sum of the first and second ionization potentials is also quite suggestive. Such a correlation may be applicable in general to metal ion inhibition that involves reaction with negative groups.

Further investigation of the above points is being undertaken in this Laboratory.

Previous work[26] has demonstrated the existence of a linear relationship between pK_{sp} and the lattice energy of the crystal. Consequently, the correlations of pI with pK_{sp} and with ϵ_{1+2} cannot be considered as independent relationships.

Although good correlation with the general chelate stability sequence was not obtained, it is still quite possible that this process is involved in metal ion inhibition. In a complex protein molecule like urease, with the large number of electron donor groups available, it would be rather surprising if chelation were not involved.

Since more detailed knowledge of true inhibition mechanism was not available, the mathematical development of the pI concept was based on the inhibited Michaelis–Menten mechanism. Within this framework the conclusions presented above appear to constitute an acceptable tentative explanation of the relative toxicity of metal ions toward the enzyme urease.

AUSTIN, TEXAS

(26) See, for example, O. K. Rice, "Electronic Structure and Chemical Binding," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 417.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Physical Chemistry of Insulin. I. Hydrogen Ion Titration Curve of Zinc-free Insulin[1-3]

BY CHARLES TANFORD AND JACK EPSTEIN

RECEIVED AUGUST 21, 1953

The hydrogen ion titration curve of zinc-free insulin indicates the presence of 4 α-carboxyl groups, 8.5 β- and γ-carboxyl groups, 4 imidazole groups, 4 α-amino groups, 10 phenolic plus ϵ-amino groups (which could not be separated in the analysis) and 2 guanidine groups per insulin monomer, in complete agreement with the best available amino acid analyses. The intrinsic pK's of these groups are, respectively, 3.6, 4.73, 6.40 (or 6.0), 7.45 (or 7.2), 9.60 and 11.9. These values suggest that all of the acidic and basic groups freely into the solvent and do not participate in intramolecular bonding. Empirical values for the electrostatic interaction energy are used to estimate rough values of the degree of association of insulin at various pH values. The results are in fairly good agreement with the molecular weight determinations of Doty and others, and difficult to reconcile with a molecular weight of 6000 in aqueous solution below pH 10. About one chloride ion is bound per insulin monomer at pH 2, and some potassium binding may occur above pH 10.

The work described in this paper is part of a long-range program to investigate the reactive groups of protein molecules, with emphasis on their interaction with other molecules or ions, and on changes in chemical structure accompanying such interaction. In the case of insulin, the primary objective has been to gain an understanding of its interaction with zinc, since insulin, as isolated from living tissue, always contains combined zinc. However, prerequisite to an investigation of this interaction is a knowledge of the behavior of the reactive groups of the protein in the absence of zinc and of other ions with which they may combine. The present study was therefore undertaken, using a preparation from which all or most of the zinc had been removed.

Experimental

Insulin.—The insulin used was lot no. 190-4B-213A, and was donated by Eli Lilly and Co. It contained 0.027% of zinc or less, and had an activity of 27 u./mg., indicative of a high order of purity. The sample was completely amorphous, and dissolved in water to give a pH of 3.31, and in 0.075 M KCl to give a pH of 3.48. From the final assignment of the end-points of the titration curve, the latter pH value was found to correspond to 7.5 bound hydrogen ions per insulin monomer.[4] From e.m.f. measurements with silver–silver chloride electrodes (see below) a water solution was found to contain 8.0 ± 1.0 chloride ions per insulin monomer, *i.e.*, a number equal to the number of bound hydrogen ions. It was therefore concluded that the sample supplied was an insulin hydrochloride (Insulin, 7.5HCl). The insulin was stored in a stoppered bottle below 0°. Samples were allowed to come to equilibrium in a room at constant temperature and humidity before being weighed. Under the conditions used the moisture content, determined by heating to constant weight at 105°, was found to be 5.7%. Correction for this, and for an ash content of 0.37%, was made in all calculations.

Other Reagents.—Hydrochloric acid solutions were prepared by weight from a stock solution of constant boiling HCl.[6] Potassium hydroxide solutions were prepared by the method of Kolthoff,[7] and standardized against HCl. Potassium chloride solutions were prepared by weight from reagent grade KCl of low iodide and bromide content (J. T. Baker Chemical Co., Phillipsburg, N. J.). Conductivity water was used throughout.

Solutions for Measurement.—Solutions for all measurements were prepared by weight. About 0.05 g. of insulin was used for each determination, and the other reagents and water were added so as to attain a final ionic strength of 0.075 in each case.

Determination of pH.—All pH measurements were made with a Beckman model G pH meter, using an external glass electrode of type 1190-80. The instrument was standard-

(1) Presented at the 124th meeting of the American Chemical Society, Chicago, Ill., Sept. 6–11, 1953.

