XXXVI. THE HYDROGEN ION DISSOCIATION CURVE OF THE CRYSTALLINE ALBUMIN OF THE HEN'S EGG.

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THIS paper is a report upon a somewhat extensive investigation of the hydrogen electrode titration of crystalline egg albumin. The work was undertaken with the object of examining the limitations of the method as a means of establishing a quantitative definition of the amphoteric properties of a protein. Crystalline egg albumin was chosen because it is probably the best accredited example of a molecularly homogeneous protein. The extensive work of Sørensen and of his colleagues, of Svedberg and of others indicates that of the common protein preparations egg albumin exhibits the greatest degree of constancy in composition, molecular weight and solubility under varying conditions of preparation and treatment. This protein has the further advantage that it is soluble in water over the whole titratable range of p_H.

The data which will be considered derive from observations gathered over a period of five years upon five distinct preparations of the protein. These preparations had been submitted to considerable variations in treatment prior to titration. Each was crystallised four times—one by the original method of Sørensen [1917], the others by a modification of this method in which sodium sulphate replaced ammonium sulphate as the salting-out agent. Two of the latter preparations were employed in the electrometric work without having been reduced to the dry state. Two were converted into a dry powder and stored for some time before use. One of the dry preparations was dissolved in water and titrated without further purification, the other after one more recrystallisation. Some details of the method of preparing crystalline egg albumin in the dry state with the aid of sodium sulphate will be found in the experimental section.

Each product was prepared for titration as a stock solution which had been dialysed in distilled water until the sulphate ion could not be detected in a dialysate after sixteen hours' contact with the solution. Varying amounts of dilute HCl or of NaOH were added to equal volumes of the stock solution and the mixtures were then diluted to a predetermined concentration of protein. The majority of the observations relate to systems containing 22–25 g. of protein in 1000 g. of water. In a few cases this value was reduced to 15 g. and in a few others raised to 45 g. The p_H of each mixture was determined in a rocking hydrogen electrode [Clark, 1928] at 25°, using a saturated calomel half-cell and a saturated potassium chloride liquid junction. The calomel half-cell was calibrated with the aid of $0.1 N$ HCl, for which we assumed a p_H of 1.075 [Scatchard, 1925]. The interval between the preparation of a mixture and the determination of its p_H did not exceed 1 hour.

Calculations.

In the calculation of the hydrogen ion combined with the protein we made the following assumptions:

1. The albumin reacted to the addition of acid by reversible combination with hydrogen ions and to addition of base by reversible dissociation of hydrogen ions.

2. HCl and NaOH were completely ionised in the range of concentrations encountered. The activity coefficient of the hydrogen ion in any mixture containing HCl was the same as in a pure solution of HCl of the same concentration of chloride ions. The activity coefficient of the hydroxyl ion in any mixture containing NaOH was the same as the mean activity coefficient of NaOH in a pure solution of NaOH of the same concentration of sodium ions.

3. No diffusion potential existed at the liquid junctions.

The two latter assumptions are undoubtedly incorrect. The limitations which they impose upon the validity of the results will be discussed in a later section. The first assumption is the hypothesis whose utility in the analysis of titration data is under examination.

Symbols.

[Cl⁻] and [Na⁺] The respective molal concentrations of added acid or base in a reaction mixture.

[H⁺] and [OH⁻] The respective molal concentrations of hydrogen or of hydroxyl ions in the reaction mixtures at equilibrium.

g The mass of protein in grams in that volume of the mixture which contained 1000 g. of water.

h, H The equiv. of hydrogen ion combined with 1 g. and with 1 g.mol. (34,500 g.) respectively of the protein.

h_0 The equiv. of hydrogen ion combined with 1 g. of the protein in a mixture containing no added acid or base.

It was assumed that the isoelectric point was p_H 4·90 [Sørensen, 1925–28] and that at this p_H, $h=0$. A negative value of h indicates the dissociation of that amount of H⁺ from isoelectric protein. The introduction of h_0 is necessitated by the fact that the stock solutions were each somewhat removed from the isoelectric condition. That is to say they possessed individual initial values of h. It is these which we have represented by h_0.

