48. ON THE *p*H AT THE SURFACE OF OVALBUMIN MOLECULES, AND THE PROTEIN ERROR WITH INDICATORS

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THE *p*H in a surface phase is frequently different from that in a bulk phase in equilibrium with the surface. The surface *p*H may be calculated from the Gibbs-Donnan equilibrium [Danielli, 1937] using the equation

$$\frac{(\alpha_{Na})_s}{(\alpha_{Na})_b}=\frac{(\alpha_H)_s}{(\alpha_H)_b}, \tag{1}$$

where α denotes activity and suffixes s and b denote surface and bulk phases. If the various ionic species considered in any one phase all have the same activity coefficient, or if a given ion has the same activity coefficient in both phases, then the activity coefficients will cancel, giving

$$\frac{[Na]_s}{[Na]_b}=\frac{[H]_s}{[H]_b}, \tag{2}$$

where the square brackets refer to concentrations. Or, taking logarithms, and using the suffix D to denote this method of calculation,

$$(pH_s)_D-pH_b=-\log\frac{[Na]_s}{[Na]_b}. \tag{3}$$

If we had considered a diffusible anion instead of a cation, e.g. Cl^- instead of Na^+, we should have

$$(pH_s)_D-pH_b=+\log\frac{[Cl]_s}{[Cl]_b}. \tag{4}$$

Equation (3) or (4) can thus be taken as one method of defining the difference between pH_s and pH_b. Harvey & Danielli [1938] pointed out that these equations predicted that the *p*H at the surface of protein molecules is different from the bulk *p*H.

Linderstrøm-Lang [1924] pointed out that the concentration of ions at the surface of a colloidal particle is different from that in the surrounding bulk phases, and that the concentration ratio will be determined by the electrical potential between the surface of the colloid and the bulk phase. Linderstrøm-Lang predicted that the titration curves of proteins would be straight lines in the region of the iso-ionic point, and that the addition of salt would change the slope of these lines. This prediction was confirmed experimentally by Linderstrøm-Lang & Lund [1926]. But Linderstrøm-Lang did not calculate the actual *p*H at the surface of a colloidal particle.

Hartley & Roe [1940] have shown that pH_s may also be calculated from the electrokinetic potential ζ, using the equation

$$(pH_s)_E-pH_b=\frac{\zeta}{60}=0{\cdot}325u \tag{5}$$

for small particles at 25°. u is the mobility in μ/sec./volt/cm.

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Both methods of calculating $p\mathrm{H}_s$ give the average value of $p\mathrm{H}_s$ in the surface phase. Actually variation in $p\mathrm{H}_s$ must occur within the surface phase, as a function of distance from the charged groups of the surface. There is some theoretical difference between $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$, since $(p\mathrm{H}_s)_D$ can, strictly speaking, give only the *activity* of the H^+ ion in the surface phase, and $(p\mathrm{H}_s)_E$ can only give the *concentration* of H^+ in the surface phase.

This paper deals with (1) the calculation of $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$ at the surface of ovalbumin molecules, (2) the correction of titration curves for the difference between bulk and surface pH, (3) the nature of the 'protein' error with indicators.

(1) *The* p*H at the surface of ovalbumin molecules*

Fig. 1 shows results for ovalbumin. $[(p\mathrm{H}_s)_E - p\mathrm{H}_b]$ is plotted against $p\mathrm{H}_b$, using the mobility measurements of Abramson [1934]. On the acid side of the isoelectric point the pH at the surface of the protein is greater than in bulk and on the alkaline side is less than in bulk.