(2) This investigation has received its principal support from a research grant 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 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) Throughout this paper the term *insulin monomer* will be used to refer to the molecular unit of molecular weight about 11,500, consisting of the four polypeptide chains described by Sanger (ref. 5). It appears to be fairly well established that this is the lowest molecular weight unit of insulin which exists in aqueous solution, at least below pH 8.

(5) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353, 366 (1953); F. Sanger and H. Tuppy, *ibid.*, **49**, 463, 481 (1951).

(6) C. W. Foulk and M. Hollingsworth, THIS JOURNAL, **45**, 1220 (1923).

(7) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1922).

ized with Bureau of Standards potassium acid phthalate pH standard (pH 4.005). The apparatus used and detailed procedure have been previously described.[8] The electrode vessels were jacketed and measurements were made at 25.0°. The greatest possible care was taken to avoid contamination by carbon dioxide.

Results

The experimental results of the present study are summarized in Table I. The low solubility of insulin between pH 4 and pH 7 presented a problem not present in other proteins for which accurate hydrogen ion titration curves have been obtained, and pH measurements in this pH region had to be made on suspensions containing precipitated insulin. No difficulty in obtaining reproducible results was, however, encountered. The points obtained are plotted in Fig. 1, together with a computed curve which will be discussed below.

Stability and Reversibility.—One of the most important questions concerning a titration curve of the kind presented here is whether or not it represents equilibrium between the protein and hydrogen ion. This appears to be the case in the titration curve of zinc-free insulin. Acid solutions and suspensions in the neutral region did not change in pH over a 24-hour period, and even the most alkaline solutions drifted downward in pH only very slowly. Although irreversible changes must slowly be occurring in this region, pH readings could apparently be taken before such changes were appreciable. Most significant is the fact that insulin samples exposed for about 10 minutes to pH 11.0 returned, upon the addition of acid, to precisely the same points on the titration curve as would have been obtained had only the net amount of acid or base been added (*cf.* Fig. 1). In addition to proving that there is no irreversible uptake of base at the high pH, these experiments shed considerable light on the nature of the precipitate in the region of pH 4 to 7, suggesting (as appears also, by way of confirmation, from microscopic examination) that the precipitate is highly hydrated, allowing free passage of hydrogen, hydroxyl and other ions. No unusual adsorption of ions, as envisaged by Mommaerts and Neurath[9] was found to occur.[10]

Stoichiometry.—For a protein molecule as small as insulin, containing only about 30 titratable groups, it is possible to obtain directly from the titration curve an accurate estimate of the number of groups ionizing in the various pH regions.[11] The numbers of groups so obtained from Fig. 1 are summarized in Table II. They are accurate to about ±0.5 group. That the figure given for the carboxyl groups agrees with the analyses discussed in the footnote to Table II should therefore not be regarded as rigorous experimental support for these analyses. The number of guanidine groups has been assumed to be two. While some additional groups are evidently being titrated above pH 12, the points are too scattered for an estimate of their number to be made. (The corresponding region of the titration curve of zinc insulin, described in the following paper, shows less scatter, and does appear to support the presence of two guanidine groups.) Table II also contains the intrinsic pK values of the various groups, which are discussed in a later section of this paper.

Isoionic and Isoelectric Points.—Since the total number of cationic groups per insulin monomer is

TABLE I

TITRATION DATA, T = 25.00°, μ = 0.075

Moles insulin monomer/ kg. H_2O × 10^4	H^+[a] added moles/ kg. H_2O × 10^4	pH	H^+ free moles/ kg. H_2O × 10^4	H^+[b] bound moles/ insulin monomer	Moles H^+[c] dissociated/ insulin monomer
10.15	425.33	1.58	305.5	11.8	0.2
8.36	267.83	1.825	175.4	11.0	1.0
8.00	216.33	1.965	128.5	11.0	1.0
6.46	148.03	2.17	80.35	10.5	1.5
4.85	82.24	2.535	35.08	9.7	2.3
4.90	60.50	2.87	16.41	9.0	3.0
4.68	35.11	3.48	4.12	6.6	5.4
6.40	46.15	3.485	4.07	6.6	5.4
4.97	14.16	4.35	0.55	2.7	9.3
5.53	10.05	4.72	0.23	1.8	10.2
5.72	5.20	5.12	0.09	0.9	11.1
	OH^-[d] added moles/ kg. H_2O × 10^4		OH^- free moles/ kg. H_2O × 10^4	OH^- bound moles/ insulin monomer	
5.54	0.47	5.715[e]	0.00	0.1	12.1
4.96	1.53	5.765	.00	0.3	12.3
4.74	1.93	5.89	.00	0.4	12.4
5.96	6.79	6.233	.00	1.1	13.1
4.58	9.06	6.565	.00	2.0	14.0
4.85	18.03	7.033	.00	3.7	15.7
5.10	20.24	7.12[e]	.00	4.0	16.0
5.13	21.75	7.268	.01	4.2	16.2
5.29	29.45	7.775	.01	5.6	17.6
7.92	52.60	8.133	.02	6.6	18.6
4.65	34.28	8.495	.04	7.4	19.4
6.34	48.76	8.69	.07	7.7	19.7
4.87	37.35	8.765	.08	7.6	19.6
5.78	49.52	9.12	.19	8.5	20.5
6.42	59.67	9.385	.35	9.2	21.2
6.10	61.32	9.71	.73	10.0	22.0
5.48	64.22	10.125	1.90	11.4	23.4
7.02	85.72	10.265	2.62	11.8	23.8
5.96	83.05	10.538	4.91	13.1	25.1
7.95	146.33	11.125	18.97	16.0	28.0
7.00	182.85	11.62	59.29	17.6	29.6
7.27	305.34	12.07	167.10	19.0	31.0
8.70	324.00	12.078	170.20	17.7	29.7
9.70	391.38	12.175	212.8	18.4	30.4
11.66	529.13	12.327	302.0	19.5	31.5
12.31	561.15	12.365	329.6	18.8	30.8
7.41	481.11	12.38	341.2	18.9	30.9