For electrical neutrality in any mixture

$$[Na^+]+[H^+]+g\,(h-h_0)=[Cl^-]+[OH^-] \qquad \ldots(1).$$

The values of [Na⁺] and of [Cl⁻] were calculated from the measured additions of acid and of base. This procedure involves the assumption that the protein does not combine with significant quantities of either of these ions. The values of [H⁺] and of [OH⁻] were obtained from the observed values of p_H with the aid of the relations:

$$p_H = -\log[H^+]-\log\gamma_{H^+}=\log[OH^-]+\log\gamma_{OH^-}-\log k_w \qquad \ldots(2),$$

where $k_w = 1\cdot005 \times 10^{-14}$ [Lewis *et al.*, 1917]. Scatchard [1925] has made observations of the mean activity coefficient of HCl in solutions of this acid and from them has calculated a series of estimated values for γ_{H^+}. Our values for the latter have been taken from a curve drawn from Scatchard's results. We are not aware of any evaluation of γ_{OH^-}. We, therefore, assumed that, in the range of concentration with which we were concerned, it was substantially equal to $\sqrt{\gamma_{Na}\gamma_{OH^-}}$ in

corresponding solutions of NaOH. For the latter we used a curve drawn from the results of Harned [1925].

Equation (1) was solved for $h - h_0$. This was then plotted as a function of p_H, a separate curve being drawn for each stock solution. The value of $h - h_0$ on each curve at p_H 4·90 was determined from the curve. This, by definition, was the value of h_0 in the corresponding stock solution. Finally, all observations were brought together by plotting h as a function of p_H (Fig. 1). The figure includes
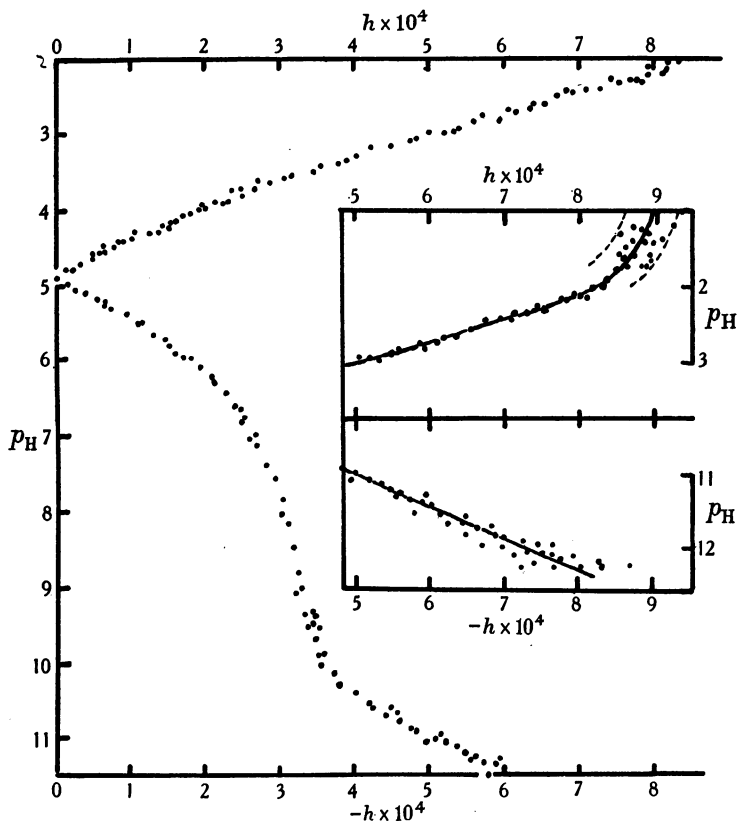

Fig. 1. H$^+$ ion combination curve of egg albumin. h = equiv. H$^+$ per g. protein.

about 180 points—observations on as many independent reaction mixtures. The only observations made in the course of this work which are not shown are the very few which through defects in the electrode system failed to give stable potentials. We have not attempted to indicate either the particular preparation or the particular concentration of protein to which each point refers. A careful inspection failed to reveal any distinct correlation between these variables and the deviations of individual points from the mean. Space does not permit the reproduction of even a representative selection of the data. We have therefore chosen a graphic analysis of the results. On a large reproduction of Fig. 1 a free hand curve was drawn through the assembled points and the ordinates of this curve were measured at small increments of p_H. These are recorded in Table I. Within the p_H range 2–11·5 no observations deviate from this mean curve by