Calculation of $(p\mathrm{H}_s)_D$ is more complicated. We need to know the molar concentration of Na^+ or Cl^- in the surface phase of the protein. We can obtain an approximate value (to be corrected later) by assuming that, when the protein is ionized as an acid, no Cl^- enters the surface phase, and that when ionized as a base no Na^+ enters the surface phase. Then the number of ions in the surface phase of one protein molecule will be approximately equal to the valency z of the protein molecule. The valency can be obtained from the titration curve. The theory of Debye & Hückel enables us to calculate the thickness δ of the surface phase. Then the volume of the surface phase is $V = \frac{4}{3}\pi[(r+\delta)^3 - r^3]$, where r = radius of the protein molecule. For ovalbumin $r = 2{\cdot}75 \times 10^{-7}$ cm. [Adair & Adair, 1940] and in $N/100$ uni-univalent electrolyte $\delta = 3{\cdot}1 \times 10^{-7}$ cm. Hence $V = 7{\cdot}5 \times 10^{-19}$ ml. per molecule. Then the concentration of ions in the surface phase is

$$\frac{z \times 1000}{V \times 6{\cdot}06 \times 10^{23}} = 2{\cdot}2z \times 10^{-3} \text{ g. mol./l.} \qquad (6)$$

This assumes that none of the mobile ions neutralizing the fixed charges on the protein surface can penetrate into the interior of the protein molecule. Having obtained this first approximation to the concentration in the surface, we can now use the Gibbs-Donnan equation:

$$\frac{[\mathrm{Na}]_s}{[\mathrm{Na}]_b} = \frac{[\mathrm{Cl}]_b}{[\mathrm{Cl}]_s} = \text{constant} \qquad (7)$$

and the condition for electrical neutrality in the interface:

$$[\mathrm{Na}]_s + [\mathrm{Cl}]_s + [\mathrm{Pr}]_s = 0 \qquad (8)$$

to calculate the true value of $[\mathrm{Na}]_s$ by successive approximations. $[\mathrm{Pr}]_s$ = concentration of ions due to groups fixed on the protein; e.g. for the case where $z = 10$, the protein is an anion and the bulk phase is $N/100$ NaCl, we have as a first approximation $[\mathrm{Na}]_s = 10 \times 2{\cdot}2 \times 10^{-3} = 0{\cdot}022$. Then from equation (7):

$$\frac{0{\cdot}022}{0{\cdot}01} = \frac{0{\cdot}01}{[\mathrm{Cl}]_s}$$

or

$$[\mathrm{Cl}]_s = 0{\cdot}0045.$$

Hence a better approximation to $[\mathrm{Na}]_s$ is $0{\cdot}022 + 0{\cdot}0045 = 0{\cdot}0265$. Then this value of $[\mathrm{Na}]_s$ can be substituted in (7) to get a more accurate value of $[\mathrm{Cl}]_s$. By a series of such successive approximations we obtain values for $[\mathrm{Cl}]_s$ and $[\mathrm{Na}]_s$ which are accurate. Having obtained $[\mathrm{Na}]_s$, and knowing $[\mathrm{Na}]_b$ and $p\mathrm{H}_b$,

we can now calculate $(pH_s)_D$ from equation (3). The results of such calculations are shown in Fig. 1.

In the above calculation there are three sources of error, whose magnitude may be estimated. (1) The possibility of some of the mobile ions in the surface phase being inside the micelle has been neglected. Suppose that the mobile ions can penetrate readily into the whole of the protein molecule—this will be the greatest possible degree of penetration. Then we must regard as the volume in which the mobile ions are distributed not only the external layer, given by the Debye-Hückel theory, but also a layer inside the molecule of the same thickness, i.e. $3{\cdot}1 \times 10^{-7}$ cm. in $N/100$ solutions. This is greater than the radius of the ovalbumin molecule and so in practice we must simply add to the previously obtained value of V the volume of the ovalbumin molecule, obtaining