[a] Including 7.5HCl per monomer unit present in the insulin preparation. [b] Referred to the isoionic point (pH 5.60). [c] Referred to the point of maximum acid binding. [d] Excluding 7.5KOH per monomer unit required to neutralize the HCl present in the insulin preparation. [e] Reversed points. The amount of H^+ or OH^- added is given as the net amount, *i.e.*, the difference between the actual amounts of KOH and HCl added.

(8) C. Tanford, *Symposium on Electrochemistry in Biology and Medicine*, in press.

(9) W. F. H. M. Mommaerts and H. Neurath, *J. Biol. Chem.*, **185**, 909 (1950).

(10) The titration curve of zinc-insulin (see following paper) is irreversible in the region of precipitation, but not for the reason suggested by Mommaerts and Neurath.

(11) C. Tanford, THIS JOURNAL, **72**, 441 (1950), Table IX.

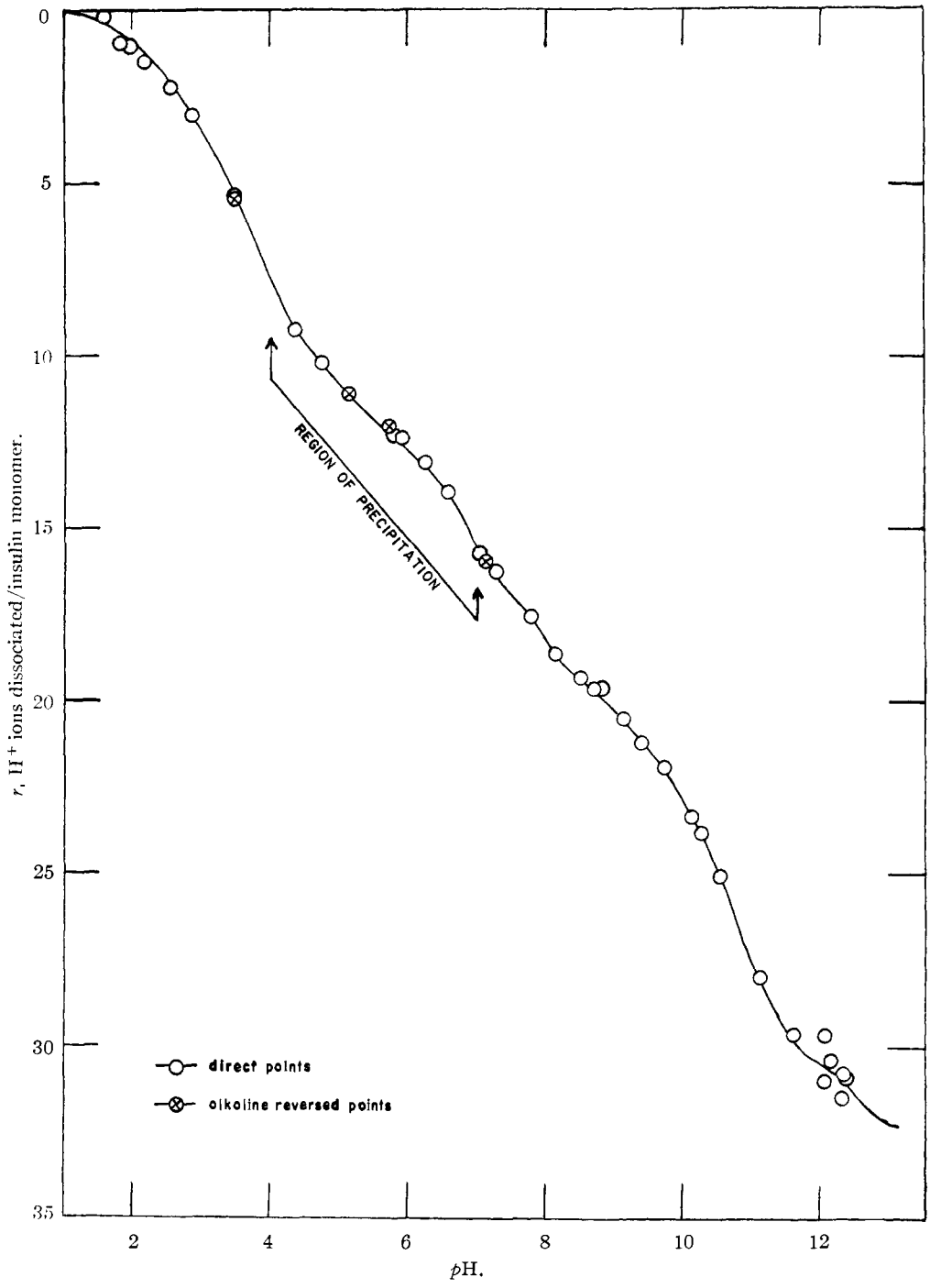

Fig. 1.—Hydrogen ion titration curve of zinc-free insulin at 25°, μ = 0.075. The crossed circles represent experimental points obtained after 10 minutes' exposure to *p*H 11. The curve drawn is a computed one, as discussed in the text.

12, the isoionic point is located 12 groups from the acid end-point, *i.e.*, at *p*H 5.60. As will be shown subsequently, there is no evidence for binding of either K^+ or Cl^- ions at this *p*H; the isoionic point should therefore be independent of ionic strength, and equivalent to the isoelectric point, in KCl solutions.

Chloride Binding.—In order to compute the molecular charge, required for the calculations discussed below, the extent of chloride binding by insulin in 0.075 *M* chloride solutions had to be determined. The method used was that of Scatchard, Scheinberg and Armstrong,[12] involving e.m.f. measurements with silver–silver chloride electrodes between protein solutions of known chloride content and standard KCl solutions. In view of the fact that most of the protein solutions used were of low *p*H, a correction for liquid junction potentials was estimated from e.m.f. measurements between HCl–KCl mixtures and KCl solutions.

