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Hydrogen Ion Titration Curves of β-Lactoglobulin[1]

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This paper consists of three parts. (1) It is shown that the isoionic point of β-lactoglobulin in pure water is at *p*H 5.39 and that it decreases when KCl and $CaCl_2$ are added. This indicates that the protein binds K^+ and Ca^{++} ions. Quantitative estimates of the extent of binding are given. (2) The titration curve is shown to be reversible between its acid end and *p*H 9.7. Within this range it is experimentally indistinguishable from that reported by Cannan, Palmer and Kibrick in 1942, but its quantitative interpretation is somewhat different because of the discovery of small ion binding, and because of the configurational change which has been shown to occur at *p*H 7.5. (3) A titration curve extrapolated to zero time has been obtained above *p*H 9.7. It agrees quite well with the curve expected for a native (rigid and compact) molecule in this range. The curve changes rapidly with time, however, approaching the kind of titration curve which is typical of a flexible, polyelectrolyte-like molecule.

In 1942, Cannan, Palmer and Kibrick[3] published the results of a detailed study of the dissociation of hydrogen ions from β-lactoglobulin. This work, together with a comparable study of ovalbumin,[4] has been the prototype on which subsequent titration studies of other proteins have been based.

The present reinvestigation of this problem was decided upon because of the availability of new techniques which provide auxiliary information. One of these is the ability, by means of ion exchange, to determine the true isoionic point of a protein. The second is the development of techniques for rapid *p*H measurement which permit extension of the titration curve of the native protein to the alkaline *p*H range, where slow denaturation interferes with the usual techniques. Information gained from application of these new techniques, together with other recent developments in the chemistry of β-lactoglobulin, necessitates a reinterpretation of some of the results obtained by Cannan, Palmer and Kibrick. It is important to note, however, that the experimentally observed titration curves remain unchanged, up to the point at which denaturation sets in.

(1) A preliminary account of this work was presented at the 134th meeting of the American Chemical Society, Chicago, Illinois, September, 1958.

(2) On leave of absence from the National Hygienic Laboratory, Tokyo, Japan.

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

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

Experimental

Crystalline β-lactoglobulin (lot 4809) was purchased from Pentex, Inc., Kankakee, Illinois. Stock solutions were prepared by dissolving these crystals in dilute KCl. The resulting solutions were clarified by centrifugation or filtration. The concentration of protein in the final solutions was determined by measurement of the optical density at 278–279 mμ in a Beckman spectrophotometer, using a value of $E_{1\,cm.}^{1\%}$ of 9.4 (where $E = \log I_0/I =$ optical density). This value of $E_{1\,cm.}^{1\%}$ was obtained as the average of a number of absorption measurements on solutions whose protein concentration was determined by drying at 107–108°. Two kinds of solution were employed, salt-free deionized solutions of low concentration and regular stock solutions containing KCl. When the latter were employed the KCl content was kept small and the amount present was known with sufficient accuracy to permit evaluat on of the protein content with a probable error which was smaller than the probable error of light absorption measurements.

The technique just outlined turned out not to be as satisfactory as had been hoped. It was found that β-lactoglobulin is highly susceptible to surface denaturation, resulting in the formation of thread-like insoluble particles. The quantitative dilution and multiple transfers required for spectrophotometric measurements were inevitably accompanied by such denaturation and the reproducibility of such measurements therefore turned out to be no better than 1%. The value of $E_{1\,cm.}^{1\%}$ given above thus has an uncertainty of ±0.1, and its use to determine the concentration of protein solutions results in a probable error of 1.5% for a given stock solution. Most of the work of this paper was performed on two or more different stock solutions, the results reported being the average of all of these.

Standard HCl was prepared by dilution of constant-boiling HCl. Standard KOH was prepared by an ion-exchange method patterned after that used by Powell and Miller.[5]

(5) J. E. Powell and M. A. Miller, *J. Chem. Ed.*, **34**, 330 (1957).

Reagent grade KCl and $CaCl_2$ were used without further purification. Solutions usually were made up volumetrically, acid or base being delivered from calibrated microburets of the Agla or Gilmont types.[6]

Three *p*H meters were used for *p*H measurements, Beckman meters, models G and GS, and a Radiometer TTT 1a autotitrator.[7] Some of the data were obtained by the point-by-point method, using the apparatus described previously.[8] At other times a continuous titration method was used, employing a jacketed vessel equipped with a calomel reference electrode which had a narrow U-bend at the tip, which was in direct contact with the solution.[9] The glass electrodes were of the Beckman all-purpose type, with shielded leads. Carbon dioxide was excluded from the measuring cells by a steady stream of purified nitrogen. Calibration depended ultimately on the use of Bureau of Standards potassium acid phthalate *p*H standard (*p*H 4.005 at 25°).

When the autotitrator was used as a *p*H-stat its relay was connected to a second relay, which operated a clutch connecting a continuously running motor so as to turn the micrometer screw of a Gilmont microburet. The volume delivered was read off at intervals from the counter with which these microburets are equipped.[10]

Spectrophotometric measurements were made using a Beckman Model DU or a Cary recording spectrophotometer. All measurements were made at 25°.

