TABLE II

CALCULATED APPARENT EQUILIBRIUM CONSTANTS FROM SEDIMENTATION

$[Ag]_0$[a]	$[Ag]_{app.}$[b]	$[Ab]_0$[a]	$[AgAb]_0$[a]	K_i
		$K_t = 10^4$		
2	1	10	2	3.2×10^4
5	4.3	4	2	1.9
10	9.5	2	2	1.7
		$K_t = 10^5$		
1.04	0.41	2.60	2.7	4.1×10^5
1.65	1.18	1.65	2.7	2.3
2.60	2.27	1.04	2.7	1.9
		$K_t = 10^6$		
0.38	0.068	0.95	3.6	9.0×10^6
0.60	0.38	0.60	3.6	2.6
0.95	0.80	0.38	3.6	2.0

[a] $[Ag]_0$, $[Ab]_0$, $[AgAb]_0$ are the molar concentrations $\times 10^5$ of free Ag, free Ab and of aggregate initially present in equilibrium in a solution. [b] $[Ag]_{app}$ is the apparent molar concentration $\times 10^5$ of free Ag which would be observed in the ultracentrifuge pattern.

I indicate that the average value of $K_i = 1.0 \pm 0.5 \times 10^4$ obtained in the accompanying paper[2] is, if anything, too small, but by no more than a factor of about $1/3$. At the present time, such a factor is not much greater than the experimental error. Second, the calculations also reveal that re-equilibration effects should produce opposite results in the ascending electrophoresis compared to the ultracentrifuge patterns: the free Ag area in the former should be greater than in the latter, with the true equilibrium value in between. No significant differences were found in the free Ag areas obtained by the two methods in the experimental system. We conclude, therefore, that the apparent K_i values calculated from the electrophoresis data in the accompanying paper are close to the true values. This conclusion was reached independently[2] from the close correspondence of the equilibrium constants calculated from electrophoresis and light scattering experiments.

That a difference is expected between the free Ag areas in the electrophoresis and ultracentrifuge patterns is a result that is generally useful. It is valid independent of the Gilbert–Jenkins model and applies to multivalent Ag–Ab systems as well as to the simpler BSA-S-R_1:anti-R case. It is the result of the fact that Ag–Ab aggregates, whatever their composition or distribution, always have an electrophoretic mobility between that of free Ag and free Ab,[16] and always have a sedimentation constant greater than that of free Ag and free Ab. If re-equilibration occurs to any significant extent in the ascending electrophoresis limb[16] the aggregates must always react to produce more than the original equilibrium amount of free Ag, whereas in the ultracentrifuge, the free Ag must always be diminished below the equilibrium amount by reactions producing aggregates.

With two multivalent Ag–Ab systems, BSA:anti-BSA[5] and RBSA:anti-R,[7] electrophoretic apparent free Ag areas were found to be not greater than ultracentrifuge values. In fact, in the former system, the latter were slightly larger, probably due to the Johnston–Ogston anomaly. We may conclude therefore that in these systems as well, the apparent K_i values are close to the true ones.

Acknowledgment.—We are grateful to Dr. R. C. Ll. Jenkins for helpful correspondence and advice.

(16) If $|V^E_{Ag}| < |V^E_{Ab}|$, the roles of the ascending and descending limbs are reversed as compared to the BSA-S-R_1:anti-R system, where $|V^E_{Ag}| > |V^E_{Ab}|$.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Thermodynamics of the Ionization of the Lysyl Residue of Insulin[1,2]

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RECEIVED JANUARY 24, 1959

The thermodynamics of the ionization of the ϵ-amino group of the single lysyl residue of insulin have been studied in order to determine whether or not this group is interacting with some other group in the protein. In order to separate the region of ionization of the lysyl residue from that of the four tyrosyl residues, the insulin has been iodinated. All of the tyrosyl residues have been converted to diiodotyrosyls, with no other apparent modification of the protein. By means of titrations in the alkaline pH range at four temperatures and by the direct measurement of the change of pH with temperature, the enthalpy of ionization of the lysyl ϵ-amino group of iodinated insulin has been found to be approximately 13 kcal./mole. For purposes of comparison, the thermodynamics of ionization of the amino groups of n-butylamine, lysine and alanyllysylalanine have been studied by the same methods. The enthalpy of ionization of the ϵ-amino group in the protein agrees well with the values for the model compounds. The apparent pK's and entropies of ionization also agree as well as can be expected, in view of the uncertainties in the electrostatic corrections. If the lysyl group in the protein were involved as the donor in a strong hydrogen bond with some other group, increases of approximately 5 kcal./mole in the enthalpy and 13 e.u. in the entropy over the values observed in model compounds would be expected. These increases are not at all in evidence, indicating either that no lysyl hydrogen bond is present in iodinated insulin or that the bond is too weak to be detected by the methods employed.

Introduction

The location of intramolecular interactions between specific side-chain groups of a protein molecule could be of value in elucidating the spatial configuration of the protein in solution. Insulin is a good subject for this type of study because its amino acid sequence, including the location of the

(1) Presented, in part, before the Division of Biological Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

(2) This investigation was supported by research grant No. E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, Public Health Service.

(3) National Science Foundation Predoctoral Fellow, 1953–1957.

disulfide bonds, has been determined by Sanger and co-workers.[4] The presence of three intramolecular disulfide bridges in the insulin monomer[5] places a considerable restriction on the possible spatial configurations of the molecule. Evidence has also been presented[6,7] for the existence of a tyrosyl-carboxylate ion hydrogen bond in the insulin molecule, which would further restrict the number of possible spatial configurations.

The insulin monomer contains a single lysyl residue, at the B-29 position.[4] This offers the opportunity of studying for the first time the properties of a single naturally-occurring ionizable group in a protein and of pinpointing the location of a hydrogen bond or other specific interaction if the lysyl group is involved in one. Several models for the spatial structure of insulin, based on the Sanger sequence,[4] have been proposed.[8-10] The possible interactions of the lysyl residue vary from one model to another, so that the discovery of a lysyl residue interaction might furnish evidence for or against some of the proposed spatial structures of insulin. The purpose of this investigation was to determine whether or not the lysyl group does interact with some other group.

Method of Study

Theory.—Since the ϵ-amino group of the lysyl residue is positively charged in aqueous solutions at pH's up to 9.5 or higher,[11] it could act as a donor in a hydrogen bond with some acceptor in the insulin molecule.[12] The existence of such a hydrogen bond would affect the thermodynamic parameters for the dissociation of the proton from the lysyl ϵ-amino group.

A general theory for the effect of hydrogen bonding on the behavior of polar R-groups of proteins has been presented.[13] This theory will be used to predict the behavior of a hydrogen-bonded lysyl group. The notation will be that of reference 13, with K_{ij} representing the equilibrium constant for the formation of a hydrogen bond between an ith donor and a jth acceptor and with the other thermodynamic constants for the hydrogen bond having similar subscripts. The enthalpy of formation of a hydrogen bond has been estimated by Pauling[14] to be -6 to -8 kcal./mole, but Schellman[15] estimates that in aqueous solution this is reduced to about -1.5 kcal./mole due to hydrogen bonding to the solvent. A reasonable value for ΔH^0_{ij} in proteins would appear to be about -6 kcal./mole in aqueous solutions. This value has been used to account for the data on the polymerization of fibrin monomer[16] and on the ionization of tyrosyl and carboxyl groups in bovine serum albumin.[13,17] It has also received confirmation from direct and spectrophotometric titrations of salicylic acid.[18] The entropy change associated with the formation of a hydrogen bond, due mainly to the loss of rotational freedom about single bonds in the side-chains of the participating groups, is estimated to be between -2.5 and -7.5 e.u. for each single bond rotation frozen in.[13] Hence, for a hydrogen bond involving a lysyl residue (which itself has 5 single bonds), $-\Delta S^0_{ij}$ would be expected to be quite large. However, it is likely that in such a long chain not all the single bond rotations would be completely frozen in and $-\Delta S^0_{ij}$ would be somewhat reduced. Loeb and Scheraga[17] calculate that K_{ij} is 4 for the tyrosyl hydrogen bond in serum albumin. By assuming this value for a lysyl hydrogen bond, together with $\Delta H^0_{ij} = -6$ kcal./mole, a value of $\Delta S^0_{ij} = -17$ e.u. at room temperature is obtained.