The results, reported in Fig. 2, indicate that no appreciable binding of chloride occurs above *p*H 3. Even below that *p*H, the binding is small. The data are obviously

(12) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

TABLE II

NUMBER OF IONIZABLE GROUPS PER INSULIN MONOMER

Ionizing group	No. expected from amino acid content: Sanger[6]	Craig[13]	No. found from titration curve	Intrinsic pK
α-COOH	4	4	12.5	3.6 ± 0.3
β,γ-COOH	8[a]	8.5[a]		4.73 ± 0.10
Imidazole	4	4	4	6.40 (6.0)[b]
α-NH_2	4	4	4	7.45 (7.2)[b]
ϵ-NH_2	2	2	10	9.60 ± 0.10
Phenolic	8	8		
Guanidine	2	2	(2)	11.9 ± 0.2

[a] Craig has found that Lilly insulin contains about 75% of a component A identical in composition with the Boots insulin studied by Sanger, and 25% of a component B containing two extra carboxyl groups per insulin monomer, derived from hydrolyzed amide groups. This accounts for the presence of 8.5, on the average, rather than 8 β- and γ-carboxyl groups per insulin monomer in Craig's analysis. Since Lilly insulin was used in this work, our observed figures should agree with Craig's rather than Sanger's. As stated in the text, the difference is actually about equal to the probable error of our observations. [b] See text for discussion of alternative values.

quite inaccurate: to obtain data suitable for the evaluation of binding constants much lower concentrations of chloride would have to be used. The extent of binding in zinc–insulin is also given in Fig. 2. Since no difference could be observed (nor would any be expected at low pH), a single curve has been drawn through all the data.

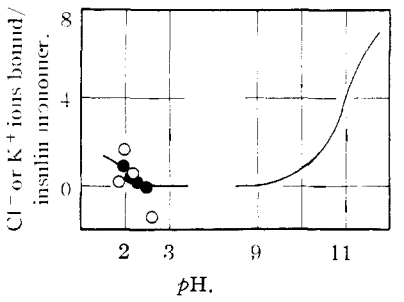

Fig. 2.—The binding of chloride and potassium ions in 0.075 M KCl. Solid and open circles represent experimental data for chloride binding for zinc–insulin and zinc-free insulin, respectively. The curve for potassium ion binding shows the number of such ions which must be bound to account for the decreased electrostatic interaction at high pH if no change in the parameter jw occurs in that region. It represents *maximum* values for K^+ binding; *cf.* Fig. 4.

