	0·85
	$(x_1 - x_2)_{max.}$	pH	$(x_1 - x_2)_{max.}$	pH
Experimental	0·37	6·15	0·50	6·35

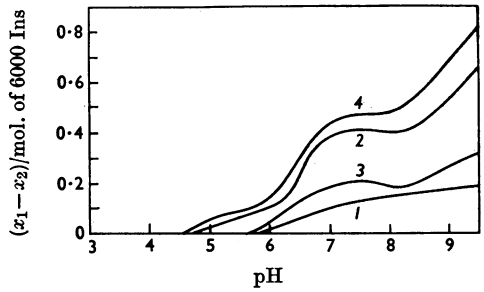

Fig. 6. Curves similar to those of Fig. 3 for Pb^{2+} ions.

2 Zn^{2+}/mol. of 36000 Ins a value close to that corresponding to $K_2 = 2 \times 10^5$. The pH differences are 0·85 and 0·68 respectively. From Fig. 3 the maximum values are found to be 6·15 and 6·35 respectively, indicating that the pK values of the group involved are 7·00 and 7·03 respectively. This excludes the histidine groups and points to the α-amino groups, as suggested by Marcker (1960).

The pK value estimated in this way is lower than that normally accepted; this is attributable to the omission of the *jwZ* term and of a correction for the ionic strength. Since both of these corrections would increase the pK by about 0·2pH unit, the correct pK is about 7·40. The exact magnitude of these corrections is unimportant; we can accept the value of 7·0 as a lower limit and compare this with histidine (pK 6·40) and α-amino groups (pK 7·45).

The conclusion is that in dilute solution the Zn^{2+} ions are bound primarily to the α-amino groups. This does not, of course, imply that these are the binding sites in crystals, where the metal ions may very well be bound to histidine, as suggested by Tanford & Epstein (1954*b*) and Brill & Venable (1966, 1967).

Fig. 4 gives the differences obtained in the same manner from the curves by Tanford & Epstein (1954*b*, Fig. 1). Comparison of Figs. 4 and 3 suggests that my curves are the same as the 'reversed' curves of Tanford & Epstein (1954*b*). The direct curves differ from my curves, and the present calculations imply that the binding must be different in the two cases.

The results of similar experiments with Cu^{2+} and Pb^{2+} ions are presented in Figs. 5 and 6. From Fig. 5 it is seen that the curves for copper show a higher maximum value at a lower pH. Curve *1* in Fig. 5 shows a maximum value of 0·45 situated at pH 5·7, corresponding to a binding constant for the metal of 10^7 and pK $5{\cdot}7 + 1{\cdot}5 = 7{\cdot}2$ (see Fig. 2), which indicates binding to the same groups as Zn^{2+} ions. Fig. 6 demonstrates that no binding occurs comparable with the binding of the other metal ions.

Thanks are due to Steen Højlund Simonsen, polytechnic student, for skilled technical assistance.

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APPENDIX

Calculation of the acid fraction

(*A*) Titration in the absence of metal ions:

$$K_1 = \frac{[H^+][\geqslant N]}{[\geqslant NH^+]}$$

(eqn. 3 of main paper).

Let:

$$[\geqslant NH^+] = C$$

$$[\geqslant NH^+] + [\geqslant N] = C_0$$

and

$$[H^+]/K_1 = h$$

and the acid fraction:

$$\frac{[\geqslant NH^+]}{[\geqslant NH^+] + [\geqslant N]} = \frac{C}{C_0} = x_1$$

we find:

$$[\geqslant N] = C_0 - C$$

$$1 + h = \frac{[\geqslant N] + [\geqslant NH^+]}{[\geqslant N]} = \frac{1}{1 - x_1}$$

and

$$x_1 = h/(1+h)$$

(eqn. 4 of main paper).

(*B*) Titration in the presence of metal ions:

$$K_1 = \frac{[H^+][\geqslant N]}{[\geqslant NH^+]}$$

(eqn. 3*a* of main paper).

$$K_2 = \frac{[\geqslant N\text{–}Me^{2+}]}{[Me^{2+}][\geqslant N]}$$

(eqn. 5 of main paper).

Let:

$$[\geqslant N] + [\geqslant NH]^+ + [\geqslant N\text{–}Me^{2+}] = C_0$$
$$[\geqslant NH^+] = C$$
$$[\geqslant N\text{–}Me^{2+}] = C_m$$

and the acid fraction:

$$\frac{[\geqslant NH^+]}{[\geqslant N] + [\geqslant NH^+] + [\geqslant N\text{–}Me^{2+}]} = \frac{C}{C_0} = x_2$$

Eqns. (3*a*) and (5) now are written:

$$h = \frac{C}{C_0 - (C + C_m)} \tag{3b}$$

$$K_2 = \frac{C_m}{[Me^{2+}][C_0 - (C + C_m)]} \tag{5a}$$

Multiplying eqns. (3*b*) and (5*a*) and rearranging the resulting equation gives:

$$\frac{K_2 C}{h} = \frac{C_m}{[Me^{2+}]} \tag{6}$$

From eqn. (3*b*) we find:

$$C_m = C_0 - C - \frac{C}{h} \tag{7}$$

which introduced in eqn. (6) gives:

$$\frac{K_2 C}{h} = \frac{C_0 - C - \frac{C}{h}}{[Me^{2+}]}$$

or:

$$\frac{C}{C_0}(K_2[Me^{2+}] + h + 1) - h = 0$$

or:

$$x_2(K_2[Me^{2+}] + h + 1) - h = 0 \tag{8}$$

Let the initial metal concentration be m_0, and let us assume three equal binding sites on one metal ion, then:

$$[Me^{2+}] = 3m_0 - C_m \tag{9}$$

Eqn. (7) together with eqn. (9) gives:

$$[Me^{2+}] = 3m_0 - \left(C_0 - C - \frac{C}{h}\right) \tag{10}$$

Now we call:

$$3m_0/C_0 = M$$

(see the Theory section of the main paper), which, since $C = C_0 x_2$, is introduced in eqn. (11):

$$[Me^{2+}] = C_0\left(M - 1 + x_2 + \frac{x_2}{h}\right) \tag{11}$$

Inserting eqn. (10) in eqn. (8) gives:

$$x_2 = \frac{h}{K_2 C_0\left(M - 1 + x_2 + \frac{x_2}{h}\right) + h + 1} \tag{12}$$

Eqn. (12) is transformed into:

$$x_2^2\left(K_2 C_0 + \frac{K_2 C_0}{h}\right) + x_2(K_2 M C_0 - K_2 C_0 + h + 1) - h = 0$$

Solving of this equation gives:

$$x_2 = \frac{-K_2 M C_0 + K_2 C_0 - h - 1 \pm \sqrt{(K_2 M C_0 - K_2 C_0 + h + 1)^2 + 4h\left(K_2 C_0 + \frac{K_2 C_0}{h}\right)}}{2\left(K_2 C_0 + \frac{K_2 C_0}{h}\right)}$$

(See the Theory section of main paper).