The changes which will be observed in the thermodynamic parameters of the lysyl residue, if it is involved as the donor in a hydrogen bonding interaction, can be estimated from equations I-38 through I-41 of reference 13. Assuming that $\Delta H^0_{ij} = -6$ kcal./mole, $\Delta S^0_{ij} = -17$ e.u. and $K_{ij} = 4$

$$pK_{obsd} - pK = \log (1 + K_{ij}) = 0.7 \quad (1)$$

$$\Delta F^0_{obsd} - \Delta F^0 = RT \ln (1 + K_{ij}) = 1 \text{ kcal./mole} \quad (2)$$

$$\Delta H^0_{obsd} - \Delta H^0 = -\frac{K_{ij}}{1 + K_{ij}} \Delta H^0_{ij} = 5 \text{ kcal./mole} \quad (3)$$

$$\Delta S^0_{obsd} - \Delta S^0 = -\frac{K_{ij}}{1 + K_{ij}} \Delta S^0_{ij} + R\left[\frac{K_{ij}}{1 + K_{ij}} \ln K_{ij} - \ln (1 + K_{ij})\right] = 13 \text{ e.u.} \quad (4)$$

where pK_{obsd} is the value observed for the donor group in the protein, pK is the value which would be observed in the absence of hydrogen bonding, and the other thermodynamic functions are derived from pK_{obsd} and pK in the usual manner. It should be pointed out that the estimates made in equations 1–4 are for a fairly strong hydrogen bond and would be considerably smaller for a weaker bond.

In principle, a comparison of any one of these thermodynamic functions for the ϵ-amino group in insulin with the value for the same group in low molecular weight model compounds should give an indication of the presence or absence of a hydrogen bond between the lysyl residue and some other residue in insulin. However, there are other factors

(4) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955), and earlier references cited therein.

(5) The term *insulin monomer* will be used to refer to the unit of molecular weight 5,733, consisting of one A-chain and one B-chain as described by Sanger.[4]

(6) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga, *Biochim. Biophys. Acta*, **19**, 581 (1956).

(7) S. J. Leach, M. Laskowski, Jr., and H. A. Scheraga, Abstracts of 131st Meeting, American Chemical Society, Miami, Florida, April, 1957, p. 48-C.

(8) K. Linderstrøm-Lang, "Symposium on Peptide Chemistry," The Chemical Society, London, 1955, Special Publication No. 2, p. 1.

(9) H. Lindley and J. S. Rollett, *Biochim. Biophys. Acta*, **18**, 183 (1955).

(10) B. W. Low, unpublished work quoted in "Proceedings, International Congress of Biochemistry, Brussels, 1955," Academic Press, Inc., New York, N. Y., 1956, p. 114.

(11) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163, 2170 (1954).

(12) A. E. Mirsky and L. Pauling, *Proc. Natl. Acad. Sci., U. S.*, **22**, 439 (1936), postulated that hydrogen bonds between side-chain carboxyl and amino groups might exist in proteins. H. A. Saroff, *J. Phys. Chem.*, **61**, 1364 (1957), has postulated that lysyl amino groups function as donors in hydrogen bonds in bovine serum albumin.

(13) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(14) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Natl. Acad. Sci. U. S.*, **37**, 205 (1951); L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1944, p. 323.

(15) J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, **29**, 223, 230 (1955).

(16) J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, THIS JOURNAL, **77**, 6168 (1955).

(17) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1633 (1956).

(18) J. Hermans, Jr., S. J. Leach and H. A. Scheraga, in preparation.

which affect the thermodynamic properties of the lysyl residue in insulin which might not be present in the model compounds. The most important of these is probably the electrostatic contribution. Equation 1 can be rewritten in terms of pK^0, the pK in the absence of hydrogen bonding and electrostatic interaction, as

$$pK_{obsd} = pK^0 + \log (1 + K_{ij}) - \frac{2wZ}{2.3} \qquad (5)$$

The last two terms on the right side of equation 5 represent the contributions of the hydrogen bonding term and the electrostatic interaction term, respectively. The latter depends on Z, the net charge of the protein, which is difficult to determine because it depends not only on the number of protons dissociated but also on the number of ions bound to the protein. Furthermore, the electrostatic factor w is difficult to determine without numerous assumptions, particularly for insulin because this protein tends to polymerize.[19] The contribution of the electrostatic correction in the region of the lysyl ionization in insulin can be of the order of one pK unit, while, as was found in equation 1, the hydrogen bonding term is only about 0.7 pK unit for $K_{ij} = 4$. Therefore, in view of the large uncertainty in the electrostatic correction term, it would be difficult to detect a hydrogen bond from the difference between the pK observed in the protein and that found in model compounds.

The effect of the electrostatic term on the observed enthalpy of ionization can be examined by applying the van't Hoff relationship to equation 5 to yield

$$\Delta H^0_{obsd} = (\Delta H^0)^0 - \frac{K_{ij}}{1 + K_{ij}} \Delta H^0_{ij} - 2R \frac{d(wZ)}{d(1/T)} \qquad (6)$$

where $(\Delta H^0)^0$ is the enthalpy corresponding to the ionization constant K^0. The electrostatic term can be estimated by assuming an expression for w. For a sphere of uniform surface charge[20,21]

$$w = \frac{Ne^2}{2DRT}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \qquad (7)$$

where N is Avogadro's number, e is the electronic charge, D is the dielectric constant of the medium, b is the radius of the protein, a is the radius of exclusion of the protein and the hydrogen ion, and κ is the inverse of the radius of the ionic atmosphere. If the net charge Z on the protein can be kept constant with changing temperature, the electrostatic contribution to ΔH_{obsd} will be

$$\Delta H_{elec} = -2RZ \frac{dw}{d(1/T)} = -ZNe^2\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \frac{d(1/DT)}{d(1/T)} + \frac{ZNe^2}{DT} \frac{d}{d(1/T)}\left(\frac{\kappa}{1 + \kappa a}\right) \qquad (8)$$

assuming that a and b are independent of temperature. Since the variations with temperature of the quantities κ and (DT) in aqueous solutions are both small, the electrostatic contribution to the enthalpy change for the lysyl ionization will be less than 400 cal./mole.[22] The hydrogen bonding contribution on the other hand was calculated in equation 3 to be about 5 kcal./mole for $\Delta H^0_{ij} = -6$ kcal./mole and $K_{ij} = 4$. Hence, an investigation of the enthalpy of ionization of the lysyl ϵ-amino group in insulin and in model compounds appears to offer promise as a means of determining whether or not this group is hydrogen bonded in the protein. The entropy of ionization could also be studied, but, in addition to the electrostatic term, it is likely that specific solvent effects make a sizable contribution to the entropy change in aqueous solution. Thus, the deviation of the enthalpy change from normalcy appears to be the best indication of the existence of hydrogen bonds.

Two methods for determining the enthalpy of ionization of the lysyl residue in insulin have been employed. First, the titration curve of the protein in the region of lysyl ionization has been determined at several temperatures. The value of pK_{obsd} then was determined as a function of temperature and ΔH^0_{obsd} was calculated by means of the van't Hoff relationship. With this method, a value for ΔF^0 as well as for ΔH^0 is obtained, but, due to difficulties in curve fitting to be discussed later, the uncertainty in ΔH^0 is rather large. Second, the change in pH with temperature at a fixed degree of ionization x of the lysyl group has been studied directly. For the ionization of a single group[21]

$$pH = pK_{int} + \log \frac{x}{1 - x} - \frac{2wZ}{2.3} \qquad (9)$$

where pK_{int} is the sum of pK^0 and the hydrogen bonding contribution, in case there is one. The enthalpy is obtained by applying the van't Hoff relationship to equation 9 (recalling that $\log x/(1 - x)$ is fixed and that the temperature variation of the electrostatic factor is negligible) to give

$$\Delta H^0 = -2.3RT^2\left(\frac{\partial pK_{int}}{\partial T}\right)_x \cong -2.3RT^2\left(\frac{\partial pH}{\partial T}\right)_x \qquad (10)$$

and ΔH^0 includes the contribution of the hydrogen bonding term if there is one. The enthalpy of ionization is obtained more directly by this second method, but the free energy of ionization cannot be calculated because the degree of ionization is not determined.

Isolation of the Lysyl Ionization.—Titration curves of insulin indicate that there is considerable overlapping of titratable groups in the region of lysyl ionization. Tanford and Epstein[11] were unable to separate the lysyl from the four tyrosyl groups per insulin monomer and assigned to both an intrinsic pK of 9.60 at 25°. They assigned intrin-

(19) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

(20) K. Linderstrøm-Lang, *Compt. rend. trav. Lab. Carlsberg*, **15**, no. 7 (1924).