Table I. *Hydrogen ion combining capacity of egg albumin.*

	Equiv.			Equiv.	
p_H	$\times 10^5$ per g.	Per g.mol.	p_H	$\times 10^5$ per g.	Per g.mol.
1·5	89·0	30·7	6·50	− 23·0	− 7·9
1·75	86·0	29·7	6·75	− 24·8	− 8·5
2·00	81·2	28·0	7·00	− 26·2	− 9·0
2·25	74·5	25·7	7·25	− 27·4	− 9·5
2·50	66·4	22·8	7·50	− 28·5	−9·8
2·75	58·5	20·2	7·75	− 29·8	− 10·3
3·00	49·2	17·0	8·00	− 30·8	− 10·6
3·25	40·6	14·0	8·50	− 32·0	− 11·0
3·50	32·2	11·0	9·00	− 32·8	− 11·3
3·75	25·0	8·6	9·50	− 34·5	− 11·9
4·00	19·0	6·6	10·00	− 37·0	− 12·8
4·25	13·0	4·5	10·25	− 39·4	− 13·6
4·50	7·0	2·4	10·50	− 42·5	− 14·7
4·75	2·0	0·7	10·75	− 46·0	− 15·9
5·00	− 2·2	−0·8	11·00	− 50·4	− 17·4
5·25	− 6·0	−2·1	11·25	− 55·7	− 19·2
5·50	− 10·4	−3·6	11·50	− 61·0	− 21·0
5·75	− 14·0	−4·8	11·75	− 67·0	− 23·0
6·00	− 17·8	−6·2	12·00	− 74·0	− 25·7
6·25	− 20·5	−7·1			

more than ± 0·5 equiv. per g.mol. of protein. The loss of precision which appears outside these limits will be considered below. In the meantime we submit that Table I defines a curve, which within the range p_H 2–11·5 represents a physical property of the protein reproducible within an error which is certainly less than one equiv. per g.mol. of protein. One qualification must be entered. The curve reported is valid only for systems from which electrolytes other than the reactants have been excluded. In a later communication we hope to report on the rather profound effects of changes in ionic strength due to addition of neutral salts.

A number of electrode titrations of egg albumin are recorded in the literature. Unfortunately the majority of these are reported in the form of small scale curves with which precise comparison is impossible. In many cases moreover the curve is modified by the presence in the protein preparation of significant concentrations of ammonium sulphate. Cohn [1925] collected the results available at that time and showed that they were in general conformity with one another and with unpublished observations of his own. Of the results quoted by Cohn we have taken the curves of Loeb [1920] and of Hitchcock [1920]. After readjustment of the isoelectric point we find that the results of Hitchcock which cover the range p_H 2–5 are in satisfactory agreement with Table I. Loeb's results extend from p_H 2 to 11. From the acid extreme to p_H 8 they do not deviate from our curve by much more than 0·5 equivalent per g. mol. The alkaline end of the curve however departs seriously from ours, a discrepancy which may in part be attributed to the presence of ammonia. Recently Prideaux and Woods [1932] have published an extensive series of observations which should have proved a valuable basis of comparison. Unfortunately they lack consistency with each other to a degree which is entirely contrary to our experience and which forbids any attempt at analysis.

The submission of a standard hydrogen ion dissociation curve for a protein requires proof that the observations relate to strictly reversible equilibria. We have repeatedly demonstrated to our satisfaction that the electrode potentials of reaction mixtures rapidly attained stable values. These remained constant (within a few tenths of a millivolt) for periods up to 48 hours, provided that the mixture was within the p_H range 2·5–11·0. We have found moreover that within

these limits a system may be moved up and down the curve in a strictly reversible manner. In making such tests it is important that the electrolyte concentration be maintained substantially constant. Precautions must also be taken to insure reproducibility of liquid junctions and to prevent diffusion of potassium chloride from them into the reaction mixtures during the periods of observation.

Below p_H 2·0 and above p_H 11·5 it is possible to demonstrate slow but consistent drifts in potential with time. In acid solutions these are in the direction of mounting values for p_H. In alkaline solution they proceed in the opposite direction. The changes are irreversible and must be assumed to be due to irreversible changes in the hydrogen ion dissociating capacity of the protein. Now the first recognised change which takes place in egg albumin under the influence of acid or of alkali is the formation of metaproteins. We are at present engaged upon a study of these products and find that separated acid metaprotein does dissociate considerably more H^+ and separated alkali metaprotein does combine with considerably more H^+ than the native albumin. Preliminary measurements have been made of the magnitude of these irreversible changes and of their rates as a function of p_H. The results, which will be published shortly, are such as to indicate that titrations may not be conducted beyond the p_H limits of 2 and 11·5 without danger of distortion from these irreversible processes.