The molecular weight of β-lactoglobulin has been taken to be 35,500.[11]

The Isoionic Point and Cation Binding

An isoionic protein solution is one which contains no small ions other than H^+ or OH^-, and in which the protein molecules bear an average net charge ($\bar{Z}$) just sufficient to neutralize the charge due to the excess of H^+ or OH^- ions which is inevitably present when the isoionic *p*H differs from *p*H 7.0. The simplest method for attaining the isoionic state is to pass a solution of the protein down an ion-exchange column of the type described by Dintzis.[12] It was not expected that this procedure could be applied to β-lactoglobulin, since its solubility in water is very low. It was found, however, that solutions containing 2.5 g. of the protein per liter could be passed successfully through a Dintzis column without precipitation, to form supersaturated isoionic solutions. Crystals of the protein separated from these solutions, but did so only slowly. No precipitation appeared to occur in the ion-exchange column, nor in the emerging solution during the time normally required to collect a workable amount.

The *p*H of the isoionic protein solution was found to be at 5.39 at 25°. Even at the low concentrations used, the corresponding value of $\bar{Z}$ is less than 0.1 (negative charge) per molecule. Since most of our measurements cannot claim an accuracy of 0.1 in the number of groups titrated, it is henceforth assumed that $\bar{Z}$ of the isoionic protein, in the absence of added salt, is zero.

(6) "Agla" micrometer syringe, Burroughs Wellcome and Co., London, England; Gilmont micropipet-buret, Emil Greiner Co., New York 13, New York.

(7) Radiometer Co., Copenhagen, Denmark.

(8) C. Tanford in "Electrochemistry in Biology and Medicine," T. Shedlovsky, editor, John Wiley and Sons, New York, N. Y., 1955.

(9) Patterned after electrodes described by C. F. Jacobsen, K. Linderstrøm-Lang and M. Ottesen, in "Methods of Biochemical Analysis," D. Glick, editor, Vol. IV, Interscience Publishers, Inc., New York, N. Y., 1957, p. 171.

(10) The principle of the *p*H-stat is described by Jacobsen, *et al.*, ref. 9.

(11) F. R. Senti and R. C. Warner, THIS JOURNAL, **70**, 3318 (1948).

(12) H. M. Dintzis, Ph.D. thesis, Harvard University, 1952.

Figure 1 shows the effect of added KCl or $CaCl_2$ on the *p*H of the initially isoionic protein solution. It is seen that there is *decrease* in *p*H with increasing salt concentration. Identical results were obtained using protein solutions which, before being passed down the Dintzis column, were more acidic and more basic than the isoionic solution.

The effect observed in Fig. 1 may be ascribed to two separate phenomena[13]: (a) a purely electrostatic effect of ionic strength on the equilibrium between protein molecules of charge 0, +1, +2, . . . , −1, −2, . . . , and (b) the preferential binding of one of the ions of the added electrolyte to the protein. Both these effects are discussed in detail by Scatchard and Black.[13] The first effect may be computed from the titration curve (using equation 18 of Scatchard and Black) and is found to represent a negligible fraction of the observed effect. The latter must therefore be ascribed almost entirely to ion binding. Where the initial charge of the protein is zero we may use equation 5 of Scatchard and Black, which gives for the difference in *p*H (ΔpH) between the initial solution and that at any concentration of added salt

$$\Delta p\mathrm{H} = -0.868w \sum_i \nu_i z_i \qquad (1)$$

where w is the usual electrostatic interaction parameter, appropriate to the final solution, ν_i is the number of ions of type i bound in the final solution and z_i their charge. Since ΔpH was found to be negative, cations must be preferentially bound (in contrast to serum albumin,[13] where anions are preferentially bound). It has been shown by Carr[14] that β-lactoglobulin does not bind chloride ion in the *p*H region of interest here. We shall therefore assume that the cation is the only ion bound, *i.e.*, we replace the sum in equation 1 by a single term $\nu_+ z_+$.

The values of w used for these calculations are computed ones, using the same procedure as was employed for serum albumin by Scatchard and Black.[13] The molecular volume is assumed to be $(M/N)(\bar{v} + 0.2)$ where M is the molecular weight (35,500), N is Avogadro's number and $\bar{v}$ the partial specific volume (0.75 cc./g.). The added 0.2 cc./g. represents an effective hydration. The radius b of a sphere with this volume is computed, and w is calculated by the Linderstrøm-Lang equation[15]

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (2)$$

where ϵ is the electronic charge, D the dielectric constant, k Boltzmann's constant, T the absolute temperature, and κ the usual Debye–Hückel constant. The parameter a is the radius of exclusion of small ions, taken as $b + 2.5$ Å.

Figure 2 shows the extent of binding of K^+ and Ca^{++} ions which one computes from Fig. 1 in this way. It is seen that both K^+ and Ca^{++} are bound to a considerable extent.

(13) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(14) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 417 (1953).

(15) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg* **15**, No. 7 (1924).