(21) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(22) Equation 8 can be used to calculate the order of magnitude of ΔH_{elec} for insulin in 2 M KBr at 25° in the region of lysyl ionization, assuming the protein to be dimerized. Letting $\mu = 2.0$ moles/l., $Z = -17$ at the pH of lysyl ionization, $b = 15.6$ Å. and $a = b + 2.5$ Å. for the insulin dimer,[11] and $\frac{T}{D}\frac{dD}{dT} = -1.37$,[23] gives

$$\kappa = \sqrt{\frac{8\pi Ne^2\mu}{1000\ DkT}} = 4.64 \times 10^7\ \text{cm.}^{-1}$$

$w = 0.053$ and $\Delta H_{elec} = -320$ cal./mole. This is likely to be a maximum value for ΔH_{elec} since the value of -17 assumed for Z is reduced probably by the binding of positive ions such as potassium ions. Tanford and Roberts[24] calculated that for bovine serum albumin at 0.15 ionic strength $\Delta H_{elec} < 1$ kcal./mole even if $Z = -100$.

(23) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd Ed., Reinhold Publ. Corp., New York, N. Y., 1950, p. 118.

(24) C. Tanford and G. L. Roberts, Jr., THIS JOURNAL, **74**, 2509 (1952).

sic pK's of 7.45 and 11.9 to the two α-amino groups and to the single guanidinium group of arginine, respectively. The ionization of these latter groups may also overlap somewhat with that of the ϵ-amino, but the overlap would be much less than that due to the tyrosyl residues since the separation in pK's between the ϵ-amino and the α-amino and guanidinium groups is of the order of two units in each case.

In order to more nearly isolate the lysyl ionization in insulin, it is therefore necessary to eliminate, as much as possible, the overlapping with the tyrosyl residues.[25] By converting tyrosine to 3,5-diiodotyrosine, it is possible to lower the pK of the phenolic hydroxyl group by approximately three pK units.[26] The enthalpy of ionization is also reduced from 6 to 0.8 kcal./mole.[27,28] Hence, if the tyrosyl residues in insulin can be fully iodinated without otherwise modifying the protein, it should be possible to study the lysyl ionization without interference from the tyrosyl groups. It should be pointed out that if there are tyrosyl residues involved as hydrogen bond donors in insulin as some evidence[6,7] suggests, such bonds would probably be broken in the iodinated derivative at the pH of lysyl ionization. Hence, it is possible that iodination may cause a change in the configuration of the insulin molecule, although if the formation of the tyrosyl hydrogen bonds is reversible the change in configuration, if any, should not be great.

Harington and Neuberger[29] prepared a fully iodinated insulin derivative and compared its electrometric titration curve in the alkaline range with that of the native protein. Their results indicate that the specific iodination of the tyrosyl residues in insulin is feasible and that the overlap of the ionization of the tyrosyls with that of the lysyl residue can be considerably reduced, although perhaps not completely eliminated.

Iodinated Insulin and Model Compounds

Iodinated Insulin.—All the insulin used in this work was Lilly crystalline beef zinc insulin,[30] lot No. 535664. It was iodinated by a slight modification of the procedure described by Oster and Malament,[31] using iodine labeled with radioactive I^{131} tracer. Analyses of the iodinated insulin for I^{131} showed it to be 94 to 100% iodinated, on the basis of two iodine atoms per tyrosyl residue (or eight iodine atoms in each insulin monomer). Therefore, all the insulin could be assumed to be 100% iodinated within the experimental error (about 5%) in the analyses, and a molecular weight of 6740 has been assumed for the iodinated insulin monomer throughout this work. Studies of paper electrophoresis, sedimentation velocity, DNP-end groups, paper chromatography of the acid hydrolyzate, ultraviolet absorption spectra and spectrophotometric and electrometric titration curves indicate that the iodinated insulin is homogeneous and fully iodinated on the tyrosyl residues, with no other apparent modifications. A more detailed characterization of the iodinated derivative may be found elsewhere.[32]

Model Compounds.—If the criterion for the existence of a lysyl hydrogen bond in insulin is to be the deviation from "normalcy" of the thermodynamic parameters for ionization of the lysyl ϵ-amino group, then it is necessary to determine what the "normal" values are. It was decided, therefore, to study the ionization of amino groups in low molecular weight model compounds under the same conditions as would be used for the study of the protein. The range of normal thermodynamic constants thus found in model compounds should provide a valid basis for comparison with the thermodynamic constants of the lysyl ϵ-amino group in the protein.

The various model compounds studied are listed: *n*-Butylamine, purchased from Carbide and Carbon Chemicals Co., dried with anhydrous sodium sulfate and calcium sulfate and then used without further purification. This is a good, simple model for the lysyl residue since it has a primary amino group on a four carbon chain. L(+)Lysine monohydrochloride purchased from Mann Laboratories, Inc. was used without further purification. The overlapping between the ionization regions of the α- and ϵ-amino groups in this amino acid is similar to that in iodinated insulin. Alanyllysylalanine monohydrochloride (3L), sample[33] number S-200, was used without any further purification; this peptide has a sequence very similar to that in insulin (which has Pro.Lys.AlaOH at the C-terminal end of the B-chain).[4] Thus, the immediate surroundings in the chain are almost the same for the lysyl group in insulin and in this peptide, making this an excellent model for study. The overlap with the α-amino group is much smaller than that in lysine, so that the ionization of the ϵ-amino group can be separated out and studied more readily.

Titrations

In order to determine the values of the thermodynamic constants for the ionization of the ϵ-amino group of the lysyl residue, titrations of iodinated insulin and the model compounds have been carried out. A continuous titration procedure has been employed in order to conserve material. To reduce the spreading of the protein titration curve due to electrostatic interactions (see equation 9), 2 M KBr has been used as the solvent.

Materials.—The iodinated insulin and model compounds have been described above. The concentrations were approximately 0.006 M for *n*-butylamine and alanyllysylalanine as determined from the titration curves, between 0.01 and 0.05 M for lysine as determined by direct weighing, and approximately 1.2% (0.002 M) for iodinated insulin as determined from the dry weight after heating at 104°.

All chemicals were reagent grade unless otherwise indicated. Carbonate-free potassium hydroxide was prepared according to the method of Kolthoff.[34] It was made 1 M in KBr and was standardized against potassium acid phthalate. The standard buffer was 0.01 M borate, prepared as described by Bates.[35]

Apparatus.—The pH meter used for most of the experiments was a Beckman Model GS calibrated at 10 mv. intervals according to the method of Bates.[36] One duodial division was found to correspond to 0.197 mv. Beckman No. 1190-80 general purpose glass electrodes were used for all measurements. Special calomel electrodes with a long side-arm with asbestos fiber tip, filled with 3.15 N KCl, were prepared. This KCl concentration avoided crystal forma-

(25) The subtraction of the spectrophotometric titration curve of the tyrosyl residues from the over-all titration curve of insulin was considered unsatisfactory for two reasons. The subtraction of the curve for four groups from that for five would involve a large experimental error. Furthermore, because of the ionization of the tyrosyl groups, the net charge on the protein would vary considerably over the pH range of lysyl ionization, as well as varying with temperature at a fixed degree of lysyl ionization. This would make the determination of the pK and ΔH for ionization of the lysyl ϵ-amino group quite difficult.

(26) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(27) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, Chap. 4, pp. 75–115.

(28) J. B. Dalton, P. L. Kirk and C. L. A. Schmidt, *J. Biol. Chem.*, **88**, 589 (1930).

(29) C. R. Harington and A. Neuberger, *Biochem. J.*, **30**, 809 (1936).

(30) We are indebted to Dr. O. K. Behrens of the Eli Lilly Co. for gifts of zinc insulin.

(31) G. Oster and S. Malament, THIS JOURNAL, **76**, 3441 (1954).

(32) L. Gruen, M. Laskowski, Jr., and H. A. Scheraga, *J. Biol. Chem.*, in press.

(33) The alanyllysylalanine was kindly supplied by Dr. Bernard F. Erlanger of Columbia University.

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

(35) R. G. Bates, "Electrometric pH Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, pp. 74–83, 118–121.

(36) R. G. Bates, ref. 35, pp. 287–289.

tion and subsequent clogging of the electrode at low temperatures. The long side-arm permitted the calomel electrode to be maintained at room temperature even when the solution being measured was at some other temperature. To assure temperature equilibration, both the calomel and glass electrodes were kept at the proper temperature for at least 24 hr. before use.