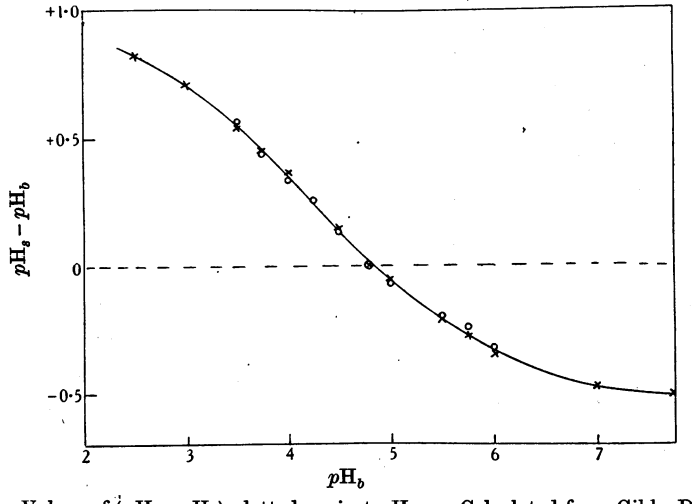

Fig. 1. Values of $(pH_s - pH_b)$ plotted against pH_b. × Calculated from Gibbs-Donnan equilibrium; o calculated from electrokinetic mobility.

$V' = 8{\cdot}37 \times 10^{-19}$ ml. per molecule instead of $7{\cdot}5 \times 10^{-19}$ ml. as the volume in which the z mobile ions are distributed. Using V' instead of V in equation (6) will lead to a reduction in $[(pH_s)_D - pH_b]$ of not more than 0·04 pH units. This is the maximum possible error introduced by ignoring penetration of ions into the interior of the ovalbumin molecule.

(2) The values of z have been taken from the results of Kekwick & Cannan [1936] for salt-free solutions of ovalbumin. In 0·01 M NaCl this value may be up to 10 % too low [Cannan, 1938]. Hence the value of z used in equation (6) may be too low by this amount, and $(pH_s)_D - pH_b$ will be too low by not more than 0·04 pH unit. Errors (2) and (3) are of opposite sign.

(3) Adsorption of ions by processes other than those considered here, i.e. specific adsorption, would also introduce an error. The results of Adair & Adair [1940] suggest that this effect is quite small for ovalbumin.

From this discussion it follows that these errors may lead to the values of $[(pH_s)_D - pH_b]$ given in Fig. 1 being in error by a factor of not more than $\pm 0{\cdot}04$ pH unit. We may therefore conclude that the values of the pH at the surface of ovalbumin molecules in 0·01 M NaCl solution, calculated by the two methods of equation (3) and equation (5), are in approximate agreement, since the points calculated by the two methods fall approximately in the same place.

(2) *The influence of ionic strength on surface* p*H and on the titration curve of ovalbumin*

We may write equation (4), for molecules on the acid side of the isoelectric point in the form

$$(pH_s)_D = pH_b + \log [Cl]_s - [Cl]_b. \qquad (4a)$$

From this it follows that pH_s is a function of the salt concentration in the bulk phase. The actual degree of dissociation in the surface phase depends primarily on the pH_s, and not on pH_b: hence it follows that, at the same pH_b, a protein will be dissociated (as an acid or base) to different degrees when different amounts of salts are present. In other words, the titration curves, that is, curves of acid or alkali combined with the protein plotted against pH_b, will be different

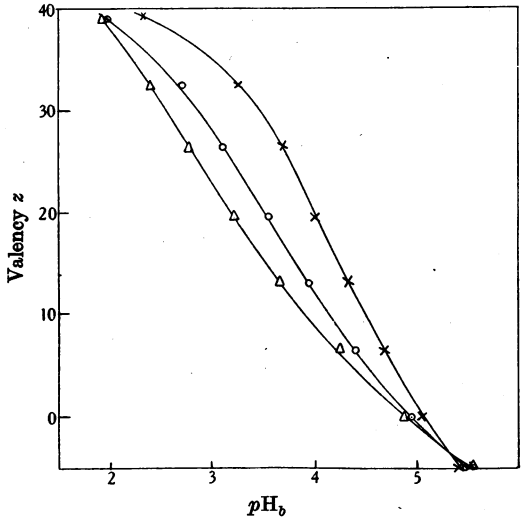

Fig. 2.