Theory

If it is assumed that all ionizable groups of a given type (*e.g.*, all α-amino groups) are intrinsically identical, the degree of dissociation of such groups at any pH is given by[8]

$$\log \frac{x_i}{1 - x_i} = p\mathrm{H} - (pK'_{\mathrm{int}})_i + 0.868Zw \qquad (1)$$

where x_i is the degree of dissociation of groups of the i^{th} kind, $(K'_{\mathrm{int}})_i$ is their intrinsic dissociation constant at the ionic strength used, Z is the net charge on the protein molecule at the given pH, and w is an empirical parameter, depending, at constant temperature and ionic strength, only on the size and shape of the protein molecule, and defined so that $2RTwZ$ is the electrostatic work which must be done to remove a hydrogen ion from the surface of the protein molecule to infinity. If there are n_i groups of a given type per insulin monomer, the degree of dissociation, x_i, is equal to r_i/n_i, where r_i is the number of dissociated groups of the given type per insulin monomer. The ordinate of Fig. 1 is then equal to $r = \Sigma r_i$, where the summation extends over all the types of ionizable groups present.

The application of equation 1 to insulin is complicated by the fact that its molecular weight changes with pH, and that precipitation occurs over a portion of the titration curve. This does not interfere with the estimation of x_i, which is an experimental parameter independent of the true molecular weight, but does enter into the assignment of a value for Z, and the interpretation of the parameter w. The following modification of equation 1 has accordingly been made. The charge per insulin monomer, Z', can be obtained directly from the experimental data. If the degree of polymerization of insulin at any pH is equal to j, the true charge per molecule is jZ', and equation 1 becomes

$$\log \frac{x_i}{1 - x_i} = p\mathrm{H} - (pK'_{\mathrm{int}})_i + 0.868jwZ' \qquad (2)$$

This equation is identical with (1) except that the product jw has replaced the single empirical parameter w. Equation 2 can be used to evaluate intrinsic dissociation constants in exactly the same way as equation 1 is used for proteins of constant molecular weight. The same calculations also yield values for the empirical parameter jw.[8]

The interpretation to be put upon empirical values of w for proteins of known and constant molecular weight is relatively simple. If the protein molecule is spherical, and its charged groups are evenly distributed over its surface, w is given by[11]

$$w = \frac{N\epsilon^2}{2DRT}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \qquad (3)$$

where b is the radius of the protein sphere (including usually 10 or 20% of water of hydration), a is its radius of exclusion (usually assigned a value 2.5 Å. greater than b in KCl solutions), κ is the familiar constant occurring in the Debye–Hückel theory, and D is the dielectric constant of water. If the protein molecule is not spherical, if unfolding occurs to increase its extension in space, or if it swells by increased hydration, w will fall to lower values than would be predicted by equation 3. For the few globular proteins studied so far, values smaller than the calculated ones have been observed for ovalbumin[14] and for β-lactoglobulin[15,8]; for serum albumin[8] the observed value of w approaches the calculated one in neutral solution, but lower values are found with decreasing pH. Values of w higher than calculated by equation 3 have so far not been found.

By means of equation 3, values of the parameter jw for hypothetical spherical insulin molecules can be calculated, as shown in Table III, for different possible values of the true molecular weight. In mak-

(13) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952); L. C. Craig, papers presented at Sixth and Seventh Lilly Insulin Symposia, May, 1952, and 1953.

(14) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(15) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

ing the calculations, we have used a partial specific volume of 0.72 and have assumed the presence of 10% by weight of water of hydration, as suggested by Oncley and Ellenbogen.[16]

TABLE III

CALCULATED VALUES OF jw

j	Mol. wt.	b, Å.	w	jw
0.5	5,750	12.4	0.151	0.075
1	11,500	15.6	.107	.107
2	23,000	19.6	.0745	.149
3	34,500	22.4	.0601	.180
4	46,000	24.7	.0513	.205

If, now, the molecular weight of insulin at any pH were known with certainty (*i.e.*, if j were known), observed values of jw could be used as a measure of the extent of deviation of the molecule at that pH from a compact, spherical shape. Unfortunately, there is still considerable dispute among various workers concerning the true molecular weight of insulin under various conditions. For example, average molecular weights ranging all the way from 6000 to 24000 have been reported for insulin in aqueous solutions in the region of pH 2 to 3.

In the present work considerable variation in observed values of jw has been found. As a first approximation, this has been taken to reflect largely variation in true molecular weight, rather than in shape or hydration. Molecular weights obtained in this way turn out to be not unreasonable.