The buret was a Gilmont ultramicroburet with glass plunger, of one-ml. capacity, graduated in 5000 divisions. Linearity was checked by titrating strong acid *vs.* base, using about 0.3 ml. of solution from the buret for each titration, and no difference could be detected over various parts of the scale. The titration cell was a 30-ml. lipless beaker enclosed in a water jacket through which water from the appropriate constant temperature bath could be circulated. The 25 and 35° baths were regulated to within 0.01°, and the 10 and 0° baths were kept constant to within 0.1° or better. It was necessary to ground the *p*H meter and the water in the constant temperature baths because the flowing water could otherwise build up charges and cause erratic behavior of the *p*H meter.

A piece of iron wire enclosed in a glass tube, small enough to stir the solution without hitting the electrodes, was used in conjunction with a magnetic stirrer. The latter had to be removed before each *p*H reading because of its effect on the *p*H meter. Nitrogen which had passed through a soda lime column and been bubbled through solvent at the proper temperature was passed over the solution being titrated. The electrodes, nitrogen inlet and buret were inserted into the titration cell through a rubber stopper.

Procedure.—The *p*H meter was standardized with about 5 ml. of borate buffer, which was placed in the titration cell; the cell was in the same position as during titrations, so that any stray effects due to location of the cell could be canceled out. The cell was cleaned and a 5-ml. sample of the solution to be titrated was placed in it. The same pipet was used for the solution and for the solvent blank. The stirring bar and the stopper with the electrodes were placed in the cell and nitrogen was passed over the solution with stirring for about 0.5 hr. to allow for temperature equilibration and removal of carbon dioxide. With the nitrogen still flowing (except for butylamine titrations, in which the nitrogen was turned off to reduce loss of solute by evaporation), the buret was inserted into the cell through the rubber stopper and the addition of increments of base was begun. The size of the increments of base added was such as to cause a *p*H change of one or two tenths of a *p*H unit. The magnetic stirrer was removed from under the cell after each addition of base and the *p*H was read. At the end of the titration, the cell was cleaned, the buffer put in, and the standardization of the *p*H meter was checked. If the standardization was off by more than 0.01 *p*H unit, the titration usually was discarded.

Calculations.—During the course of a continuous titration, the total volume of solution changes because of the addition of base. This has been corrected for by assuming that the volumes of solution and of added base are additive. Since the ionic strength of the base had been adjusted with KBr to make it the same as the solvent, such an assumption appears to be quite reasonable. The slight changes in ionic strength due to the neutralization of part of the added base have been neglected.

In order to calculate the titration curve from the raw data, *p*H (or duodial readings) *vs.* ml. of standard base added to the solution of known volume and concentration, it is necessary to establish a relationship between the *p*H and the *concentration* of hydrogen or hydroxyl ion in the solution. This can be done by means of an *apparent* activity coefficient, γ', determined from a titration of the pure solvent, which for hydroxyl ion is defined as

$$pK_w - pH = pOH = -\log (OH^-) - \log \gamma'_{OH^-} \quad (11)$$

where (OH^-) refers to the concentration of hydroxyl ion in the solution. This is an apparent activity coefficient because it includes, in addition to the thermodynamic activity coefficient, any errors in the hydrogen ion response of the glass electrode and any liquid junction potentials which have not been canceled out.[37] The use of the apparent activity coefficient in the pure solvent for the calculation of the protein titration curve involves the usual assumption[38] that the presence of the protein does not affect the value of the activity coefficient. With these assumptions the titration curve, *i.e.*, the value of r, the moles of hydrogen ion dissociated per mole of protein (or other substance being titrated), at each *p*H can be calculated readily.

The apparent pK for the ionization of a group is the *p*H at which that group is half ionized. This can be determined from the titration curve by matching with a theoretical curve for the ionization of a single group, or with several such curves which may overlap if more than one group is ionizing. The apparent pK's for the model compounds have been determined from the titration curves in this manner. For *n*-butylamine, which has only a single ionizable group, and for alanyllysylalanine, in which the two ionizable amino groups are reasonably well separated in pK, the procedure is quite simple. However for lysine, in which the ionization regions of the two amino groups overlap considerably, the matching becomes more difficult. The method of Speakman[39] as described by Peek and Hill[40] has also been used to determine the apparent pK's of the amino groups of lysine; the results by the two methods agree within one or two hundredths of a *p*H unit.[41]

The matching of the iodinated insulin titration curves with theoretical curves is more difficult: In spite of the lowering of the phenolic hydroxyl pK's by iodination, there is still some overlapping of the lysyl ionization with groups of lower pK, possibly the α-aminos. There is also overlapping on the high *p*H side of the lysyl ionization, presumably with the ionization of the guanidinium group of the arginyl residue. The absolute value of r is not known, and the overlapping of groups makes it difficult to determine the exact value of r (which we shall arbitrarily call $r = 0$) at which the ϵ-amino of the lysyl group starts to dissociate its proton. The titration curves of iodinated insulin at four temperatures have been matched vertically (*i.e.*, on the r-scale) by making use of data on the variation of *p*H with temperature which will be discussed in the next section and by requiring that the enthalpy of ionization remain constant[42] at any fixed value of r.

(37) See, for example, R. G. Bates, ref. 35, Chaps. 3 and 8; C. Tanford in T. Shedlovsky (Ed.), "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955, pp. 248-265.

(38) E. J. Cohn and J. T. Edsall, ref. 27, Chap. 20.

(39) J. C. Speakman, *J. Chem. Soc.*, 855 (1940).

(40) H. M. Peek and T. L. Hill, THIS JOURNAL, **73**, 5304 (1951).

(41) The ionization constants obtained by the method of Speakman are not defined in the same manner as those obtained from matching with curves for individual groups, but the difference in pK would be less than 0.02 *p*H unit for groups as widely separated as the amino groups of lysine. See, for example, H. S. Simms, *ibid.*, **48**, 1239, 1251 (1926).

(42) The ΔC_p^0 for amino groups is quite small. King[43] finds $\Delta C_p^0 = -5$ cal. mole^{-1} deg.$^{-1}$ for the amino group of γ-aminobutyric acid, and Everett and Pinsent[44] estimate that $\Delta C_p^0 = 8 \pm 2$ cal. mole^{-1} deg.$^{-1}$ for the ionization of primary amines. Hence, the maximum variation in ΔH with temperature in the interval from 0 to 35° would