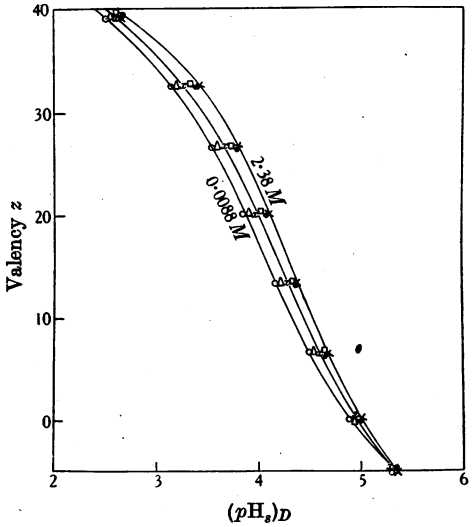

Fig. 3.

Fig. 2. Titration curves of ovalbumin in 2·38, 0·067 and 0·0088M KCl solution. Valency z plotted against pH_b. ×—×—× 2·38M, △—△—△ 0·0088M.

Fig. 3. Titration curves of ovalbumin in various dilutions of KCl. Valency z plotted against pH_s. The 3 curves are for 2·38, 0·067 and 0·0088M KCl. ×—×—× 2·38M, •—•—• 0·67M, □—□—□ 0·133M, ×—×—× 0·067M, △—△—△ 0·033M, o—o—o 0·0088M.

for different salt concentrations. If, however, we were to plot the amount of combined acid or alkali against pH_s, we should eliminate the effect of variation in pH_s with salt concentration. Of course, pH_s cannot be determined directly, but it can be calculated by either of the methods given above.

Consider ovalbumin: Cannan [1938] has published titration curves for this protein in nine different concentrations of KCl. Fig. 2 shows his curves for 0·0088, 0·067 and 2·38M KCl. The pH_b at which a given amount of acid is combined with the protein, i.e. at which the protein has a given valency z, varies by up to 0·9 pH unit between 0·0088 and 2·38M KCl; as would be predicted from equation (4a), the more dilute the bulk phase, the more acid is the pH_b at which a given valency is achieved.

Fig. 3 shows the same data, plotted against pH_s as calculated from equations (3) and (4). The greater part of the variation with salt concentration is eliminated, and instead of the family of curves of varying form of Fig. 2, we get a

series of curves which are substantially the one curve displaced along the $(pH_s)_D$ axis. The method by which $(pH_s)_D$ was calculated was as follows. (1) Approximate values for the surface concentration C_s of the ion of opposite sign to the protein were calculated for various dilutions of KCl. This involved calculation of (*a*) δ, the thickness of the double layer, (*b*) V, the volume of the double layer, and (*c*) the approximate value of C_s as the valency z multiplied by a constant typical of a given salt concentration. Table 1 shows this calculation. The next step (2) was to calculate the approximate values of C_s for a given z but different KCl dilutions from the values in Table 1, then correct this value of C_s by the method of successive approximations. Then (3) $(pH_s)_D$ was finally calculated from equation (3) or (4). A typical example of such calculations is shown in Table 2. Values of $\Delta pH = (pH_s)_D - pH_b$ are also given and will be observed to increase with increasing dilution of KCl. The individual values of $(pH_s)_D$ are subject to an error not greater than $\pm 0{\cdot}05$ pH unit, apart from any systematic error.

Table 1. *Calculation of the approximate concentration* C_s *in the surface phase of the ion of opposite sign of charge to that of ovalbumin*

C_s is given in the last column as a function of the valency z of ovalbumin. The radius r of ovalbumin is taken as $2{\cdot}75 \times 10^{-7}$ cm. $\delta = 3{\cdot}1 \times 10^{-8} \div \sqrt{C_b}$.