A further question presents itself with regard to the pH region of incomplete solubility. The values of jw in this region are certain to be higher than any listed in Table III: precise calculation would require knowledge of the extent of hydration of the precipitate. The intrinsic dissociation constants of some of the dissociating groups may also be different in the precipitate and in dissolved insulin, if these groups are involved specifically in intermolecular bonds holding the precipitate together. The calculations following show that it is not necessary to assume that this is so, *i.e.*, no inconsistencies occur if the intrinsic pK's of all the groups are taken to have the same, or nearly the same values on dissolved insulin molecules and on precipitated insulin. It is of interest to note that in crystalline zinc insulin (see following paper) the titration curve requires the participation of imidazole groups in the crystal structure.

Calculations and Discussion

The Carboxyl Groups.—Figure 3 (curve 1) shows a plot of $\log x_i/(1 - x_i)$ for the carboxyl groups of zinc-free insulin *versus* the value of Z' calculated from the titration curve and the chloride binding data of Fig. 2. Actual experimental points have been used, except below pH 2.5, where a smooth curve was drawn through the somewhat scattered points. In estimating x_i from the observed values of r for the most alkaline points, a small correction was applied for the number of imidazole groups titrated at each pH. The correction was based initially on estimated values of jw and of the intrinsic pK of the imidazole groups. Subsequent insertion of the final values for these constants did not significantly alter the results. The curve shows two regions of curvature, one at the most acid and the other toward the neutral side. The first of these is a downward curvature. As previously pointed out[8] this indicates that the groups chosen for analysis are not all intrinsically identical. This is, of course, not surprising since four of the carboxyl groups per insulin monomer are α-carboxyl groups which should have a different intrinsic pK.

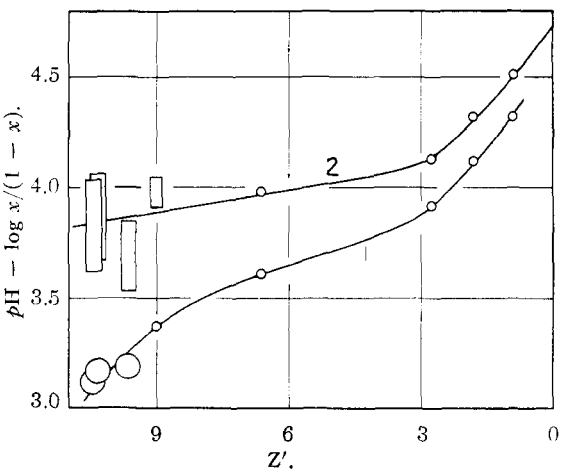

Fig. 3.—Titration of the carboxyl groups. In curve 1 all carboxyl groups are assumed identical. Curve 2 represents the titrations of β- and γ-carboxyl groups after correction for the presence of 4 α-carboxyl groups with an intrinsic pK of 3.6. For the points at lowest pH (highest Z) the difference between observed values of r and calculated contributions from the α-carboxyl groups is small, so that the experimental error becomes large.

Curve 2 of Fig. 3 shows a plot of the same kind in which correction has been made for the presence of these α-carboxyl groups. A reasonable intrinsic pK and reasonable values of jw were chosen, and the number of α-carboxyl groups dissociated at each pH was computed. This was subtracted from the observed values of r, leaving a net value due only to the β- and γ-carboxyl groups, derived from the aspartyl and glutamyl side chains. The intrinsic pK of the α-carboxyl groups was varied until the downward curvature was eliminated. The residual curvature at the neutral end of the curve is due to variation in the parameter jw.[8] Assuming that the remainder of the carboxyl groups are intrinsically identical (as is most reasonable to expect[8]), the intercept of this new curve at $Z' = 0$ represents the intrinsic pK of these groups. New values of jw, at other values of Z' were then obtained by substitution of this intrinsic pK into equation 2, and the entire calculation was repeated, using these values of jw. A second repetition was necessary, after which consistent values were obtained. (Curve 2 represents the final curve arrived at in this way.) The intrinsic pK required for the α-carboxyl groups to straighten curve 1 of Fig. 3 depends very strongly on how the titration curve is drawn through the most acid points. A value of 3.6 was needed for a reasonable curve giving equal weight to all points

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

below pH 2.5, but the required value differed by as much as ± 0.3 if somewhat different curves were drawn through the experimental points. The value of 3.6 is in good agreement with the intrinsic pK of α-carboxyl groups deduced experimentally by Ellenbogen.[17]

The intrinsic pK of the β- and γ-carboxyl groups was found to be 4.73. It was found to be quite insensitive to the corrections made for the α-carboxyl groups. Even if no correction is made at all, about the same value is obtained (*cf.* curve 1 of Fig. 3). The value of the intrinsic pK is very close to the value expected for normal carboxyl groups extending from a protein molecule.[18] A value of 4.60 has been obtained for β-lactoglobulin.[18] A lower value of 4.03, obtained for the carboxyl groups of both human and bovine serum albumin,[11,18] is indicative of some strong intramolecular interaction of these groups in the serum albumins. No such interaction appears to occur in insulin.

