CLXXI. THE DETERMINATION OF THE ISOELECTRIC AND ISOIONIC POINTS OF HAEMOGLOBIN FROM MEASUREMENTS OF MEMBRANE POTENTIALS.

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HARDY [1899] showed that the electrical charge of protein particles is negative in alkaline solutions and positive in acid solutions. The isoelectric point may be defined as the effective concentration or activity of hydrogen ions at which the protein is uncharged or possesses an equal number of positive and negative charges. Many of the properties of proteins, including solubility, osmotic pressure and cataphoresis, are correlated with the charge on protein ions and have been used in measurements of their isoelectric points, recently reviewed by Pauli and Valkó [1933].

The suggestion that the isoelectric point of a protein could be determined by measurements of membrane potentials has been made by Loeb [1924], Taylor [1924], Adair [1928] and Henriques [1929], but hitherto this has not been realised in practice. Loeb showed that acid solutions of gelatin are positive, whereas alkaline solutions are negative, but he was unable to obtain a definite value for the isoelectric point. The difficulties he encountered were partly due to the fact that gelatin is sparingly soluble at the isoelectric point and partly to the very low buffer value of the dilute acids and alkalis he used as dialysates. Taylor used haemoglobin and reported that the experiments were unsuccessful on account of precipitation of the protein at the isoelectric point. His solutions were acidified by the direct addition of acids. Adair [1928] obtained haemoglobin at the isoelectric point without precipitation by dialysis against buffer mixtures and stated that the isoelectric point in phosphate buffers is on the acid side of the isoionic point, defined by Sørensen *et al.* [1927] as the activity of hydrogen ions at which the protein neither gives off nor takes up hydrogen ions.

According to Henriques [1929] the "Donnan potential" of haemoglobin is zero at p_H 5.5, a figure much more acid than the value p_H 6.8 determined by the method of cataphoresis [Michaelis and Davidsohn, 1912]. Theoretical conclusions based on this work [Bolam, 1932] are of doubtful significance, as Henriques did not determine the equilibrium concentrations of phosphates and bases or the isoionic point of the protein, and the material used, a mixture of corpuscles and saponin, dialysed for 24 hours, was probably not strictly comparable with more highly purified preparations. There is a discrepancy between Henriques' experimental data and the results obtained by Adair [1928] and by Roche *et al.* [1932].

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The sources of error in measurements of membrane potentials have been studied by Adair and Adair [1934], who devised a method adapted for systems with small potentials. The experiments described in the present communication show that it is possible to determine the isoelectric point of a protein by a similar method, and by correlating the measurements of membrane potentials with studies of the distributions of diffusible ions, made by chemical methods, the isoelectric point may be compared with the isoionic point of the protein.

1. *Preparation of CO-haemoglobin.*

Horse CO-haemoglobin was crystallised by a modification of the method of Ferry [1923], based on the fact that this protein is sparingly soluble at the isoelectric point. The haemoglobin of the sheep, like that of man and of the ox, is highly soluble at the isoelectric point and is therefore adapted for experiments which require a wide range of protein concentrations. In the preparation of proteins for measurements of isoelectric or isoionic points, a high degree of purification is advisable, but impurities must be removed under conditions which cause minimum changes in the protein molecule. In the present investigation, comparisons of preparations of sheep CO-haemoglobin, made by different methods, have been made, in order to reduce the risks of error due to impurities and denaturation products.

Method 1. This depends on the use of freshly purified ether and of sodium chloride to separate haemoglobin from the stroma-proteins and from lipins. Laboratory ether generally contains peroxides which cause methaemoglobin formation; the removal of peroxides can usually be effected by five extractions with *N*/10 sodium hydroxide, followed by four extractions with water. Even with purified ether, it is necessary to cool the mixture to 0° as soon as possible and to remove the ether by dialysis, as described below. The disadvantages attending the use of ether are compensated by a much more complete removal of stroma-proteins and of lipins than is accomplished by alternative methods based on the use of alumina cream [Walker and Williamson, 1920], centrifuging or certain processes of crystallisation.