C_b	$\delta \times 10^7$	$(r+\delta) \times 10^7$	$(r+\delta)^3 \times 10^{21}$	$V \times 10^{20}$	$C_s \times 10^{-2}$ (1st approximation)
2·38	0·21	2·96	25·9	2·18	7·6z
1·19	0·28	3·03	28	3·06	5·4z
0·67	0·38	3·13	30·6	4·15	3·99z
0·27	0·60	3·35	37·5	7·05	2·35z
0·133	0·85	3·60	46·5	10·8	1·53z
0·067	1·20	3·95	61·5	17·1	9·7z
0·033	1·70	4·45	88	28·2	5·85z
0·017	2·38	5·13	134	47·5	3·49z
0·0088	3·3	6·05	222	84·5	1·95z

Table 2. *Calculation of* $(pH_s)_D$ *from the data of Table 1, and equations* (7), (8) *and* (4a)

Case of $z = 26{\cdot}3$.

C_b	C_s 1st approximation	C_s corrected	pH_b	$(pH_s)_D = pH_b - \log C_b + \log C_s$	$\Delta pH = (pH_s)_D - pH_b$
2·38	2·0	3·58	3·70	3·88	0·18
1·19	1·42	2·1	3·55	3·81	0·26
0·67	1·048	1·38	3·5	3·81	0·31
0·27	0·62	0·72	3·35	3·78	0·43
0·133	0·402	0·44	3·22	3·74	0·52
0·067	0·255	0·27	3·10	3·71	0·61
0·033	0·154	0·161	2·88	3·61	0·73
0·017	0·092	0·095	2·80	3·55	0·75
0·0088	0·051	0·053	2·75	3·53	0·78

The fact that, when z is plotted against $(pH_s)_D$ we get not one but several curves, shows that variation in ionic strength at constant pH_b has other effects in addition to affecting pH_s. There are probably many factors involved. The liquid junction potential involved in the measurement of pH_b varies slightly with ionic strength. The dissociation constants even of monocarboxylic acids vary slightly with change in ionic strength. In addition, it has been assumed in the above calculations that the surface area of ovalbumin molecules is in-

dependent of ionic strength: this may well not be true. Also the possibility that ions may enter the interior of the protein molecule has been neglected.

Hartley & Roe [1940] suggest that curves of z plotted against pH_s, rather than pH_b, should be used in determining the nature of the ionizing groups of proteins. It is certainly true that Fig. 3 presents a picture much more susceptible to analysis than is Fig. 2. If, as Cannan suggests, the titration curve of ovalbumin between *p*H 2 and *p*H 7 is that of a polycarboxylic acid, the apparent *p*K of this acid, from Fig. 3, lies between 4·15 and 4·4.

To carry out the suggestion of Hartley & Roe involves calculating pH_s either from the electrokinetic mobility or from the Gibbs-Donnan equilibrium, using the data of the titration curve itself, as has been done above for ovalbumin. Systematic studies of variation in mobility with wide variation in *p*H and ionic strength have not yet been made, so that the method of Hartley & Roe is not always available. On the other hand, calculation from the Gibbs-Donnan equilibrium involves knowledge of the shape and surface area of the protein concerned—this again is seldom available. A possible solution of this difficulty is to obtain values of the electrokinetic mobility at several *p*H, and from these to calculate the charge density. Then, since the titration curve gives the valency z, i.e. total charge per molecule, multiplying the charge density by z will give the surface area, which may then be used for other *p*H values. This suggestion, however, needs very careful experimental checking.

(3) *The 'protein error' in* p*H determinations with indicators*

Many workers, e.g. Sörensen [1909], have studied the effect of proteins on colorimetric *p*H determinations. As a general rule, where the indicator and protein have opposite charges, the protein error may be large. Where the indicator and protein have the same sign of charge, the protein error is usually small.

Danielli [1937] pointed out that much of the error in *p*H determinations with indicators is due to adsorption of indicators at surfaces whose *p*H is not that of the bulk phase. The indicator, if adsorbed, tends to give the *p*H of the surface phase, not of the bulk phase. Hartley [1934] has made a wide study of the indicator error with solutions containing paraffin chain acids and bases, finding that with solutions containing micelles of basic substances (whose surfaces are more alkaline than the bulk phase) the *p*H given by the indicator is displaced from the bulk value towards the alkaline side, whereas with micelles of acidic substances the displacement is in the reverse direction. A most striking effect is that where warming of the solution causes dissolution of the micelles; as the dissolution becomes complete, the indicator colour changes sharply to that characteristic of the bulk *p*H. This change may be as large as two *p*H units.