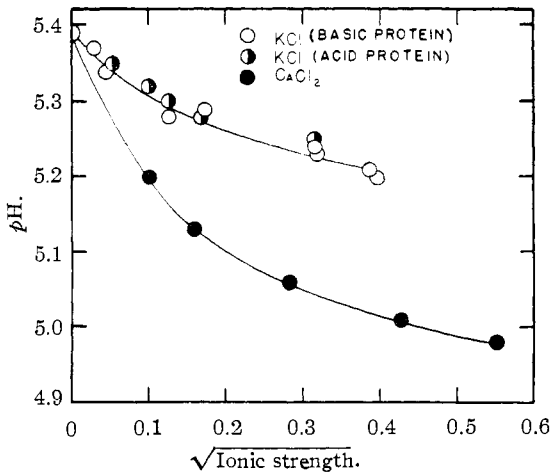

Fig. 1.—The effect of added KCl and $CaCl_2$ on the isoionic *p*H of β-lactoglobulin. The experiments with KCl were performed in two ways, with protein which was on the basic side of the isoionic point before being passed down the ion exchange column, and also with protein which was on the acidic side.

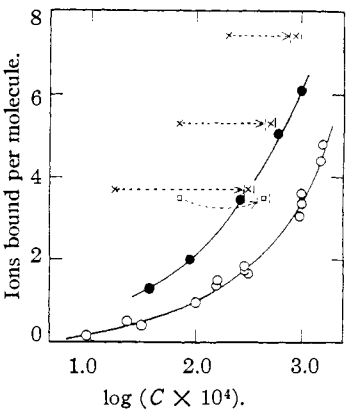

Fig. 2.—K^+ and Ca^{++} binding by isoionic β-lactoglobulin, as determined from the data of Fig. 1: O, K^+; ●, Ca^{++}; □, K^+ binding determined by Carr at *p*H 7.4; ×, Ca^{++} binding determined by Carr at *p*H 7.4. The arrows leading from Carr's data to bracketed points represent a calculated correction of his data to the conditions of the experiments of the present paper.

To check on the validity of these experiments our results may be compared with direct binding studies performed by Carr[16] on β-lactoglobulin which had been titrated to *p*H 7.4. The protein at this *p*H has lost about 11 protons (see below) and the resulting negative charge increases its affinity for cations, with the result that higher binding is expected, and Fig. 2 shows that this is indeed the case. This electrostatic effect can, however, be computed, and one can calculate from Carr's data the binding to be expected at the isoionic point. It may be assumed that there are several kinds of groups (n_i of each kind) capable of binding either cation. Each kind of group will have an associated *intrinsic* association constant k_j, and, under a given set of conditions, will bind $\bar{\nu}_i$ ions (on the average), such that

$$\log \frac{\bar{\nu}_j}{n_j - \bar{\nu}_j} = \log c + \log k_j - 0.868w(z_i\bar{Z}_H + z_i^2\bar{\nu}) \quad (3)$$

where c is the concentration of free ions in the solution, $\bar{Z}_H$ is the charge due to bound or dissociated protons, z_i is the charge of the cation (*i.e.*, 1 for K^+, 2 for Ca^{++}), and $\bar{\nu} = \Sigma\bar{\nu}_j$ is the average total number of cations bound. If two solutions with identical $\bar{\nu}$ are considered it may be assumed that all $\bar{\nu}_i$ are also identical. Choosing such solutions at *p*H 7.4 ($\bar{Z}_H = -11$) and in the vicinity of the iso-ionic point we get for the displacement along the abscissa

$$\Delta \log c = 0.868w_1(z_i^2\bar{\nu}) - 0.868w_2(z_i^2\bar{\nu} - 11z_i) \quad (4)$$

where w_2 is the value of w appropriate to the ionic strength in Carr's experiments, and w_1 that appropriate to the experiments conducted at the isoionic point. The value of $w_1 > w_2$ because of the higher salt concentration required to maintain constant $\bar{\nu}$ as the *p*H is reduced. When Carr's data are adjusted in this way, the points shown in Fig. 2 in brackets are obtained. Their agreement with our data is seen to be quite good, especially when it is considered that Carr's results represent only four experiments in a general survey of a number of proteins, and when account is taken of the arbitrary nature of the calculated w used to make our calculations.

The result here reported is at variance with the conclusion of Cannan, *et al.*,[3] that the isoionic point of β-lactoglobulin is independent of ionic strength. It should be noted, however, that Cannan, *et al.*, had no method available for the direct determination of the isoionic point.

It is possible to show that the isoionic points here reported are not the result of a change in the protein as it passes down the ion-exchange column. When the crystalline protein, as received, was dissolved in aqueous KCl, its *p*H was found to be 5.48, 5.35 and 5.33, respectively, at ionic strengths 0.01, 0.10 and 0.15. The isoionic *p*H's at the corresponding ionic strengths are, respectively, 5.32, 5.23 and 5.21. The difference at each ionic strength (0.16, 0.12, 0.12) is the change in *p*H required to titrate 1.4 groups per protein molecule, *i.e.*, the observed *p*H's are all consistent with the isoionic points reported here if the protein received carried a charge, due to dissociated protons, of −1.4.