For the preparation of sheep CO-haemoglobin, defibrinated blood was saturated with pure carbon monoxide and cooled for an hour at 0°. The plasma-proteins were removed by four centrifugings, 1.5 % NaCl being used to wash the corpuscles. A rubber-tipped glass rod was used to mix the corpuscles adherent to the bottom of the centrifuge-tube with the salt solution. The volume of the corpuscles was estimated, and for each 100 ml., 30 ml. of distilled water were used to wash the corpuscles into a flask, where laking was completed by the addition of 30 ml. of purified ether in the course of about 3 minutes' shaking. 13 g. of NaCl were then added, to shrink the swollen stromata, and the mixture was shaken until the salt had dissolved. After centrifuging (for about 10 minutes at 3000 r.p.m.) the stromata rise to the top in the form of a solid cake, and the clear solution of haemoglobin can be removed by means of a pipette. If contamination by stroma proteins occurs, it may be removed by filtration through cotton wool.

The haemoglobin solution was then placed in collodion membranes and dialysed against distilled water at 0°, the water being changed about 6 times at intervals of one hour on the first day and at three hour intervals on subsequent days. Overnight, the membranes were left in a volume of water at least 10 times as great as the volume of the membrane. In order to concentrate the solutions, the membranes were connected with reservoirs of air or carbon monoxide, under a positive pressure of about 700 mm. Hg. After dialysis against distilled water

for 3 days, the protein was dialysed against buffer solutions, as described in the following section.

Method 2. The corpuscles were laked with an equal volume of distilled water, without using ether, and 1 % NaCl was then added. A part of the stroma-protein could be removed by centrifuging, and further purification could be effected by pressure dialysis against distilled water, when a gelatinous precipitate formed on the membrane. It was generally necessary to change the membrane several times to ensure efficient dialysis and approximately complete removal of stromata.

Method 3. The solution freed from stromata and from ether and salt by method 1 was crystallised by the addition of ammonium sulphate at 0°. A 22 % solution of CO-haemoglobin was mixed with an equal volume of $M/15$ Na_2HPO_4 , since a slightly alkaline reaction inhibits methaemoglobin formation; conditions favourable for crystallisation were then determined by trials with samples containing from 1.02 to 1.2 ml. of saturated ammonium sulphate per ml. of the diluted protein solution. Thin layers of the mixture exposed to the air usually crystallise within an hour or two, and these crystals can be used to seed the samples. The stock solution was then crystallised in a bottle containing carbon monoxide, with the addition of the determined amount of ammonium sulphate and about 5 ml. of previously crystallised solution. The mixture was shaken at intervals, and if necessary, after crystallisation had started, the yield of crystals was increased by the addition of more ammonium sulphate. The crystals could be separated from the mother-liquor by filtration through a Büchner funnel; they were then washed with a mixture of 110 ml. saturated ammonium sulphate and 100 ml. $M/15$ Na_2HPO_4 or $(\text{NH}_4)_2\text{HPO}_4$, saturated with carbon monoxide. The crystals were then dissolved in the minimum volume of water and dialysed. All the above operations were carried out at 0°. The yield was about 60 % of the total haemoglobin. Recrystallisation was brought about by the same procedure.

Method 4. The mother-liquor from the crystals prepared by method 3 was placed in large membranes and concentrated by dialysis against distilled water under a pressure of about 600 mm. Hg.

2. *Experimental methods for the determination of the isoelectric point from measurements of membrane potential.*

The apparatus for the measurement of membrane potentials, including collodion membranes and saturated calomel KCl electrodes, has been described in a previous paper [Adair and Adair, 1934]. In determinations of the isoelectric point, a series of membranes was prepared, filled with protein solution and placed in pyrex tubes or beakers containing a series of buffer solutions at different p_{H} values, exemplified by the lists of solutions in Tables IV and V. The solutions and electrodes were kept in a room at $0^\circ \pm 0.2$. The increase in the stability of the proteins and the inhibition of bacterial action at low temperatures made it possible to continue equilibration for 10 days or more, with repeated changes of buffer mixtures, and to make rigorous tests of equilibrium. If the membranes were fitted to manometers, osmotic equilibrium was determined as described by Adair [1925, 2]; if the membranes were stoppered with rubber bungs, the membrane potentials were determined at intervals until constant readings were obtained. Large volumes of dialysate were required for concentrated protein solutions equilibrated with dilute buffers.