A large part of the protein error with indicators is probably due to this effect. Most of the evidence in the literature is fragmentary, but it is true of these scattered observations that with indicators which are anions, the indicator error is large and positive on the acid side of the isoelectric point of a protein and small on the alkaline side. With indicators which are cations the relationships are reversed. This is the type of variation to be expected if the indicator error is mainly due to adsorption of indicator at the protein surface: if a protein is positively charged it will tend to adsorb large organic anions but not cations, and since the *p*H at the protein surface is greater than in bulk, the 'protein error' is positive. These relationships are reversed at a negatively charged surface. St Johnston & Peard [1926] have made a study of the sulphonethalein indicators

with gelatin: their results are in agreement with the above theoretical conclusions, but, as the colour comparison was made by eye, their individual results are in error by 0·05–0·1 *p*H unit, so that the points are scattered rather widely.

Fig. 4 shows some more accurate determinations on 1 % ovalbumin solutions, made with a photoelectric colorimeter, which allows determinations to be made to ±0·02 *p*H unit on standard buffer solutions. The bulk *p*H values were measured with a glass electrode. Three indicators were used: bromocresol green and chlorophenol red being anionic, and neutral red cationic. Fig. 4 shows that with the anionic bromocresol green the protein error is positive on the acid side of the isoelectric point and almost zero on the alkaline side. With chlorophenol red the pH_b values all lay on the alkaline side and the protein error was small. But with neutral red, with which again the pH_b values were all on the alkaline side, the protein error was large and negative. Qualitatively these results are in agreement with theoretical predictions.

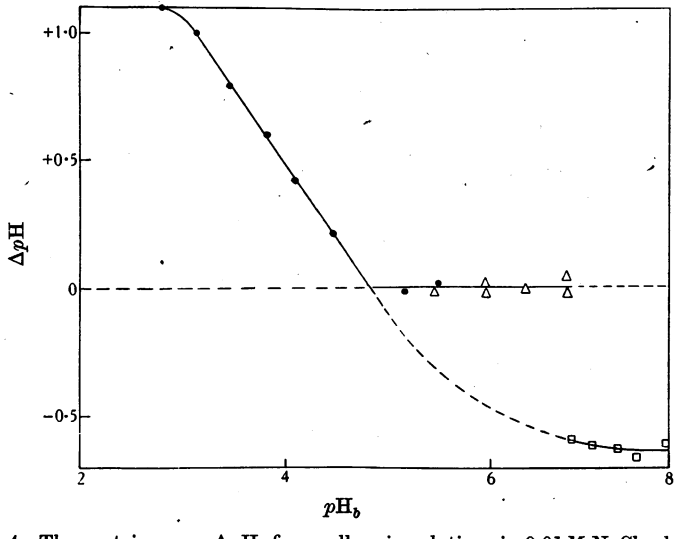

Fig. 4. The protein error, Δ*p*H, for ovalbumin solutions in 0·01 *M* NaCl, plotted against pH_b. • Bromocresol green; ▵ chlorophenol red; □ neutral red.

The ovalbumin was four times recrystallized, dialysed salt-free and diluted to a 4 % solution. NaCl was added to bring the salt concentration to 0·01 *N*. 5 ml. samples of the solution were titrated with 0·01 *N* NaOH or HCl in 0·01 *N* NaCl to suitable pH_b values, with a standard amount of indicator present, and then diluted to 20 ml. with 0·01 *N* NaCl: the final solution contained 1 % ovalbumin. The *p*H values of these solutions were measured first with a photoelectric colorimeter, then with the glass electrode, both determinations being made on the same solution, the difference between the two values being the protein error. The colorimeter was calibrated by using solutions of measured *p*H containing 0·01 *N* NaCl and 0·0002 *M* acetate buffers and the same standard amount of indicator.

