THE COMBINATION OF INSULIN WITH THIOCYANATE IONS

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Measurements of the electrophoretic mobilities of insulin reported by Hall (1, 2) are limited to regions higher than pH 7 and lower than pH 4.5, since the protein is practically insoluble in common buffer solutions of the intermediate pH range. While it has been possible to determine the electrophoretic mobility of adsorbed insulin and of suspensions of insulin crystals in the isoelectric range by the microscopic method (3), strict comparison of mobility data obtained under these two different experimental conditions is not always permissible (4). Both sets of data, however, indicate that the isoelectric point of insulin lies within the range of pH 4.9 to 5.9.

Attempts have been made in the present study to increase the solubility of insulin in its isoelectric region sufficiently to afford determinations of the electrophoretic mobility in this range by the moving boundary method. Of various ions of the lyotropic series that have been tested, thiocyanate was found to exert a marked peptizing effect. The lower pH limit of solubility just sufficient for mobility measurements was approximately pH 5, the solubility increasing with increasing pH. In the presence of thiocyanate, insulin remained insoluble, however, at all pH regions below 5, including those in which it is soluble in the absence of this ion, i.e. pH 2.

The electrophoretic data reported in this paper, together with the observed effect of thiocyanate on the solubility of insulin, suggest an interaction of this anion with certain basic groups of the protein. Considerable evidence has already been given for the binding by proteins of fatty acid anions (5–8), anionic detergents (9–13), and even anions of common buffer salts (14–16). Although some of these combinations may occur with the non-polar residue of the anion (17–18), all of them will be reflected by measurable changes in electrophoretic mobilities.

EXPERIMENTAL

Electrophoretic measurements were carried out at 1° with the Tiselius electrophoresis apparatus equipped with the Thoevert-Philpot-Svensson optical system (19). Only the mobilities of the descending boundaries were

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considered in this work. The time intervals between the initial and final photographs were generally 3 hours, and the potential gradients were within the range of 1.9 to 2.0 volts per cm.

The crystalline zinc insulin used in this work was obtained through the courtesy of the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

One series of measurements was carried out in buffer solutions containing 0.15 M NaCNS, the other in buffer solutions containing 0.15 M NaCl. Citrate, phosphate, and veronal buffers were employed. The citrate buffers were prepared by dissolving calculated quantities of citric acid and the desired amounts of NaCNS or NaCl, respectively, in 95 percent of the final volume of solution. This was titrated with NaOH to the desired pH with the aid of a Beckman pH meter, and then diluted with water to the final volume. The phosphate buffers were prepared by dissolving NaH₂PO₄ and Na₂HPO₄ in the desired ratio, together with NaCNS or NaCl respectively, in water and diluting to volume. In a like manner, the veronal buffers were prepared from diethylbarbituric acid and its sodium salt. The pH values of all buffer solutions were checked with the Beckman pH meter after their preparation.

The total ionic strength of all buffer solutions containing either 0.15 M NaCNS or 0.15 M NaCl was 0.18 ± 0.01.

The crystalline zinc insulin was dissolved in 20 cc. of buffer solution and allowed to dialyze against 500 cc. of buffer for 24 hours at 4°. The final protein concentration never exceeded 0.2 per cent. Because of the low protein concentration and high ionic strengths, the δ and ε boundaries resulting from electrophoresis were kept at a minimum.

**Results and Interpretation**

**Homogeneity of Crystalline Zinc Insulin in Buffer Solutions**—A photograph of a typical electrophoretic experiment is shown in Fig. 1. A single, practically symmetrical, boundary is evident, indicating a high degree of homogeneity. The δ boundary is negligible. These characteristics have been found in all experiments, regardless of pH and buffer composition.

**Electrophoretic Mobility in Presence of Thiocyanate**—Fig. 2 represents a plot of the electrophoretic mobility of insulin in buffers containing NaCl or NaCNS versus pH. Because of the limited solubility of the protein in buffer containing 0.15 M NaCl, measurements had to be confined to regions above pH 6.8 and below pH 4.2. In buffers containing 0.15 M NaCNS, the protein is soluble at pH 5.2 and higher but remains insoluble at all pH values lower than 5, including pH 2 at which pH it is readily soluble in buffers of the NaCl series.

It is evident from comparison of the two curves that, at equivalent pH
values, the anodic mobilities of the protein in the presence of 0.15 M NaCNS are markedly increased over those observed in the presence of 0.15 M NaCl.

Fig. 1. Descending boundary of crystalline zinc insulin in phosphate buffer containing 0.15 M NaCNS, after 3 hours migration. The total ionic strength was 0.18, the pH was 6.02, and the protein concentration was approximately 0.1 per cent. The sharp peak at the left is the starting boundary.

Fig. 2. A plot of the mobilities versus pH of insulin in buffers containing 0.15 M NaCNS (○) and in buffers containing 0.15 M NaCl (□). The curves connecting the points were drawn by visual inspection. The region in which the protein is insoluble in the buffer solution containing NaCl is indicated by the broken line.

In the pH region 5.2 to 5.8, the region in which insulin has been estimated to be isoelectric (1-3, 20), the protein, in the presence of 0.15 M NaCNS, still retains an anodic mobility of 3.0 to 3.5 units.
It seems apparent that the interaction between the insulin and thiocyanate causes an increase in the net negative charge of the protein, with the result that under the conditions of these experiments the protein remains negatively charged at all pH values at which it is soluble.