By dialysis with repeated changes of buffer mixtures, it is possible to make a series of experiments with varying p_{H} and constant phosphate concentration, or at a constant ionic strength. In the experiments of Loeb [1924], of Taylor [1924]

and of Henriques [1929], which were carried out at room temperature, a single sample of dialysate was used, in order to reduce the time of equilibration, but the equilibrium concentrations of the salts in the dialysate in such cases may differ quite markedly from the initial concentrations, and the relationship between the membrane potentials and the p_H values is more difficult to interpret, if the salt concentrations are independent variables. Comparative studies of the membrane potentials of different protein preparations in equilibrium with the same dialysate are moreover facilitated by methods in which the dialysate is renewed repeatedly. This procedure has the additional advantage that traces of acidic and basic impurities are removed more rapidly by dialysis with solutions of electrolytes than with pure water.

After equilibrium has been established, two procedures are available for the determination of the isoelectric point

1. The p_H values of the protein solutions and the dialysates are determined by the Clark hydrogen electrode or by other methods. In the more acid solutions, the p_H of the protein solution exceeds that of the dialysate; in the more alkaline solutions, the p_H of the protein is lower, as shown in Table XV, and it is possible to determine by interpolation the isoelectric point I, where both p_H values are equal, according to the definition suggested by Adair [1928].

2. The membrane potential E and the p_H of the dialysate are determined for each of the systems, and the point where $E=0$ is found by interpolation, as shown in Fig. 1. If the protein concentrations are not identical, a correction can be applied, based on the observation that the ratio E/C_v is approximately constant, when E is small [Adair and Adair, 1934].

Theoretically, the two methods should give the same results, since the membrane potentials and the p_H values of the protein solution and the dialysate, symbolised $p_{H'}$ and $p_{H''}$, are correlated by a thermodynamical formula due to Donnan and Allmand [1914], equivalent to formula 1 below, in the case of measurements at 0° , as stated by Adair and Adair [1934]:

$$E = 54 \cdot 16 (p_{H'} - p_{H''}) \quad \dots\dots(1),$$

E = membrane potential in millivolts at 0° .

In this equation and in all the formulae and tables given below where it is necessary to distinguish between the protein solution and the dialysate, the same notation is used. The symbols with two accents refer to the dialysate. Method 2 is preferable in the case of haemoglobin solutions on account of the difficulties of hydrogen electrode measurements in the protein solutions. Both methods are based on the assumption that a saturated solution of potassium chloride annuls the liquid junction potentials, and therefore the results may be regarded as conventional rather than as absolute values.

In special cases where the volume of the solution available is very small, approximate determinations may be made by a third method, in which the protein is equilibrated with a dialysate at a reaction close to the isoelectric point. The hydrogen ion concentration of the protein solution is estimated by formula 1, and the apparent valence n_p by formula 9, and if n_p be not equal to zero, a correction is applied, based on the observations recorded in Section 9, which show that over a limited range of p_H , the rate of change of valence with the p_H is approximately constant.

The membrane potentials plotted against p_H , shown in Figs. 1 and 2, lie on smooth curves, and the point of intersection where $E=0$ can be determined with an accuracy which compares not unfavourably with alternative methods for the estimation of the isoelectric point. The degree of accuracy obtainable is increased

by the use of concentrated protein solutions, although the gain is not directly proportional to the protein concentration, since certain errors, including those due to liquid junction potentials, tend to increase with the protein concentration. In buffered solutions where the ionic strength is low (0.01–0.02), but not so low that traces of carbon dioxide from the air and alkalis from the glass vessels cause significant changes in the p_H of the dialysate, it seems possible to estimate the isoelectric point of a given preparation with an accuracy of 0.01 p_H unit. If the ionic strength of the buffer be 0.1 or more, the error may be 0.05 p_H unit or even greater, as the potentials are small in concentrated salt solutions.

Measurements of isoelectric point are of little value without precise specification of the experimental conditions, including the composition of the solutions and the standardisation of the electrometric measurements. All the p_H values of the dialysates, given below, were measured with a Clark hydrogen electrode, rocked for 1 hour at 0°, and a saturated calomel electrode. In order to obtain measurements on buffer solutions at 0° comparable with figures for higher temperatures, standardised as suggested by Clark [1928], the calomel electrode was standardised on the conventional assumptions that the p_H of a solution of 0.01 *N* HCl plus 0.09 *N* KCl is 2.04, and that a saturated solution of KCl annuls the liquid junction potentials.