Discussion

The previous examination of the results for ovalbumin shows that the *p*H at the surface of ovalbumin molecules may be quite different from that in bulk solution and that it is affected by salt concentration as well as by bulk *p*H. In

the case of colloidal enzymes, as was suggested to me by Prof. J.C. Drummond in 1935, this may be a matter of considerable significance. In 0·01 N solutions, the thickness of the ionic double layer is about 30 Å.: this is considerably greater than the diameter of many substrate molecules, so that in such a solution the enzyme-substrate interaction must occur within the double layer, and the significant pH determining the rate of reaction is probably the surface pH. Varying the ionic strength of the medium at constant $p\mathrm{H}_b$ must vary $p\mathrm{H}_s$, and it seems probable that the greater part of the effect of neutral salts on enzymes is due to this variation in $p\mathrm{H}_s$. Whether this is actually so must await more systematic studies than are yet available. It is, of course, not practicable in general practice to measure $p\mathrm{H}_s$ in most cases. But if the ionic strength of the substrate is kept constant when the pH is changed a standard set of conditions will result, so that one observer can compare his results exactly with those of another; e.g. if two sets of results are obtained with an ionic strength of 0·1, they may be compared exactly, for the same colloid in both sets of experiments will have the same $p\mathrm{H}_s$ at a given $p\mathrm{H}_b$, even though the $p\mathrm{H}_s$ values cannot be measured. The simplest procedure is probably to carry out all experiments in say 0·5 or 0·1 N NaCl, and not to allow the molar concentration of buffer substances to rise above 1 % of the NaCl; under these conditions the ionic strength will be practically constant. Polyvalent ions are to be avoided as far as possible.

Both methods of calculating $p\mathrm{H}_s$ determine the average pH in the surface phase, assuming that the charges are evenly distributed over the colloid surface and it is assumed that $p\mathrm{H}_s$ is the same throughout the surface phase. It is quite possible that this is not true and that there is a considerable local variation in $p\mathrm{H}_s$. Neither method of calculation can detect such local variations. It is possible, however, that the results with indicators are partly due to local variations. The protein error ΔpH of Fig. 4 is roughly 1·4 ($p\mathrm{H}_s - p\mathrm{H}_b$) of Fig. 1, for indicators of opposite charge to the protein; i.e. although qualitatively the protein error follows the difference between the surface and bulk pH values, quantitatively it is 40 % larger than can be due to the calculated values of $p\mathrm{H}_s$, even if the whole of the indicator were adsorbed. This factor of 1·4 may be due to the ionizing groups being concentrated in patches on the protein surface. But there are many other possible explanations and no conclusion can be reached until the distribution of other ions is studied.

The views advanced here are not intended to constitute a complete theory of the protein error with indicators. Many other factors, such as the different valencies of the two forms of an indicator, and molecular interactions such as have been recently studied by Schulman & Rideal [1937], must also be involved.

Summary

1. $p\mathrm{H}_s$, the pH at the surface of a protein molecule, is different from that in the surrounding bulk phase. $p\mathrm{H}_s$ may be calculated either from the Gibbs-Donnan equilibrium, or from the electrokinetic mobility. For ovalbumin the two methods give results in reasonable agreement.

2. At constant bulk pH, $p\mathrm{H}_s$ varies with ionic strength, and consequently the acid- and base-binding powers of a protein vary with ionic strength. It is shown that this accounts for the major part of the influence of ionic strength on the titration curve of ovalbumin, between pH 2 and pH 5·5.

3. Indicators of opposite charge to a protein molecule tend to be adsorbed and give values of $p\mathrm{H}_s$ rather than bulk pH values. Results are given for ovalbumin solutions with bromocresol green, chlorophenol red and neutral red. It is

not clear whether the whole of the protein error is due to the difference between $p\mathrm{H}_s$ and the bulk pH.

4. It is suggested that $p\mathrm{H}_s$, and not $p\mathrm{H}_b$, is the significant pH in enzyme activity, and that, to facilitate comparison of results, enzyme activities should be determined in solutions of constant ionic strength.

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