If, on the other hand, the isoionic point is assumed to be independent of ionic strength, at 5.18, let us say, as stated by Cannan, *et al.*,[3] then the observed *p*H's at the three ionic strengths would correspond to molecular charges of −2.3, −1.8 and −1.6, respectively, *i.e.*, the original material would have appeared to have a different number of dissociated protons, depending on the ionic strength.

Discussion

It is of interest to note, in this connection, that one of us recently has obtained salt-free isoionic ferrihemoglobin by ion exchange. The isoionic point was found to be virtually independent of ionic strength showing that there can be essentially no K^+ or Cl^- binding by this protein, unless both are bound to an equal extent over a considerable range of concentration, a situation unlikely to occur.

(16) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 424 (1953); **62**, 476 (1956).

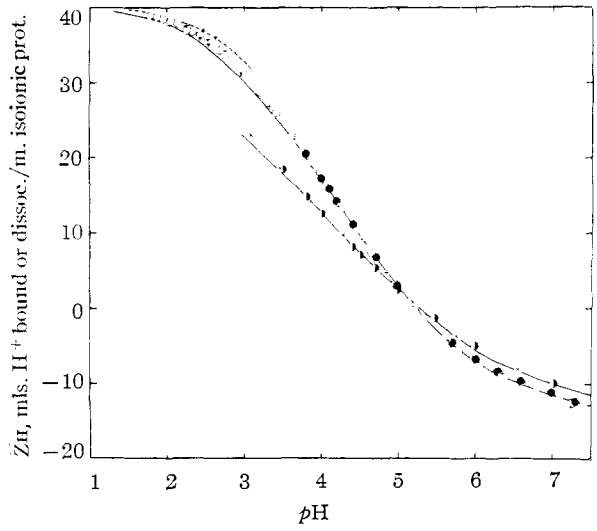

Fig. 3.—Titration curve up to *p*H 7.5: the symbols have the significance: ○, ionic strength 0.15, protein not de-ionized; ●, ionic strength 0.15, protein de-ionized; ◑, ionic strength 0.01; +, data of Cannan, *et al.*,[3] ionic strength 0.15; ×, data of Cannan, *et al.*, ionic strength 0.01. The solid lines are calculated for a molecular weight of 35,500, the dashed line for a molecular weight of 17,750.

This result again agrees with Carr's direct determinations of ion binding,[14,16] which show that hemoglobin binds neither Cl^- nor K^+ ion. There are thus three common proteins for which ion binding has been determined by both techniques, with substantial agreement between them. It is significant that one of these (serum albumin) binds Cl^- but not K^+, one (β-lactoglobulin) binds K^+ but not Cl^-, while the third (hemoglobin) binds neither. If the binding of these ions is largely an electrostatic phenomenon, this result suggests that serum albumin has several centers of unusually high positive electrostatic potential (*e.g.*, amino groups close together) while β-lactoglobulin has centers of unusually high negative potential (presumably carboxyl groups close together).

Titration Curves below *p*H 9.7

It is well-known that β-lactoglobulin is irreversibly denatured in alkaline solutions.[17] Below *p*H 9.7, however, the rate of this reaction is sufficiently slow so that no detectable denatured product appears in the time required for titration studies. Between this *p*H and the point of maximum acid binding the titration curves at two ionic strengths (0.15 and 0.01) were found to be completely reversible.

Experimental data are shown in Fig. 3. Most of the experiments made use of protein which had not been deionized, since the concentration of the deionized solution is too low (0.25%) for accurate titration studies over a wide range of *p*H. At ionic strength 0.15 the deionized material was titrated within the range of *p*H 3.8 to 8.5 ($\bar{Z}_H$ +20 to −18) and the data are seen to be identical, within experimental error, with those obtained for protein which had not been deionized.

(17) M. L. Groves, N. J. Hipp and T. L. McMeekin, THIS JOURNAL, **73**, 2790 (1951).

Figure 3 also shows the data of Cannan, *et al.*,[3] at ionic strengths 0.135 and 0.01. The original data have been converted to a molecular weight of 35,500 and have been adjusted to the same isoionic point as our data, *i.e.*, to the isoionic point determined with deionized material. There is seen to be no significant difference between our results and those of Cannan, *et al.*, at corresponding ionic strengths.

Reversed points have been omitted from Fig. 3 to avoid overcrowding of the figure. A large number of reversal experiments were performed, however, and not the slightest indication of irreversible behavior was detected.

Stoichiometry.—Figure 3 shows that the maximum acid binding, which is also equal to the total number of cationic groups, is 40 H^+ ions per molecule. This figure agrees well with that expected from amino acid analysis (Table I) and also with the corresponding number determined by Cannan, *et al.*

TABLE I

TITRATABLE GROUPS PER MOLE (35,500 G.)