The protein concentration has been determined by the method of Kjeldahl, supplemented by measurements of densities and of refractions, as described by Adair and Robinson [1930, 1] and by Roche *et al.* [1932]. In order to calculate the percentage concentration of the protein, symbolised C , in g. dry protein per 100 ml. solution, the nitrogen content of sheep CO-haemoglobin was determined as 16.75 ± 0.05 % for crystallised and for uncrystallised protein. The percentage concentration C is directly proportional to the difference between the densities of the protein solution and the dialysate, symbolised D' and D'' in the formula $D' - D'' = k_d C$, where k_d is an empirical constant, equal to 0.00257 at 0° and 0.00251 at 18°, in the case of solutions of sheep haemoglobin in equilibrium with dilute phosphate buffers. The refractive indices of the protein solution and of the dialysate, symbolised R' and R'' , are correlated with C by the empirical formula $R' - R'' = \alpha' C$, where α' is equal to 0.001945 ± 0.000005 .

The carbon monoxide capacity of the solutions of haemoglobin after equilibration was determined by the method of Van Slyke and Hiller [1928]. Estimations of diffusible substances present in the protein solutions and dialysates, namely phosphates, chlorides, ammonia and bicarbonates were made by methods referred to by Adair and Adair [1934]. Sodium was determined by electro-dialysis [Adair and Keys, 1934]. The estimations of small amounts of bases, including ammonium and sodium, in the presence of large amounts of protein are subject to appreciable errors. Determinations of the ammonia content of the protein solution by nesslerisation gave in some cases higher values than those obtained by the micro-Kjeldahl method or by electro-dialysis, some of which are included in Table VIII.

The results of analyses are usually expressed in g. mols. per litre of solution. The corrected concentrations, expressed in g. mols. per litre of solvent, may be calculated by formula 2, as described by Adair and Adair [1934]:

$$m_p = c_p \times 100 / (100 - V_1 C); [\text{NH}_4]_c' = [\text{NH}_4]' \times 100 / (100 - V_1 C) \dots\dots(2),$$

m_p = corrected concentration of the protein, of molecular weight 67,000;
 c_p = protein concentration in g. mols. per litre of solution; $(100 - V_1 C)$ = volume of solvent in ml. per 100 ml. of protein solution; C = g. dry protein per 100 ml.;
 V_1 = effective volume per g. dry protein, approximately 0.965 ml. The difference

between this figure and the specific volume of dry protein, 0.75 ml., is attributed to water combined with the protein. $[\text{NH}_4]_c'$ = corrected concentration of ammonium ions; $[\text{NH}_4]'$ = concentration of ammonium ions in g. mols. per litre of protein solution. In the dialysates, which are free from protein, the corrected concentration is the same as the observed concentration, $[\text{NH}_4]''$.

In addition to the measurements in which chemical analyses of protein solutions and dialysates were made for the purpose of comparing isoelectric and isoionic points, a number of determinations of isoelectric point were made by a simpler procedure, in which the measurements made included the membrane potentials, the refractive indices of the protein solutions and dialysates and the p_{H} of the dialysates. If the protein solutions have been equilibrated with successive changes of dialysate, and the refractive indices and p_{H} values of the final dialysate agree with those of the standard buffer mixture, it may be assumed that the composition of the dialysate is the same as that of the standard solution.

3. *The determination of the isoionic point of haemoglobin.*

Sørensen *et al.* [1927] have given a detailed description of the determination of the isoionic point of a protein in a form applicable to solutions of all protein concentrations. If, however, concentrated solutions are available, the estimation of the isoionic point can be made by simpler methods, since calculations given below show that there is little difference between the isoionic point and the hydrogen ion concentration of a concentrated solution of the protein in pure water, or in a solution containing equivalent amounts of crystalloid acids and bases.