The computed values of jw are plotted as a function of pH in Fig. 4, together with similar values from subsequent calculations. They, too, depend only weakly on the way in which the titration curve is drawn at the acid end. The increase from pH 2 to 4 is always regular, and absolute values differ by no more than 10 or 20% at any pH. Allowing for a probable error of this magnitude, the empirical values of Fig. 4 indicate that insulin exists at pH 2 either as monomer or dimer, more probably the latter if cognizance is taken of the fact that the calculated values for any given value of j in Table III are likely to be somewhat too large. A sharp increase in association is indicated between pH 3 and 4, and at pH 4 the value of jw corresponds to the

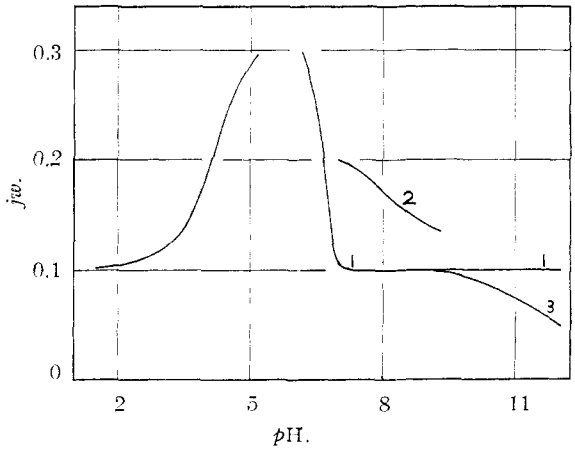

Fig. 4.—Empirical values for jw as a function of pH. On the alkaline side branch 1 represents a constant molecular weight of about 12,000, and is compatible with intrinsic pK's of 6.40 and 7.45, respectively, for the imidazole and α-amino groups; and with the potassium ion binding curve of Fig. 2. Branch 2 is compatible with intrinsic pK's of 6.0 and 7.2, respectively, for the imidazole and α-amino groups. Branch 3 gives the empirical values of jw above pH 9.5 if no potassium is assumed bound at all. In this region the true variation in jw probably lies between curves 1 and 3.

(17) E. Ellenbogen, THIS JOURNAL, **74**, 5198 (1952).

(18) Reference 8, Table III.

tetramer or even higher. These conclusions are in qualitative accord with the molecular weight determinations on the acid side made by Doty and coworkers,[19,20] or with the somewhat lower molecular weights observed by Oncley and Ellenbogen.[16] A molecular weight as low as 6000 at pH 2.7, as reported by Fredericq,[21] would be difficult to reconcile with our values of jw.[22]

It should be noted that the data of Fig. 3 extend into the precipitated region and that the ability to extrapolate into that region without introducing any inconsistency suggests that the intrinsic pK of carboxyl groups is close to the same on dissolved and precipitated insulin molecules. In this region, the values of jw increase well above 0.2 as precipitation increases above pH 4, in accord with expectation.

Imidazole and α-Amino Groups.—Because there is no pH region in which either the imidazole or the α-amino groups are essentially the only ones being titrated, the method of calculation applied to the carboxyl groups proved to be inconvenient. The procedure adopted instead was to allow jw, and the two intrinsic pK's under consideration to vary over a wide range of possible values, and to find sets of values of these parameters to fit the titration curve by means of equation 2 in the region of pH 7 to 9. Due allowance was made for the contribution to the experimental values of r by the carboxyl groups, using the pK values already determined, and also for the very small contribution due to the phenolic and ϵ-amino groups. A solution for the entire pH range was sought, which had the same pK values throughout and which did not involve an increase in jw with increasing pH (which could have implied an increase in association with pH).

Essentially two solutions can be obtained, with possible minor variations. The first solution gives intrinsic pK's of 6.0 and 7.2, respectively, for the imidazole and α-amino groups, with jw decreasing steadily from 0.20 at pH 7 to 0.12 at pH 9. These values of jw are again quite reasonable. Both Oncley and Ellenbogen[16] and Creeth[23] assign a molecular weight of 36,000 to insulin immediately above pH 7, and Creeth[23] has shown that dissociation occurs above that pH. However, the intrinsic pK values are unexpectedly low. Imidazole groups have an intrinsic pK of 6.8 in β-lactoglobulin,[15] of 6.7 in ovalbumin,[14] and of about 6.5 in serum albumin.[24] Similarly, Ellenbogen's suggested intrinsic pK for α-amino groups is 7.8.[18] Another objection to this solution for the region of pH 7 to 9 is that the intrin-

(19) P. Doty, M. Gellert and B. Rabinowitch, THIS JOURNAL, **74**, 2065 (1952).