	Amino acid analysis[a]	Titration: This paper	Titration: Cannan, *et al.*[e]
α-COOH	2[b]	2	51.5
β,γ-COOH (normal)	50	49[c]	
β,γ-COOH (anomalous)		2	5.3
Imidazole	4	4	
α-NH_2	2[b]	2	30.2
ε-NH_2	27	28	
Phenolic	7	6[d]	..
Sulfhydryl	3	..	..
Guanidyl	6	6	5.3
Total cationic	39	40	40.8

[a] W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949). The figures are adjusted to the nearest integer. [b] H. Fraenkel-Conrat, "Symposium on Structure of Enzymes and Proteins," *J. Cell. Comp. Physiol.*, **47**, supplement 1 (1956). [c] β-Lactoglobulin A has 50 and β-lactoglobulin B 48 normal side chain COOH groups. See text. [d] From the total change in the molar absorbancy at 295 mμ which accompanies ionization of the phenolic groups. [e] Reference 3. The numbers were adjusted to be integers for a molecular weight of 40,000.

Figure 3 also shows that about 50 groups are titrated in the acid region characteristic of carboxyl groups, and the usual kind of trial and error calculation indicates that 51 is the best integral value. Two of these groups are α-COOH groups and 49 are side chain COOH groups. (This figure is 0.5 more than that given in a previous paper,[18] which was based on a molecular weight of 35,000 rather than 35,500.) There are about 8 groups titrated in the neutral region, of which 4 are imidazole groups, 2 are terminal α-amino groups and 2 are anomalous carboxyl groups which have been discussed in an earlier paper.[18] As was also mentioned in that paper, Cannan, *et al.*,[3] included these anomalous groups largely with their imidazole groups. The total sum of carboxyl and imidazole groups given by Cannan, *et al.*, is 56.8 per 35,500 g. The number given here is 57.

A summary of these and other calculations which bear on the numbers of various kinds of groups

(18) C. Tanford, L. G. Bunville and Y. Nozaki, *ibid.*, **81**, 4032 (1959).

present is given in Table I, together with the corresponding figures from amino acid analysis.

It should be pointed out here that the β-lactoglobulin used in Cannan's study, as well as that used here, is obtained from the pooled milk of many different cows, and thus consists of a mixture of the two electrophoretically separable components, β-lactoglobulins A and B,[19] usually in approximately equal amounts. The sample used in the present study has been analyzed for us by S. N. Timasheff and R. Townend, and its composition was found to be 52 ± 3% B, 48% A.

Studies in progress in this Laboratory have shown that the only detectable difference between β-lactoglobulins A and B is in the number of carboxyl side chains, form A having two more than form B. (This conclusion has been reached independently, by other methods, by Timasheff.[20]) The total of 51 side chain carboxyl groups found present in our sample thus indicates that β-lactoglobulin A has 52 such side chains, whereas B possesses 50.

It also should be noted that the β-lactoglobulin molecule of molecular weight 35,500 consists of two separable polypeptide chains which possess identical terminal residues and which may be identical in all respects.[21] The data of Table I would be consistent with this idea, for the number of titratable groups of any distinguishable kind in a molecular weight of 35,500 is an *even* number for each of the two kinds of β-lactoglobulin (A and B).

Computed Titration Curve.—In view of the fact that β-lactoglobulin binds K^+ ions at its isoionic point and Cl^- ions at more acid *p*H, the titration of any one kind of group is described by Linderstrøm-Lang's equation[15] as

$$p\mathrm{H} - \log \alpha/(1-\alpha) = pK_{\mathrm{int}} - 0.868w(\bar{Z}_{\mathrm{H}} + \bar{\nu}_{K^+} - \bar{\nu}_{Cl^-}) \quad (5)$$

where α is the degree of dissociation of the kind of group considered at any *p*H, pK_{int} is its intrinsic pK, w is the customary electrostatic interaction factor (here considered as an empirical parameter), $\bar{Z}_{\mathrm{H}}$ is the average molecular charge due to bound or dissociated H^+ ions, $\bar{\nu}_{K^+}$ is the average number of bound K^+ ions and $\bar{\nu}_{Cl^-}$ is the average number of bound Cl^- ions. The term in brackets on the right side of equation 5 is, of course, the total net molecular charge.

The value of $\bar{\nu}_{K^+}$ is available only at the isoionic point (Fig. 2). The value of $\bar{\nu}_{Cl^-}$ is known to be zero at this point and has been determined at just two *p*H's and two chloride concentrations in more acid solutions. The *p*H dependence of $\bar{\nu}_{K^+}$ and $\bar{\nu}_{Cl^-}$ is thus unknown. We have assumed that their effect on the net charge will be a linear function of $\bar{Z}_{\mathrm{H}}$.[22] Thus, where $\bar{\nu}_{K^+}{}^0$ is the value of $\bar{\nu}_{K^+}$ (and also of $\bar{\nu}_{K^+} - \bar{\nu}_{Cl^-}$) at the isoionic point at any ionic strength, we write

$$\bar{\nu}_{K^+} - \bar{\nu}_{Cl^-} = \bar{\nu}_{K^+}{}^0 - B(\bar{Z}_{\mathrm{H}}) \quad (6)$$

(19) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955); **180**, 376 (1957).