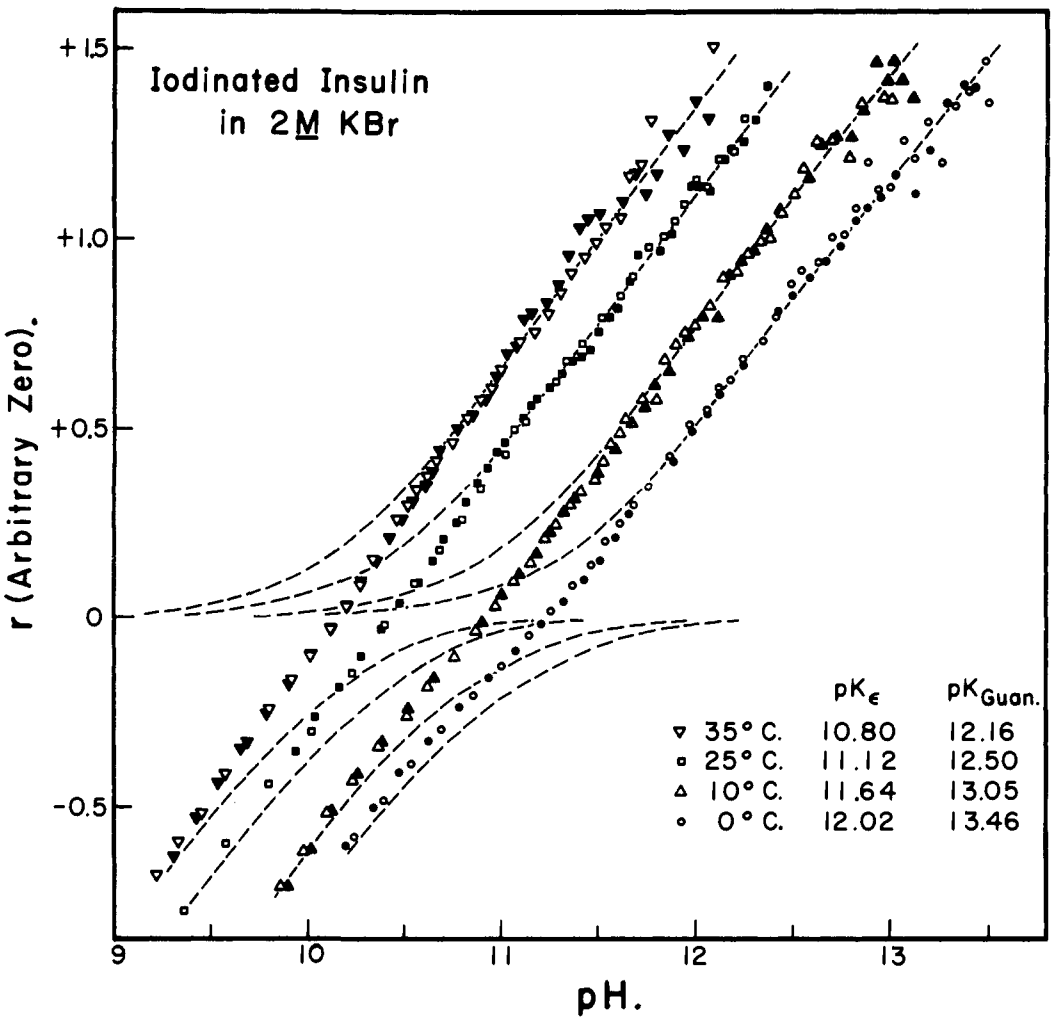

Fig. 1.—Titration curves of iodinated insulin in 2 *M* KBr at 0, 10, 25 and 35°. The filled circles are for the reversed titration (see text); the other filled symbols are for duplicate forward titrations. The upper curves are theoretical curves for two independent but overlapping groups with the apparent *pK*'s indicated. The lower dashed curves represent the difference between the experimental points and the theoretical curve.

The zero for r was determined from the break in the titration curves, which is most evident at the lower temperatures, and the curves above $r = 0$ were matched by two overlapping theoretical curves for single groups (the ϵ-amino and the guanidinium). The zero for r was chosen in such a way as to make the experimental curve remaining after subtraction of the theoretical curve flatten smoothly. The *pK*'s are apparent *pK*'s since they include the electrostatic term.

If the enthalpy is constant over the temperature range under consideration the van't Hoff relationship can be written

$$\Delta H = -2.303RT^2 \frac{dpK}{dT} = -2.303\, RT_1T_2 \left(\frac{pK_2 - pK_1}{T_2 - T_1}\right) \quad (12)$$

The enthalpies of ionization for the amino groups of the model compounds and for the lysyl ϵ-amino group of iodinated insulin have been determined from the apparent *pK*'s at three or four different temperatures by means of equation 12. For lysine, sufficient data were available to permit the determination of ΔH from the least squares slope of *pK* *vs.* $1/T$.

Results and Discussion

The apparent *pK*'s and enthalpies of ionization of the amino groups in the model compounds and in iodinated insulin, determined from the titration curves in 2 *M* KBr, are listed in Table I. The titration curves of iodinated insulin are shown in Fig. 1.

Model Compounds.—The probable error in the apparent *pK*'s for alanyllysylalanine is about ±0.07 unit, due mainly to the uncertainty in the concentration. For lysine the probable error is about ±0.03 unit in *pK*, and for *n*-butylamine it is ±0.05 unit in *pK*. The values for alanyllysylalanine at 25° in 2 *M* KBr are somewhat higher than those of 7.65 and 10.30 found by Ellenbogen[45]

be 0.3 kcal./mole, which is less than the experimental error. However, since the value of ΔH at constant r is in general a composite ΔH for overlapping groups, it could vary with temperature as the amount of overlapping varies.

(43) E. J. King, THIS JOURNAL, **76**, 1006 (1954).

(44) D. H. Everett and B. R. W. Pinsent, *Proc. Roy. Soc.* (*London*) **A215**, 416 (1952).

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

TABLE I

APPARENT pK'S AND ENTHALPIES FROM TITRATION DATA IN 2 M KBr

Group	pK 0°	pK 10°	pK 25°	pK 35°	ΔH, kcal./mole
Ala. Lys. Ala., α-amino	...	8.63	8.23	7.98	10.4
Lysine, α-amino	10.26	9.95	9.53	9.26	11.0
n-Butylamine	11.88	11.49	10.95	...	13.9
Ala. Lys. Ala., ϵ-amino	...	11.31	10.81	10.50	13.0
Lysine, ϵ-amino	11.83	11.46	10.94	10.63	13.3
Iod.Insulin, ϵ-amino	12.02	11.64	11.12	10.80	13.4
Iod. Insulin, guanidinium	13.46	13.05	12.50	12.16	14.3

for the pK's of the α- and ϵ-amino groups, respectively, at 25° and 0.1 ionic strength. The apparent pK's at 25° for lysine in 2 M KBr are also somewhat higher than Ellenbogen's values[45] at 25° and 0.1 ionic strength, which are 9.18 and 10.79 for the α- and ϵ-amino groups, respectively. For the ionization of n-butylamine, Evans and Hamann[46] found ΔF^0 = 14,498 cal./mole at 20° and ΔH^0 = 14.1 kcal./mole, which would correspond to a pK of 10.62 at 25°. A titration of n-butylamine at 25° in 0.3 M KCl gave an apparent pK of 10.82. Both of these results are lower than the pK found for butylamine at 25° in 2 M KBr. From titrations of n-butylamine in solutions of from zero to 3.2 M potassium chloride, Donovan, *et al.*,[47] find that the apparent pK increases with ionic strength from a value of about 10.7 in water to about 11.1 in 3.2 M KCl; the interpolated values of 11.0 and 10.83 for the pK's of butylamine in 2 M KCl and in 0.3 M KCl, respectively, are in good agreement with the results of the present experiments. Hence, it appears likely that the generally higher values observed for the pK's of the amino groups of the model compounds in 2 M KBr, as compared with the pK's quoted from the literature, are a result of the high ionic strength and do not represent a discrepancy between the two.

There are few good values in the literature for the ΔH's of the amino groups in amino acids and peptides. The value for n-butylamine of 14.07 kcal./mole between 20 and 40° determined by Evans and Hamann[46] is in excellent agreement with the value of 13.9 kcal./mole obtained in the present work. King[43] finds a value of 12.07 kcal./mole for the enthalpy of ionization of the amino group of γ-aminobutyric acid; the presence of the carboxylate ion probably influences the behavior of this primary amino group somewhat, although not to the extent that it would for an α-amino acid such as glycine. Cohn and Edsall[27] list values of 11.60 and 11.35, respectively, for the enthalpies of ionization of the α- and ϵ-amino groups of lysine, but these values are probably not too reliable. Calorimetric measurements by Sturtevant[48] give 10.6 and 10.84 kcal./mole for the enthalpies of ionization of the α-amino groups of glycine and alanine, respectively, which agree well with the values of 10.4 and 11.0 kcal./mole found here for the α-amino groups of alanyllysylalanine and lysine, respectively. It is evident that ionic strength has a much smaller effect on the enthalpies than on the apparent pK's, as was expected.

Iodinated Insulin.—Although a direct back titration with acid proved to be impracticable because of precipitation of the protein, the reversibility of the iodinated insulin titration curve was checked in the following manner. An iodinated insulin solution was left at pH 12.7 for 0.5 hr. and then dialyzed against distilled water to remove excess base, lyophilized and its direct titration curve compared with that of a sample of iodinated insulin which had not been subjected to high pH. The two curves are shown by the open and filled circles in Fig. 1 and can be seen to match as well as the duplicate direct titrations at the other temperatures. Hence the iodinated insulin appears not to have undergone any irreversible change at high pH.

The values obtained for the apparent pK's in iodinated insulin are much more uncertain than those for the model compounds because of the uncertainty in the zero for the r-scale of the protein titration curves. The probable error in the apparent pK's assigned to the lysyl ϵ-amino group is of the order of one or two tenths of a pH unit, and that for the guanidinium group is even higher because the uncertainty in the activity coefficient corrections at the highest pH's produces an uncertainty in r of the order of 0.1 group or more, and, in addition, the guanidinium group has been only half titrated at the highest pH's obtained. The *differences* between the pK's at various temperatures should be more reliable, however, because the zero for r was chosen in the same way for all the curves. Hence, a large part of the uncertainty in the pK's will not be reflected in the uncertainty in ΔH.