Earlier in this paper it was acknowledged that the validity of the curve which we are reporting is limited by the errors involved in the assumption respecting (a) diffusion potentials and (b) the activity coefficients in equation (2). The presence of a significant diffusion potential will be reflected in an error in the observed p_H and therefore in the calculated value of $[H^+]$. An error in the assumed value of either γ_{H^+} or γ_{OH^-} will, likewise, introduce an error into $[H^+]$. Now in acid solution $g.h$ is substantially equal to $[Cl^-]-[H^+]$ and in alkaline solution to $\frac{k_W}{[H^+]}-[Na^+]$. It follows that the sources of error under consideration will affect h increasingly the more closely $[H^+]$ approximates to $[Cl^-]$ or $[OH^-]$ to $[Na^+]$, i.e. the higher the ratio of free HCl to total chloride or of free NaOH to total sodium. This consideration, be it said, was the one which led us to choose for the activity coefficients the values obtaining in pure solutions of acid or base. The choice becomes the more nearly correct the more seriously an error in it would affect the results.

In order to determine the practical limits of p_H imposed on the method of titration by these sources of error, we will assume the possibility of a diffusion potential of about 1 mv. and an error in the assumed activity coefficients of 5 %. A simple calculation will show that if any one of these were operative alone it would lead to an error of about 5 % in $[H^+]$. Taken together a possible error of 10 % arises. Now consider a 2·5 % solution of albumin. With the aid of Table I it may be calculated that an error of 10 % in $[H^+]$ would introduce an error of about 4 equiv. per g.mol. at p_H 1·5 or 12·5 and of about 1 equiv. at p_H 2·0 or 12·0. If a precision of at least 1 equiv. per g.mol. is to be attained, it would appear that we may not safely continue the titration curve beyond the limits p_H 2–12·0. Increase in the concentration of protein may permit a slight, but only slight, extension of these limits. These considerations together with those respecting irreversible changes in the protein lead to the choice of p_H 2–11·5 as the limits within which electrometric determinations of the hydrogen ion dissociation curve are dependable.

Maximum hydrogen ion combining and dissociating capacities.

Inspection of Fig. 1 will lead to the conclusion that within the acceptable range p_H 2–11·5 there is no evidence of the attainment of either a maximum or a minimum value of h. Below p_H 2 however the curve does turn definitely in a direction which, with some plausibility, we may extrapolate to a maximum value of about 0·9 milliequiv. per g. This is indicated in the upper inset curve of Fig. 1. The continuous curve is drawn for a maximum combining capacity of 31 equiv. of H^+ per g.mol. (0·89 milliequiv. per g.). The two broken lines are the corresponding curves for 30 and for 32 equiv. per g.mol. respectively. Although theoretically this extrapolation is subject to large errors, we are inclined to accept as the maximum hydrogen ion combining capacity of egg albumin the value of 31 ± 1 equiv. per g.mol. (0·87–0·93 milliequiv. per g.).

At the alkaline extremity of the curve on the other hand there is no indication of a maximum dissociating capacity even beyond p_H 12. There are evidently present in the protein groups which dissociate hydrogen ions only in strongly alkaline media. Although there is no indication of a stoichiometric end-point at the alkaline extremity of the curve, a distinct break in the curve does appear between p_H 8 and 9. This corresponds with the dissociation of 11 equiv. of hydrogen ion per g.mol. It occurs in so readily controllable a range of p_H that it should prove a very useful point about which to orient measurements of the amphoteric behaviour of the protein.

In forthcoming papers we hope to report on the changes in the curve which accompany (a) changes in the electrolyte concentration and (b) the presence of formaldehyde.

EXPERIMENTAL.

The preparation of crystalline egg albumin with the aid of sodium sulphate.

The following method was found convenient. The preparations used in this work have been of the order of 100–400 g. each.

A solution of Na_2SO_4 was prepared by dissolving 400 g. of the anhydrous salt in 1 litre of warm water. This solution which contains 36·7 g. of salt in 100 ml. must be kept at a temperature above 30° to prevent crystallisation. Having collected the whites of fresh eggs and broken up the membranes, the volume was measured and an equal volume of the salt solution added. The mixture was stirred for some time and then after 1 to 2 hours the precipitate was removed by filtration or in the centrifuge. A solution of 0·2 N H_2SO_4 was slowly added to the filtrate whilst the latter was stirred mechanically. Titration was continued until the p_H was about 4·6–4·8. A test on a few drops of the mixture with bromocresol green proved sufficiently accurate. It was unusual for any permanent precipitate of protein to separate during the titration but, if it did do so, it was redissolved by cautious addition of water. After the desired p_H had been attained, stirring was continued and anhydrous Na_2SO_4 was added slowly until a permanent opalescence developed. When crystallisation of the protein became clearly evident the mixture was decanted from any solid Na_2SO_4 which remained undissolved and placed on one side for a day or two. The whole of the process must be conducted in a room not cooler than 20° or it will be impossible to achieve a high enough concentration of salt to effect crystallisation of the protein.