(20) S. N. Timasheff, personal communication.

(21) R. Townend and S. N. Timasheff, THIS JOURNAL, **79**, 3613 (1957).

(22) A similar assumption was made by F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952), for the effect of nitrate ion on the net charge of serum albumin.

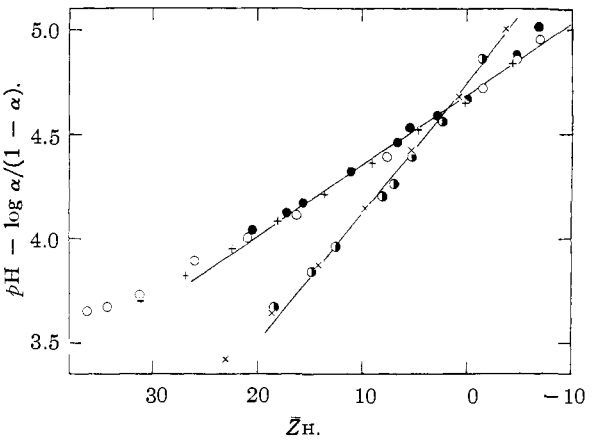

Fig. 4.—Logarithmic plot for the side chain carboxyl groups. Symbols have the same meaning as in Fig. 3.

Substitution of this equation into equation 5 then gives

$$p\mathrm{H} - \log \alpha/(1-\alpha) = pK' - 0.868w'\bar{Z}_{\mathrm{H}} \quad (7)$$

where

$$pK' = pK_{\mathrm{int}} - 0.868w\bar{\nu}_{K^+}{}^0 \quad (8)$$

$$w' = w(1 - B) \quad (9)$$

Only pK' and w' can be obtained with any certainty from the experimental data. The values of these parameters will coincide closely with those obtained for pK_{int} and w (after adjustment for the difference in assumed molecular weight) by Cannan, *et al.*, since these workers assumed no binding of salt ions. The adjustment for the difference in assumed molecular weight is made by use of equation 2 of Tanford and Epstein.[23]

$$w(\text{assumed mol. wt. } 35{,}500) = (40/35.5)\, w(\text{assumed mol. wt. } 40{,}000) \quad (10)$$

To evaluate pK' and w' one plots the left-hand side of equation 7, for a given kind of group, *versus* $\bar{Z}_{\mathrm{H}}$. This is most easily accomplished for the carboxyl groups, where correction for other groups which titrate in the same *p*H region is of least importance (*cf.* refs. 8 and 23 for a description of the procedure used). Using the data of Fig. 3 one obtains the plots of Fig. 4, which yield the values $w' = 0.072$ and 0.039, respectively, at ionic strengths 0.01 and 0.15. The corrected values of Cannan, *et al.*, at ionic strengths 0.01 and 0.135, are, respectively, 0.069 and 0.036. For pK' we get 4.75 and 4.69 at the two ionic strengths, whereas Cannan, *et al.*, obtained 4.60 at both ionic strengths. The difference in the pK values results largely from the difference in assumed isoionic points.

If the values of w' empirically observed for the acid region also apply to the neutral region, then one can determine pK' for the imidazole groups simply by finding what value is needed to fit the experimental points in the region from *p*H 6 to 7 (*i.e.*, up to the point where the structural rearrangement previously referred to occurs). The values so obtained are $pK' = 7.25$ at ionic strength 0.15 and $pK' = 7.4$ at ionic strength 0.01.

The values of pK' and w' just evaluated have been used to calculate titration curves at the two

(23) C. Tanford and J. Epstein, *ibid.*, **76**, 2163 (1954).

ionic strengths up to pH 7.5. Two terminal carboxyl groups with $pK' = 3.75$ have been included in making the calculations, as well as two amino groups with $pK' = 7.8$, though these make virtually no contribution in the pH region covered. The curves so calculated are the solid curves drawn through the experimental points of Fig. 3. It is seen that they provide an adequate representation of the experimental data except at the very acid end of the curve at ionic strength 0.15. (A corresponding deviation from linearity is seen at $\bar{Z}_H > 30$ in Fig. 4.) The deviation here is most probably due to the fact that β-lactoglobulin dissociates into half molecules (mol. wt. 17,750) at low pH.[21] The calculated value of w (using equation 2) for such half-molecules turns out to be 1.5 times the value of w calculated for a molecular weight of 35,500. The value of w which must be used to fit a titration curve based on double the true molecular weight is (using the analog of equation 10) one half of this. Assuming that the same ratios apply to w' we must use at the acid end $w' = 0.75$ (0.039) instead of the value of 0.039 used elsewhere. The curve so calculated is also shown in Fig. 3 and is seen to provide a much better fit of the approach to the point of maximum acid binding.