It can be seen from the data in Table I that the apparent pK's found for the lysyl ϵ-amino group in iodinated insulin are 0.3 unit higher than those found in alanyllysylalanine, which is presumably the best of the models. The difference readily can be explained by the electrostatic interaction between the negatively charged protein and the ionizing proton (see equation 5). Tanford and Epstein[11] assigned *intrinsic* pK's of 11.9 ± 0.2 to the guanidinium group and 9.60 ± 0.10 to the ϵ-amino group in zinc-free insulin at 25°. The apparent pK of 12.50 found in this work for the guanidinium group at 25° is only 0.6 higher than Tanford and Epstein's value, a difference which can be accounted for readily by the electrostatic term; the agreement is better than would be expected from a consideration of the probable errors. The apparent pK found in the present work for the lysyl ϵ-amino group at 25° is 1.5 units higher than Tanford and Epstein's intrinsic pK. This difference is too large to be accounted for by the electrostatic term alone, unless *local* electrostatic interactions are considered. The use of 2 M KBr in the present work probably contributes to the raising of the observed pK but not by more than a few tenths of a pH unit. Since the present work was done with iodinated insulin in which the phenolic hydroxyl groups are almost completely

(46) A. G. Evans and S. D. Hamann, *Trans. Faraday Soc.*, **47**, 34 (1951).

(47) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, in preparation.

(48) J. M. Sturtevant, THIS JOURNAL, **64**, 762 (1942).

ionized before the ϵ-amino group starts to dissociate its proton, while in Tanford and Epstein's study the ϵ-amino and phenolic hydroxyl ionizations completely overlap, it is perhaps unreasonable to expect better agreement between the pK's assigned to the lysyl ϵ-amino group. Fredericq[49] has assigned an intrinsic pK of 10.0 and an apparent pK of 10.5 to the lysyl ϵ-amino group of insulin in 0.1 M KCl at 20°. Assuming a ΔH of 13 kcal./mole, these pK's would have to be lowered by approximately 0.16 to make them correspond to 25° values. Hence, it is apparent that Fredericq's results are also lower than those found in this work for the apparent pK of the ϵ-amino group in iodinated insulin in 2 M KBr.

The enthalpies of ionization calculated from the apparent pK's for the ϵ-amino and guanidinium groups of iodinated insulin are shown in Table I. The probable error in ΔH of the lysyl group is of the order of 1 kcal./mole, while the error in the ΔH of the guanidinium group may be much higher because of the large uncertainty in the curves at very high pH, as mentioned above. The value of 13.4 kcal./mole observed for the enthalpy of ionization of the lysyl ϵ-amino group in iodinated insulin is in the range of 13.0 to 13.9 kcal./mole observed for the corresponding group in the model compounds.

Apparent entropies of ionization can be calculated from the apparent pK's and ΔH's, although the validity of such a calculation is somewhat in doubt because the electrostatic correction and extrapolation to zero ionic strength and zero concentration have not been made. However, these corrections should only change the value of ΔS by approximately 3 e.u., so that an estimate of ΔS to ± 4 e.u. (the probable error) should still be valid. The apparent entropies of ionization calculated for the amino groups of the model compounds are small negative numbers between −3 and −8 e.u., and that for the lysyl ϵ-amino group of iodinated insulin is −6 e.u., which is in the same range as the values for the model compounds.

Temperature Dependence of pH

Theory.—Because of the difficulty in matching titration curves of iodinated insulin at various temperatures, it was decided to determine ΔH by a method which would avoid this problem. If titration curves at different temperatures are compared for the *same* value of r, equation 12 can be written

$$\Delta H = -2.303\, RT_1T_2 \left(\frac{p\mathrm{H}_2 - p\mathrm{H}_1}{T_2 - T_1}\right)_{r = \text{const}} \quad (13)$$

which gives results identical with those from equation 12 if there is no overlapping in the ionization of groups at the given r. If there is overlapping of groups, equation 13 will give a composite ΔH for the two or more groups, depending on their individual ΔH's and on the relative amounts of each ionized. However, if the overlapping groups do not differ greatly in ΔH and if there is a region in which overlapping is small, the error caused by using ΔpH rather than ΔpK to calculate ΔH will not be large, and the difficult task of determining the exact pK will be avoided.

The method of determining ΔH from the change of pH with temperature is illustrated in Fig. 2. If one starts with a protein solution at a point on one titration curve (point a at 25°) and changes the temperature to shift to another curve (point a′ at 0°), one finds that the displacement is usually not just horizontal, corresponding to constant r, but that there is also some vertical displacement corresponding to an increase or decrease in r (shown in Fig. 2 as an increase, $\Delta(\mathrm{OH}^-)_{\mathrm{bd}}$). This is because a change in temperature changes the activity coefficient of the hydroxyl ion, the pK_{w}, and the pK's of the various ionizable groups of the protein. Since the total amounts of added base and of protein remain fixed, the concentration of free hydroxyl ion in the solution after all the equilibria are satisfied at the new temperature will probably not be the same as it was at the initial temperature, and hence some protons will probably either have come off or gone onto the protein, producing a change in r.

The value of the pH change at constant r which we wish to know corresponds to the distance ac in Fig. 2, while the pH change we have observed corresponds to the distance ad. To correct the observed pH change to the pH change at constant r, we can write

$$(\Delta p\mathrm{H})_{r = \text{const}} = (\Delta p\mathrm{H})_{\text{obsd}} - (\mathrm{d}p\mathrm{H}/\mathrm{d}r)\Delta r \quad (14)$$

assuming $\mathrm{d}p\mathrm{H}/\mathrm{d}r$ to be approximately constant over the change in r. For a fixed concentration of protein, equation 14 can be rewritten in terms of the concentration of hydroxyl ion "bound" (or protons dissociated) as

$$(\Delta p\mathrm{H})_{r = \text{const}} = (\Delta p\mathrm{H})_{\text{obsd}} - [\mathrm{d}p\mathrm{H}/\mathrm{d}(\mathrm{OH}^-)_{\mathrm{bd}}]\Delta(\mathrm{OH}^-)_{\mathrm{bd}} \quad (15)$$

If the change in r is small and the whole correction term is small compared to $(\Delta p\mathrm{H})_{\text{obsd}}$ we can assume that the slope of the titration curve, $\mathrm{d}(\mathrm{OH}^-)_{\mathrm{bd}}/\mathrm{d}p\mathrm{H}$, is approximately the same at both temperatures and over the whole change in r, and we can approximate the slope by a straight line between neighboring points along one of the curves. In other words, we assume that (slope at 0°) = (slope at 25°) = $(\delta(\mathrm{OH}^-)_{\mathrm{bd}}/\delta p\mathrm{H})_T$, where the subscript T indicates either temperature. Then equation 15 becomes

$$(\Delta p\mathrm{H})_{r = \text{const}} = (\Delta p\mathrm{H})_{\text{obsd}} - \left[\frac{\delta p\mathrm{H}}{\delta(\mathrm{OH}^-)_{\mathrm{bd}}}\right]_T \Delta(\mathrm{OH}^-)_{\mathrm{bd}} \quad (16)$$

where δpH and $\delta(\mathrm{OH}^-)_{\mathrm{bd}}$ represent the changes in pH and $(\mathrm{OH}^-)_{\mathrm{bd}}$ on moving a short distance along one of the titration curves near the initial value of r, while $\Delta(\mathrm{OH}^-)_{\mathrm{bd}}$ is the change in $(\mathrm{OH}^-)_{\mathrm{bd}}$ on going from the initial to the final temperature, all determined at the same fixed concentration of protein.

By using identical solutions for the measurements at the two temperatures, the matching of the curves has been taken care of automatically. Only the correction term (the second term on the right in equation 16) must be determined, although unfortunately this cannot be done without considerable uncertainty due to the assumptions made in

(49) E. Fredericq, *J. Polymer Sci.*, **12**, 287 (1954).

deriving equation 16 and to the large experimental error in determining the small differences which go into the correction term. It is obvious from equation 16 that it is not even necessary to know the protein concentration as long as it remains fixed during the measurement of both $\Delta(OH^-)_{bd}$ and $\delta pH/\delta(OH^-)_{bd}$. Once the value of $(\Delta pH)_{r=const}$ has been determined by means of equation 16, the apparent enthalpy of ionization can be calculated by means of equation 13.