The crystalline material was removed by filtration or in the centrifuge and redissolved in a volume of water about equal to the original volume of egg white. Recrystallisation was then effected by addition of solid Na_2SO_4 accompanied by stirring. From 140 to 180 g. of the anhydrous salt was required for each litre of

solution. After two further recrystallisations the final product was either brought into solution and dialysed or converted into a dry powder. In the former case it was possible by extracting the crystalline mass with ice-cold water to obtain a concentrated solution of protein which contained only about 5 % of sodium sulphate.

By spreading the mass of crystals and mother-liquor thinly in front of a fan in a warm room it was rapidly reduced to a dry powder. Little denaturation occurred during desiccation. The product was readily ground to a fine powder which sampled reproducibly and in which the protein had retained its ability to crystallise after storage at room temperature for 2 years. The compositions of two such preparations have been

	%	%
Soluble protein	63	70
Insoluble protein	3	1·8
Inorganic	28	23
Water	6	5·2
	100	100

After the first preparation had been stored for 2 years a sample was recrystallised. 93 % of the soluble protein (determined two years earlier) was recovered in the normal crystalline form.

The main advantage to us in the use of Na_2SO_4 for crystallisation was the elimination from our products of ammonia. Dialysed solutions of egg albumin which have been crystallised from $(NH_4)_2SO_4$ retain amounts of NH_4^+ which depend on the p_H of the solution. If specific action is not taken to remove this the observed titration curve of the protein will include the titration of NH_4^+ in the region of p_H 9–10. Now egg albumin is often crystallised at a p_H above 5·0. A simple calculation from Table I will show that the ammonia retained by such a protein will at p_H 10 dissociate an amount of hydrogen ion equal to 10 % of that dissociated by the protein itself. Had the initial p_H of the protein been 5·25 the error introduced would have been 20 %.

Analytical.

The concentrations of the various dialysed stock solutions were of the order of 7–10 %. A 5 ml. pipette was calibrated for the weight of the solution which it delivered under standard conditions. The dry weight, ash, total nitrogen and ammonia (none was found) in this volume of solution were determined. The dry weight was determined at 105°, the ash as sulphate and the ammonia by distillation *in vacuo* with $Mg(OH)_2$. In common with other observers we have had trouble with the determination of nitrogen in egg albumin by the Kjeldahl method. Various modifications of this method give different results and some methods are inconsistent in their behaviour. On the whole the Arnold-Gunning modification has proved the most satisfactory. In the calculations involved in the titrations we have preferred to use the ash-free dry weight rather than the protein calculated from the total nitrogen.

Electrode titrations.

The aliquots of the stock solutions for the various reaction mixtures were measured with the calibrated 5 ml. pipette. The first observation of the potential was made after 10 min. equilibration in the electrode vessel. The second reading was made after a further period of 5 min. in a fresh atmosphere of hydrogen. If these did not agree within 0·2–0·3 mv. (in the substantially unbuffered range of

p_H 8–9 a somewhat larger error was accepted) a third reading was taken. If a pair of these readings did not agree the sample was rejected and the electrode discarded. Each reading, of course, corresponded with a fresh liquid junction. The electrodes employed were thinly palladised or platinised platinum.

SUMMARY.

1. On the basis of an extensive series of observations of the hydrogen electrode potentials of the systems egg albumin-HCl and egg albumin-NaOH in dilute aqueous solution, data are submitted for the construction of a hydrogen ion dissociation curve of egg albumin.

2. An error not greater than ± 0.5 equiv. of hydrogen per g.mol. of albumin (mol. wt. 34,500) is claimed within the range of p_H 2–11.5.

3. Certain facts which limit the precision of the titration method are discussed.

4. The hydrogen ion combining capacity of egg albumin probably reaches a maximum of 30–32 equiv. per g.mol. slightly below p_H 2. At the alkaline extreme of p_H 11.5–12.0 there is no evidence of a stoichiometric end-point indicative of a maximum dissociating capacity. Such an end-point is however observed between p_H 8 and 9 and corresponds to the dissociation of 11 equiv. of hydrogen ion per mol.

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