The Region beyond pH 7.5.—As was mentioned in an earlier paper,[18] a change in molecular configuration occurs near pH 7.5. Two carboxyl groups are titrated in the process, and, possibly, a change in pK_{int} of imidazole groups may occur. A change in the extent of K^+ binding is also a possibility. No further calculations for this region of pH have been made. Work is in progress on a detailed study of the configurational transition, from which it is hoped to obtain the thermodynamic parameters for the transition and to compare them with the titration curve. Regardless of the result of this study, however, no appreciable change in w' can take place, as the calculations pertaining to Fig. 5 below will show.

The Values of w and pK_{int}.—To obtain the true values of the empirical parameter w of equation 5 it is necessary to estimate a value for the parameter B of equation 6. Assuming that $\bar{\nu}_{K^+}$ becomes zero when the pH is as low as pH 3.1 we can evaluate B by using Carr's chloride binding data at that pH,[14] together with values of $\bar{\nu}_{K^+}{}^0$ taken from Fig. 2 and of $\bar{Z}_H$ from Fig. 3. Doing so, one obtains $B = 0.2$ and 0.33, respectively, at ionic strengths 0.01 and 0.15. By equation 9, w at these two ionic strengths becomes 0.090 and 0.058, respectively. Both these values are somewhat larger than the values calculated ($w = 0.088$ and 0.046) by equation 2, which assumes that the protein ion is spherical, with an evenly distributed surface charge. As has been pointed out elsewhere,[24] such a deviation indicates that the groups being titrated are unevenly distributed with respect to other charged groups, *i.e.*, some carboxyl groups are perhaps likely to be unusually close to other carboxyl groups (these are perhaps the K^+ binding sites) while others may be quite close to cationic groups.

The value of pK_{int} can now be obtained from equation 8. For the carboxyl groups $pK_{int} = 4.83$ and 4.87, respectively, at ionic strengths 0.01 and 0.15, for the imidazole groups $pK_{int} \simeq 7.45$ at both ionic strengths. It is to be noted that pK_{int} for the carboxyl groups is a little higher than has been observed in other proteins. This is perhaps again a reflection of the presence of pairs or higher multiples of COOH groups in fairly close proximity. The value for imidazole groups is not significant. It could be changed considerably if, for instance, the number of side chain carboxyl groups were adjusted to be integral for a molecular weight of 35,000 instead of 35,500. Such a change would not have an appreciable effect on the calculated value of pK_{int} for carboxyl groups.

The Titration Curve beyond pH 9.7

Titration curves above pH 9.7 were determined at one ionic strength (0.15) by means of the Radiometer autotitrator, used as a pH-stat. The instrument was set at a given pH and the amount of base required to attain that pH (time required $\sim$ 30 sec.) and then to maintain it was recorded as a function of time. Two titration curves were constructed from the data. One was based on an extrapolation to $t = 0$, using all points measured after the desired pH was obtained. The second was based on an extrapolation to $t = \infty$. In practice the amount of base used varied only negligibly after a period of about 2–3 hours at pH 10, and a period of about 5 minutes at pH 11. Both curves are shown in Fig. 5. This figure also shows the data of Cannan, *et al.*,[3] in this pH region, and it is seen that these correspond closely to our curve at $t = \infty$.

A curve at $t = 0$ was obtained in another way by using the flow apparatus described by Lovrien and Tanford.[25] This apparatus permitted pH measurements to be made within 1 second of mixing of the base added with the protein. The curve obtained is seen from Fig. 5 to be essentially identical to that obtained with the pH-stat, indicating that no appreciable denaturation occurs during the 30 seconds required in the pH-stat measurements for attainment of the desired pH.

Figure 5 also shows a few points obtained by the addition of acid to β-lactoglobulin which had been exposed for a few minutes to pH 12.5. These points fall right on the $t = \infty$ curve, *i.e.*, they indicate that denatured β-lactoglobulin is a relatively stable molecule with a reversible titration curve. This same conclusion was reached from an earlier study of the titration of phenolic groups in the same range of pH.[26]

In order to analyze these curves it is necessary to decide what groups are being titrated. At pH 10 all carboxyl, imidazole and α-amino groups have been titrated already, and the titration of guanidyl groups would normally not be expected to contribute to the curve below pH 12. Thus only ϵ-amino, phenolic and sulfhydryl groups come into consideration. It is assumed that all of these

(24) C. Tanford, THIS JOURNAL, **79**, 5340 (1957).

(25) R. E. Lovrien and C. Tanford, *J. Phys. Chem.*, **63**, 1025 (1959).

(26) C. Tanford and S. A. Swanson, THIS JOURNAL, **79**, 3297 (1957).

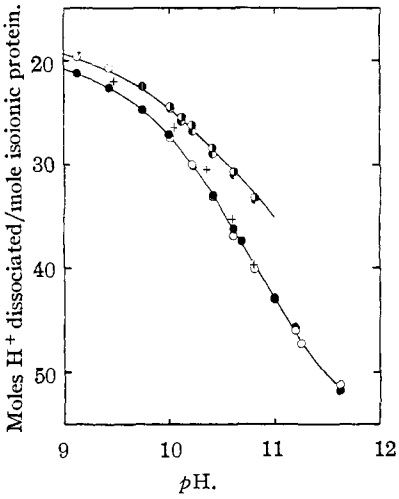

Fig. 5.—Titration curve above *p*H 9.7, at ionic strength 0.15: the symbols have these meanings: ◑, 1 second titration with flow apparatus; ◐, *p*H-stat data extrapolated to $t = 0$; ○, *p*H-stat data extrapolated to $t = \infty$; ●, reverse titration from *p*H 12.5; +, data of Cannan, *et al.*[8] The curve through the data at $t = 0$ is a calculated curve, as discussed in the text.