Estimation of Extent of Binding of Thiocyanate—An attempt has been made to correlate the pH-mobility curve of insulin in the presence of thiocyanate to the titration curve of the protein, and to estimate thereby the extent of binding of thiocyanate ions. Although titration data are available (cf. (21)), the insolubility of insulin in the isoelectric region, pH 4 to 7, precludes the use of these data for the present purpose. The analytical data by Brand (22), however, can be employed to calculate a theoretical titration curve for insulin. Using the data for the number of ionizable amino acid residues in the insulin molecule of molecular weight 45,000 and the mean estimated pK values for the ionizable groups as they occur in protein, given by Cohn and Edsall (21), one can construct a theoretical titration curve such as that given in Fig. 3. This curve has been con-
structured with the considerations that (a) at the start of the titration the protein has already bound its capacity of acid, and (b) the titration with base proceeds until the maximum base binding has been reached. At the left of Fig. 3, the titration curves for the individual ionizable amino acids in insulin are shown, including 9 arginine, 8 lysine, 30 tyrosine, 2 cysteine, 15 histidine, 23 aspartic, 5 glutamic, 21 terminal α-amino, and 21 terminal α-carboxyl residues per molecule. It has been assumed that the 21 residues of cystine in the insulin molecule yield no free ionizable groups. The large titration curve in the center of Fig. 3 is a plot of the total base bound, obtained by summation of the base bound by the individual amino acids, at various pH values, over small increments of pH. Comparison of this theoretical titration curve with the experimental titration curve (21) reveals the two to be superimposable from pH 2 to 12, exclusive of the region pH 4 to 7 for which no titration data are available.

The usual empirical procedure for comparing titration curves with pH-mobility curves consists in shifting the titration curve along the pH axis to the point at which 0 equivalent of acid or base bound coincides with isoelectric point (4, 23). Since electrophoretic mobilities near the isoelectric point could not be determined, recourse was had to an empirical conversion factor, which may be defined as the change in base equivalents bound corresponding to 1 unit change in mobility \((1 \times 10^{-3} \text{ cm sec}^{-1} \text{ volt}^{-1})\). This conversion factor was determined by selecting two adjacent, experimentally determined mobility values for reference. The difference in equivalents of base bound at the two pH values corresponding to the reference mobilities was divided by the difference in mobility units, and other points on the titration curve were compared to the mobility curve with this factor by the following relations: Let \(u_1, u_2, u_3, \ldots\) be the mobilities at pH\(_1\), pH\(_2\), pH\(_3\), etc.; \(e_1, e_2, e_3, \ldots\), are the corresponding equivalents of base bound. The conversion factor, \(F\), is then \(F = (e_1 - e_2)/(u_1 - u_2)\). The complete titration curve may then be calculated from this factor and the experimentally determined mobilities. For instance, \(e_3 = (u_3 - u_1) \times F + e_1\). In the present instance, the mobilities at pH 6.92 and 7.38 in 0.15 M NaCl and the corresponding equivalents of base bound were used as reference for the calculation of \(F\).\(^1\) The equivalents of base bound, calculated from the mobilities of insulin in the presence of 0.15 M NaCl at pH 8.42, 3.70, and 3.18 (\(e_3, e_4, e_6\)), are indicated on the titration curve of Fig. 3, together with the two reference points (\(e_1\) and \(e_2\) at, respectively, pH 6.92 and 7.38) by the

\(^1\) The value of \(F\) calculated from the present experimental data is 5.15. Calculation with the aid of the Henry theory (4, 23), the corrections subsequently introduced by Abramson et al. (4) for the "average" radius of the ions in the ion atmosphere being omitted, yields a value of \(e/u = 2.53\). The magnitude of the discrepancy between these two values is comparable to that previously found by Longsworth (23) for analogous calculations for egg albumin.
squares. It can be seen that the calculated points fit exceedingly well on the theoretical titration curve.

The same factor was used for the calculation of equivalents of base bound from mobilities determined in the presence of 0.15 M NaCNS. In this manner the change in base equivalents bound corresponding to the difference in mobility of insulin in the presence of 0.15 M NaCNS and 0.15 M NaCl, respectively, was determined. It can be seen that the points obtained for insulin in the presence of 0.15 M NaCNS are displaced in the direction of greater base binding.

From the titration curves of the individual amino acid residues it can be determined that at pH 5.8 the number of positively and negatively charged groups is equal. This pH, at which the net charge is zero, may be considered as the isoionic point. At the same pH, the equivalents of base bound by insulin in the presence of NaCNS exceed by 9 or 10 equivalents the amount of base bound by insulin in the presence of 0.15 M NaCl.

One may speculate that the binding of thiocyanate ions takes place through an electrostatic linkage with the strongly basic guanidino groups of the 9 arginine residues or with the ε-amino groups of the 8 lysine residues in the insulin molecule. This is suggested by the convergence of the mobility curves above pH 8 and by the effect of thiocyanate on the solubility of insulin in the acid pH region. It is of interest to note that the number of anionic detergent molecules bound by serum albumin in the first complex (11, 12) likewise coincides with the number of arginine residues in this protein. More direct evidence than that presented here must be forthcoming to prove this hypothesis.

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SUMMARY

The electrophoretic mobility of crystalline zinc insulin in buffers containing 0.15 M NaCNS and 0.15 M NaCl, respectively, has been investigated.

In the presence of thiocyanate, the solubility of insulin is sufficiently increased to afford mobility measurements with the moving boundary method within the isoelectric range. The increased electrophoretic mobility over that observed in the presence of NaCl corresponds to an increase in net negative change, suggesting association of thiocyanate with basic groups of the protein.
Comparison of mobility data with a theoretical titration curve of insulin calculated from summation of the base-binding capacities of the constituent amino acid residues reveals that at the isionic point of the protein a net negative charge of approximately 9 or 10 is retained in buffers containing 0.15 M NaCNS.

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