Description of Apparatus and Solutions.—The Model GS pH meter and glass electrodes were the same as were used for the titrations. The 3.15 N KCl calomel electrodes were slightly modified by replacing the asbestos fiber by a capillary tip and adding a small stopcock and reservoir to the top of the salt bridge tube. This permitted the use of a fresh liquid junction for each pH measurement while it avoided the continuous flowing of potassium chloride into the solutions. The cells in which the pH measurements were made were 30-ml. lipless beakers placed inside brass water jackets which were connected to the same ground as the pH meter. The stirrer and buret were the same as for the titrations, except that a 0.1-ml. plunger and tip were used on the buret for the addition of small increments of base.

The standard buffer, base and other reagents were as previously described, except as otherwise indicated. The standard base used in the experiments in 0.15 M KCl was approximately 0.15 N KOH with no added salt. Solutions of *dl*-alanine, lysine and *n*-butylamine, at concentrations from 0.004 to 0.05 M, were prepared in quantities of 25 to 100 ml. using 2 M KBr or 0.15 M KCl as the solvent. Because of lack of material, a neutralized combined solution of the alanyllysylalanine used in the three titration experiments, which had been stored in the refrigerator for several months, was used. A titration of this material showed no evidence of hydrolysis or other changes; the curve agreed well with that obtained originally on the freshly prepared solution. Iodinated insulin solutions of approximately 30 mg./ml. were prepared in quantities ranging from 11 to 33 ml. by dissolving the dry material in 2 M KBr or in 0.15 M KCl, with the addition of small amounts of base if necessary. To conserve material, many of the solutions were combined and reused for more than one point, and differences between results on used and fresh solutions were within the experimental uncertainty. No solution was re-used more than four times. Solutions for the determination of activity coefficients were prepared by adding standard base from a buret to a known volume of 2 M KBr or 0.15 M KCl.

Procedure.—About 11 ml. of the solution to be studied was brought to the desired pH by the addition of the calculated amount of base. Duplicate samples of approximately 5 ml. of borate buffer and of exactly 5 ml. of the solution being studied were placed in 30-ml. lipless beakers, tightly stoppered and one of each placed in the 0 and 25° constant temperature baths to bring them to the proper temperatures. After about 0.5 hr., the buffer and then the sample at 25° was placed in the brass cell at 25° and the pH's were measured on the duodial of the pH meter. With the sample still in the cell and with nitrogen flowing, a small increment of base was added, with stirring, from a buret inserted through an opening in the rubber stopper. After removal of the buret and magnetic stirrer the pH was measured. Following this the borate buffer sample was again measured. The electrodes at 25° then were unplugged from the pH meter and replaced by the 0° electrodes and the whole series of measurements was repeated on the 0° samples. The duplicate sets of glass and calomel electrodes were stored at 25 and 0° when not in use.

Because of the time required to obtain steady pH readings, 1.5 hr. usually elapsed between the reading of the pH of the sample at 25° and the reading at 0°. No difference in the results was observed when the 0° samples were measured before the 25° samples.

Calculations.—The apparent activity coefficient of hydroxyl ion at each temperature was determined from pH measurements of solutions of known hydroxyl ion concentration in the appropriate solvents, using equation 11. The concentration of free hydroxyl ion in the solution at each temperature, both before and after the addition of the increment of base, then was calculated from the pH's. The difference in initial free hydroxyl ion concentration between the solutions at the two temperatures is $\Delta(OH^-)_{bd}$, and the difference between the amount of base added as an increment and the *change* in amount free at a single temperature is $\delta(OH^-)_{bd}$ (see Fig. 2). The difference between the initial pH's at the two temperatures is ΔpH_{obsd} and the difference between the pH's before and after the addition of the increment of base at a single temperature is δpH. These quantities were used in equation 16 to calculate $\Delta pH_{r=const}$, which in turn was used in equation 13 to calculate ΔH. A value of the correction term in equation 16 was calculated from the slope at each temperature and the average of the two values was used to correct ΔpH_{obsd}. Since the increments of base added to the 25 and 0° samples were identical, the final pH's at the two temperatures (points b and b′ in Fig. 2) could be used to calculate values of ΔpH_{obsd} and $\Delta(OH^-)_{bd}$, giving a value of ΔH at a slightly higher pH.

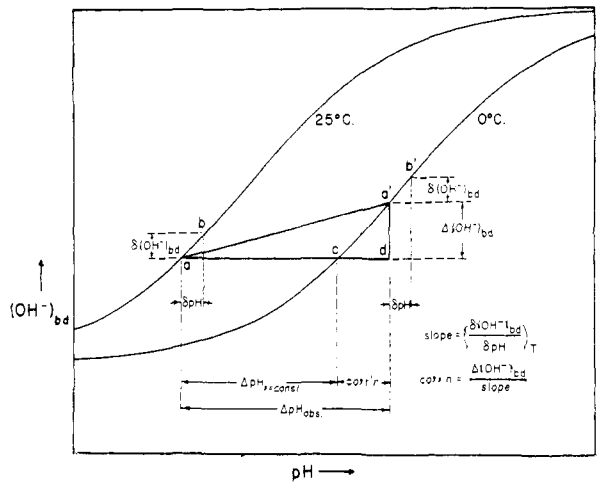

Fig. 2.—Schematic diagram showing the quantities involved in the determination of $\Delta pH_{r=const}$ from equation 16. The two curves are theoretical titration curves for a single group at two temperatures, plotted as $(OH^-)_{bd}$, which is directly proportional to r, *vs.* pH. The differences (such as ea′ and a′b′) have been exaggerated for clarity.

Results and Discussion

As was mentioned above, the error in the correction term is large. In a series of measurements in which six correction terms were calculated at both 0 and 25°, the differences between corresponding terms at the two temperatures were about 20%. In unfavorable cases, *i.e.*, at high pH's or in regions of poor buffering by the ionizing group (due to low concentration or to the pH's being too far from the pK), the error in the correction term may be as large as 100%. It is possible for the per cent. error in the correction term to be even greater for groups ionizing near neutrality, but in this pH region the correction term makes a negligible contribution to ΔpH. In these experiments on the amino groups, the correction term was not more than a few per cent. of ΔpH as long as the pH was not too high

and was close to the pK of the group being studied, and as long as this group was present in sufficiently high concentration. Thus, under these conditions, the error in ΔpH will be much smaller than that in the correction term.

The alanine α-amino group, which has a pK of 9.87 at 25°,[27] appeared to be a good group on which to test this direct method of determining ΔH's, because its thermodynamic parameters are similar to those of the amino groups being studied in the present work. Sturtevant[48] has determined its enthalpy of ionization by calorimetric measurements and found $\Delta H^0 = 10.838$ kcal./mole. The solutions studied were 0.05 and 0.01 M *dl*-alanine in 2 M KBr and 0.005 M *dl*-alanine in 0.15 M KCl, and the value of ΔH appeared to be independent of the concentration of alanine and of the solvent. The over-all average value for the enthalpy of ionization of the α-amino group of alanine was found to be 10.9 ± 0.4 kcal./mole, in good agreement with Sturtevant's value.[48]

The average values of ΔH obtained by the direct method for the amino groups of the model compounds and iodinated insulin are listed in Table II together with an estimate of the probable experimental errors. The *n*-butylamine was measured at approximately 0.1 pH unit intervals in a range of approximately 1.5 pH units about the pK. Alanyllysylalanine was measured at eight pH's covering the ionization of the last quarter of the α-amino group through the first half of the ϵ-amino group. Lysine solutions were measured at intervals of 0.2 pH unit from pH 9 to pH 11.4. The values in 2 M KBr below pH 10 were assigned to the α-amino group and those above pH 10.5 to the ϵ-amino group; in 0.15 M KCl the divisions were made below pH 9.8 and above pH 10.3 because the apparent pK's of the amino groups are probably a few tenths lower in 0.15 M KCl than in 2 M KBr. The values obtained for the ΔH's of the model compounds in 2 M KBr agree well with the results from titrations, and the results in 0.15 M KCl agree with those in 2 M KBr.