(37 in number, *cf.* Table I[27]) are titrated in the denatured protein, *i.e.*, in the curve at $t = \infty$. That the phenolic groups are titrated has been shown by spectrophotometric measurements reported earlier.

Sulfhydryl groups are assumed not to be titrated in the $t = 0$ curve, since they are quite unreactive in native β-lactoglobulin.[28] To decide whether phenolic groups are titrated, rapid optical density measurements of alkaline solutions were made on a Cary recording spectrophotometer. Neutral protein solution was placed in the spectrophotometer cell, base was added by means of a syringe, and absorption measurements were made at 295 mμ, beginning just a few seconds after mixing. In each such experiment the initial optical density was essentially the same as that finally attained, *i.e.*, since the final optical densities represent the normal titration of the phenolic groups of the denatured protein, it can be concluded that these groups were titrated before the onset of denaturation.[29]

Figure 6 shows the plot of *p*H-log $\alpha/(1 - \alpha)$ obtained for the titration of an assumed total of 34 amino plus phenolic groups at $t = 0$. The steep increase with increasing negative charge, typical of native proteins, is observed. Assuming both phenolic and amino groups to have the same pK' value, the slope of this plot can be intepreted as $0.868w'$ (equation 7), yielding a value of $w' =$

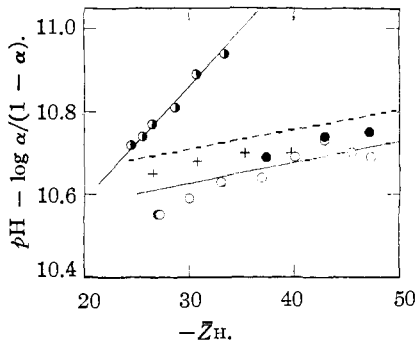

Fig. 6.—Logarithmic plot of the data of Fig. 5. Symbols have the same meaning as in Fig. 5. The dashed line is a similar plot for titration of phenolic groups, taken from an earlier paper.[26]

0.0315. This is somewhat less than the value of 0.039 observed for the carboxyl titration from Fig. 4, but is sufficiently close to it to indicate that the $t = 0$ titration curve is that of a molecule which is still rigid and relatively compact. Extrapolation of the straight line of Fig. 6 to $Z = 0$ yields $pK' = 9.95$, which is a reasonable value for amino and phenolic groups.

Actually a reasonable fit of the $t = 0$ curve can be obtained even if the value $w' = 0.039$ is retained. The value of pK' required is 9.85. The calculated curve based on these parameters is shown in Fig. 5. The number of side chain carboxyl groups used in this calculation is, of course, 51 rather than 49 because of the two extra carboxyl groups which appear near *p*H 7.5.[18]

Figure 6 also shows the corresponding logarithmic plot for the $t = \infty$ curve, assuming that 37 groups are being titrated.[30] The plot stands in sharp contrast to that for the $t = 0$ curve, the slope being much smaller. This behavior is typical of a flexible, polyelectrolyte-like molecule.[31] The titration curve at $t = \infty$ thus confirms the conclusion already reached from the spectrophotometric titration of phenolic groups in this *p*H range. The dashed line of Fig. 6 is taken from our earlier paper[26] on this subject. The line drawn, being for comparison with data at ionic strength 0.15, is taken as half-way between the experimental curves at ionic strengths 0.08 and 0.27. It is seen to be essentially parallel to the plot obtained from the $t = \infty$ curve, but it is located about 0.1 higher. This indicates that the phenolic groups have an intrinsic pK about 0.1 higher than that of the ε-amino groups.

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(27) The analytical figure for sulfhydryl groups must be incorrect, of course, if the β-lactoglobulin molecule consists of identical halves, However, the exact number does not matter for the calculations to be made here (see footnote 30).

(28) B. L. Larson and R. Jenness, THIS JOURNAL, **74**, 3090 (1952).

(29) It should be observed that these experiments are quite different from the *p*H-stat experiments in which the extent of titration of all groups increases at constant *p*H. Here the *p*H is not constant, but falls with time. If phenolic groups were inaccessible to titration at $t = 0$ they would become ionized with time, partially at the expense of other titratable groups.

(30) The assumptions which have been made concerning the accessibility of sulfhydryl groups are really quite unimportant. The logarithmic plots of Fig. 6 depend only in a minor way on whether 34 or 37 is chosen as the total number of titratable groups.

(31) C. Tanford, in "Symposium on Protein Structure," A. Neuberger, editor, Methuen & Sons, London, 1958.