(20) P. Doty and G. E. Myers, *Discs. Faraday Soc.*, No. 13, 51 (1953).

(21) E. Fredericq, *Nature*, **171**, 570 (1953).

(22) It should be pointed out that these calculations are not particularly sensitive to the number of α-carboxyl groups assumed present. Essentially similar results, with only slightly lower pK's and slightly higher values of jw, are obtained if two, instead of four, α-carboxyl groups are assumed present.

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

(24) The pK of 6.1 previously given for human serum albumin (ref. 11) was obtained by assuming the presence of nine α-amino groups per mole. It now appears that only one such group is present. This has the effect of increasing the intrinsic pK of the imidazole groups. Recent investigations show that for both human and bovine albumin the intrinsic pK is about 6.5.

sic pK's found do not give the correct isoionic point, *i.e.*, different values (6.4 and 7.4, respectively) would have to be used for the imidazole and α-amino groups when they are present in the precipitated region. That this should be the case is inherently not impossible: it could occur, for example, if imidazolium–carboxyl bonds contributed toward the stability of the precipitate. In that case, however, the carboxyl groups should exhibit a corresponding difference, which, as shown above, is not in fact observed.

The second set of parameters which can fit the titration curve between pH 7 and 9 are intrinsic pK's of 6.40 and 7.45, respectively, for the imidazole and α-amino groups, and a constant value for jw of 0.10 throughout the region. These pK values are much more reasonable, and, in addition predict the correct isoionic point, *i.e.*, the same values can be used in the precipitated region. The value of jw, however, suggesting that insulin exists as a monomer throughout this region, appears to be in conflict with the best molecular weight determinations, cited above. This is probably a reflection of the crudeness of calculation of the electrostatic correction. It is also possible, however, that the molecular weight of zinc-free insulin in solution above pH 7 may be smaller than that of zinc insulin, which was used in the molecular weight determinations cited above.

The Alkaline Region.—Above pH 9, the charge per insulin monomer, Z', is no longer known with certainty, since it is likely that potassium ions will be bound by the protein. Potassium ion binding in serum albumin at high pH has been demonstrated by recent studies by Carr.[25] For this reason, the calculation of intrinsic constants in this region has not been made as thoroughly as in the lower pH regions. The 2 ϵ-amino groups, which dissociate in this pH range, have arbitrarily been assigned the same intrinsic pK as the 8 phenolic groups, by which they are overshadowed. It turns out, however, that to avoid an increase in jw with increasing pH, or an oscillatory variation in this parameter, the choice of intrinsic pK's for these groups, and for the two guanidine groups, is quite limited. The intrinsic pK for the phenolic and amino groups cannot be varied by as much as 0.1 from the value of 9.60. This is precisely the value to be expected for the phenolic groups.[11] For ϵ-amino groups, intrinsic pK's of 9.4 and 10.1 have been reported for serum albumin[11] and ovalbumin,[18] respectively. For the guanidine groups an intrinsic pK of 11.9 gives a reasonable fit of the data.

The variation in electrostatic free energy occurring in the alkaline region can be interpreted in two ways. If jw is assumed to remain constant at 0.10 between pH 9 and 12, it is necessary to use negative charges smaller than those calculated from the titration curve alone, *i.e.*, it is necessary to assume that K^+ ions are bound. The number of K^+ ions which must be bound to satisfy the experimental data are plotted as a function of pH in Fig. 2. If, alternatively, it is supposed that no potassium ions are bound, a sharp decrease in jw must occur as shown by the dotted line in Fig. 3. This would be indicative of an unfolding or swelling of the insulin molecules in that region, or of dissociation of the monomer to smaller fragments. Such dissociation might occur by means of interchange of disulfide bonds, the possibility of which has been reported by Sanger[26] and by Huggins, Tapley and Jensen.[27] It might be mentioned that our data do not give support to Fredericq's contention[22] that the molecular weight falls to a value of 6000 at a pH as low as 9.

Conclusion.—All of the intrinsic pK values derived above are tabulated in Table II, and the titration curve computed from them and from the values of jw of Fig. 3 is plotted in Fig. 1. It is seen to agree completely with the experimental data. The pK values derived suggest that all of the acidic and basic groups of zinc-free insulin extend freely into the solvent, and do not participate in intramolecular bonding. This is in contrast to zinc insulin, discussed in the following paper, and also to serum albumin and ovalbumin. In all of these proteins some of the acidic or basic groups have definitely been shown to participate in intramolecular bonds, such participation leading to abnormal pK values or to inability to titrate the groups involved reversibly.

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(25) C. W. Carr, personal communication

(26) F. Sanger, *Nature*, **171**, 1025 (1953).

(27) C. Huggins, D. F. Tapley and E. V. Jensen, *ibid.*, **167**, 592 (1951).