TABLE II

ΔH'S FROM $\Delta p\mathrm{H}_r$ = const BETWEEN 0 AND 25°

Group	ΔH (kcal./mole) 2 M KBr	0.15 M KCl
Alanine, α-amino	11.0 ± 0.4	10.9 ± 0.4
Ala. Lys. Ala., α-amino	10.4 ± .5	.....
Lysine, α-amino	10.9 ± .4	10.6 ± 0.8
n-Butylamine	14.0 ± .4	.....
Ala. Lys. Ala., ϵ-amino	13.1 ± .6	.....
Lysine, ϵ-amino	13.5 ± .5	13.1 ± 0.5
Iod. insulin, ϵ-amino	12.7 ± 1	12.7–13.2 ± 1

For iodinated insulin in 2 M KBr, measurements were made only at pH's below the apparent pK of the lysyl ϵ-amino group because the experimental uncertainty becomes too great at higher pH's for the dilute solutions (about 0.004 M) studied. Therefore, the observed values for ΔH may be somewhat low because of the overlapping between the ϵ-amino group and groups of lower ΔH such as the α-amino, zinc complex[11] and phenolic hydroxyl groups. The average ΔH for iodinated insulin at pH's above 10.4 (25°) is 12.7 ± 1 kcal./mole. This ΔH can be assigned to the lysyl ϵ-amino group since the overlapping with groups of lower ΔH at pH's above 10.4 should cause an error of less than 1 kcal./mole. The agreement with the results for model compounds and with the result obtained from the titration data is as good as can be expected in view of the overlapping of the ionization of groups in iodinated insulin.

In order to make sure that the 2 M KBr used as the solvent in most of these experiments does not break a lysyl hydrogen-bond or other interaction in iodinated insulin, the enthalpy of ionization of the ϵ-amino group of the lysyl residue was also determined using 0.15 M KCl as the solvent. As has been noted above, the enthalpies of ionization of the amino groups of the model compounds do not change in going from 2 M KBr to 0.15 M KCl as the solvent.

The enthalpy of ionization of iodinated insulin from pH 8 to pH 11.3 was determined for a 3% solution in 0.15 M KCl. The values obtained for ΔH range from about 5.5 kcal./mole at pH 8 to about 13 kcal./mole at pH 11.3. The average value at pH's above 10.6 is 12.7 ± 0.7 kcal./mole and probably corresponds to the ionization of the ϵ-amino group of the lysyl residue. The enthalpy increases with pH even above pH 10.6, presumably increasing as the amount of overlapping of the ϵ-amino group with groups of lower ΔH decreases. Therefore, the value of 13.2 kcal./mole observed above pH 11.2 is probably closer to the true value of ΔH for the lysyl ϵ-amino group in iodinated insulin. In any case, it is obvious that the value for the enthalpy of ionization of the ϵ-amino group in iodinated insulin does not change significantly when the solvent is changed from 2 M KBr to 0.15 M KCl. Hence, the apparent "normalcy" of the lysyl ϵ-amino group of iodinated insulin in 2 M KBr is not due to the disruption by the bromide of any lysyl group interactions which would occur in 0.15 M KCl and which would affect the enthalpy of ionization.

General Discussion

Methods.—Two methods have been employed to determine the thermodynamics of the ionization of the lysyl ϵ-amino group of iodinated insulin. In order to determine their relative merits, it is necessary to consider in more detail the condition of a protein solution at various temperatures. For a concentrated protein solution near neutrality, the total number of protons dissociated will be almost constant with temperature because the change in free hydrogen and hydroxyl ion with temperature will be negligible in comparison with the protein concentration. At higher pH's, however, the value of r may change considerably with temperature for a given protein solution, as was pointed out above. Therefore, it is not possible to match titration curves at different temperatures by assuming that the value of r is the same at the start of each titration, unless the titrations have been started on a solution at a pH near neutrality. Because of the insolubility of iodinated insulin near neutrality and because of the limitation of a three pH-unit range[50] for the duodial of the

(50) The range could be extended to cover six pH units if it were not necessary to check the meter standardization *both* before and after

Model GS pH meter, the titration curves of iodinated insulin were started at fairly high pH's. Moreover, because of the lability of protein solutions on standing, it was not possible to use identical starting solutions of iodinated insulin at each temperature. Therefore, it was necessary to match the 0 and 25° titration curves by use of the corrected ΔpH's obtained by the second method and then to match the 10 and 35° curves to these visually, a subjective procedure at best.

Simply matching the titration curves at different temperatures does not locate the region of ionization of the lysyl residue. A reference point on the r-scale must be known. The absolute value of r at the beginning of the titration is undetermined because the initial state of ionization of the protein is unknown. The maximum value of r cannot be determined because the pK of the guanidinium group is too high to permit it to be completely titrated. Hence, neither end of the titration curve can be used as a reference point. It was hoped that the lowering of the pK of the phenolic hydroxyl groups by iodination would produce a distinct flattening of the titration curve at the low pH end of the lysyl ionization. Although the flattening is not as pronounced as had been hoped, there is evidence of a break in the titration curves, especially at the lower temperatures, and this has been used as the reference point for the start of the lysyl ionization. The uncertainty in this point is of the order of $\pm$ 0.1 group, which produces a corresponding uncertainty of $\pm$ 0.15 in the value of the apparent pK. The uncertainty in ΔH is not as large because the value of ΔH is determined mainly by the matching of the curves at various temperatures and is only slightly affected by the uncertainty in the reference point for r. It might have been possible to determine the region of lysyl ionization more precisely by making a further chemical modification of the protein. However, the more the protein is modified, the less likely it is that the configuration will be the same as the native.

The direct method of determining ΔH has several advantages. By the use of identical solutions at the two temperatures, it avoids some of the uncertainties in curve matching encountered in the titrations, although the region of ionization of the group of interest must still be determined. Neither the total amount of base added nor the exact concentration of protein need be known, provided that ΔH does not depend on the concentration. The activity coefficient of the hydroxyl ions, the volume of the sample and the volume of the increment of base added enter only into the correction term. The method also has some disadvantages, however. It cannot be used at very high pH's or in very dilute solutions where the correction term becomes large, because the correction term itself is too uncertain under these conditions. The amounts of time and material required for a measurement at a single point are relatively large, and it is very difficult to exclude completely carbon dioxide. Probably the chief disadvantages of this method as compared with the study of titration curves are that the apparent pK's cannot be determined and that the value of ΔH obtained in a region of overlapping groups is a composite ΔH for all the groups which are ionizing at the given point. This represents the price which must be paid for avoiding the difficulties of curve matching and curve fitting.

The best method for accurately determining the enthalpy of ionization of overlapping groups in a protein appears to be a combination of the two methods described. The direct change of pH with temperature should be used to match the titration curves at different temperatures. The latter may then be used to determine pK's, enthalpies of ionization, and other thermodynamic parameters for the ionizing groups in the protein.

Results

Equations 1, 3 and 4 predicted increases over the normal values of approximately 0.7 unit in pK, 5 kcal./mole in ΔH and 13 e.u. in ΔS if the lysyl residue in the protein were involved in a strong hydrogen-bonding interaction. It was pointed out that the change in the enthalpy probably would be the most reliable indication of the existence of a hydrogen bond. Normal values of the thermodynamic constants for both α- and ϵ-amino groups have been determined from studies of low molecular weight model compounds, using the same methods that were used for the study of the protein. All of the thermodynamic constants determined for the ionization of the lysyl ϵ-amino group in iodinated insulin fall within the range of the normal values for the corresponding group in the model compounds. It has been shown that this apparent "normalcy" of the ϵ-amino group in iodinated insulin is not due to the disruption of a hydrogen bond or other specific interaction by 2 M KBr, because the same results are obtained from experiments in 0.15 M KCl. It is possible that iodination has disrupted a lysyl residue interaction, but the experimental evidence[32] indicates that only the phenolic groups of the protein have been affected by iodination and that no extensive changes have taken place in the molecule.

The evidence presented shows that the single lysyl ϵ-amino group in iodinated insulin behaves thermodynamically as an essentially free group and appears not to be involved in any strong intergroup interaction.[51]

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the titration, but the stability of the pH meter was not sufficiently high to permit reliance on a single standardization.

(51) An examination of the Lindley–Rollett model[9] of the insulin monomer shows that there are no polar groups in the molecule in a position to satisfy the steric requirements[52] for the formation of a hydrogen bond with the ϵ-amino group of the lysyl residue. Hence, the results of the present experiments support the Lindley–Rollett model. It must be emphasized, however, that these results do not rule out the possibility that the lysyl residue is in a sterically favorable position for hydrogen bonding. It may be that the entropy change for formation of the hydrogen bond is much more negative than the -17 e.u. estimated, making the equilibrium constant for formation of the bond so small that the hydrogen bond would be too weak to be observed by the present methods.

(52) J. Donohue, *J. Phys. Chem.*, **56**, 502 (1952).