Theoretically, such a solution is neither isoionic nor isoelectric, because the addition of a pure protein such as egg-albumin to water increases the acidity, so that the protein may lose hydrogen ions. In the case of egg-albumin, where the p_{H} of pure solutions is approximately 4.8, the concentration of hydrogen ions given off is of the order of 2×10^{-5} equivalent per litre, or 0.01 equivalent per mol. of protein, if the protein concentration be 0.002 *M*. The titration curves of Sørensen *et al.* [1927] show that egg-albumin is a protein with a relatively high buffer value, since one molecule (34,500 g.) gives off more than 8 hydrogen ions, if the p_{H} of the solution be increased by 1 unit by the addition of alkali.

The loss of 0.01 equivalent of hydrogen ions per mol. corresponds to an increase of 0.0012 unit in the p_{H} , and therefore the p_{H} of the pure solution may exceed the isoionic point by 0.0012 unit, a correction which can be disregarded in comparison with the experimental errors. At low protein concentrations, where the differences between the isoionic point and the p_{H} value of the pure protein solution are more important, the correction cannot be estimated in the simple manner referred to above, and the determination must be made by the method of Sørensen.

The determinations of the isoionic point of haemoglobin made by previous workers have been obtained at various temperatures; the values may be reduced to 0°, using the temperature coefficient of 0.018 p_{H} unit per degree [Adair *et al.*, 1929; Stadie and Martin, 1924]. The titration curves of Hastings, Van Slyke *et al.* [1924] show that the reduced haemoglobin of the horse has an isoionic point of 6.81 at 38° (7.495 at 0°), and the values for oxyhaemoglobin are 6.70 at 38° (7.385 at 0°), with a maximum deviation of 0.07 p_{H} unit. Terroux [1931], who used the glass electrode, obtained the figure 6.79 at 22° (7.176 at 0°) for sheep haemoglobin; Errera and Hirschberg [1933] give 6.55 at 18–20° (6.87–6.91 at 0°) for horse haemoglobin. The results of different workers are thus not in agreement. The

data of Hastings, Van Slyke *et al.* [1924] are of special value as they refer to analysed solutions of known CO₂ and oxygen content.

In the present investigation, three methods have been used for the determination of the isoionic point. By method A, a number of measurements were made by a procedure similar to that of Sørensen *et al.* [1927] and of Van Slyke *et al.* [1922]. A concentrated solution of sheep CO-haemoglobin was dialysed for a week against distilled water, then against water containing CO₂ for one or two days, to remove bases combined with the protein, and finally re-dialysed against distilled water. Samples of the solution were then mixed with water, dilute HCl and dilute NaOH, and the p_H values were determined with the Clark hydrogen electrode. The CO₂ contents of samples equilibrated with hydrogen were then measured. The calculations of the acid or base in combination with the protein were then made by formula 3, as described by Van Slyke *et al.* [1922] and Hastings, Van Slyke *et al.* [1924]. Within the range of concentrations of the protein and of electrolytes used in this work, the calculations of the acid bound made by these methods are in agreement with the more rigorous methods described by Sørensen *et al.* [1927]:

$$A_p c_p = [\text{Total anions}] - [\text{Total cations}] \quad \dots\dots(3).$$

A_p = equivalents of acid, *i.e.* hydrogen ions combined with one molecule of the protein. c_p = molar concentration of the protein. The terms in square brackets denote the total concentrations of anions and cations in equivalents. Where anions are present in excess, as in mixtures of sodium chloride and protein chloride, A_p is positive; where cations are present in excess, as in sodium proteinate, A_p is negative, since the protein gives off hydrogen ions in alkaline solution.

Observations made by this method, recorded in Table XII and Fig. 6, indicate that the isoionic point, or more exactly, the p_H value where $A_p = 0$, is approximately 7.4 at 0°.

In method B, devised to facilitate comparisons of isoionic and isoelectric points under the same conditions, the calculations of the acid bound are based on chemical analyses of the dialysate and of the protein solution, samples of the latter being removed from the membrane just before the measurement of the potentials. Experiments were carried out with a range of buffers, so that the point where $A_p = 0$ could be determined by interpolation, as shown in Fig. 5.

In calculations of A_p for solutions of haemoglobin buffered with ammonium phosphates, it must be remembered that the analytical results are obtained in terms of total ammonia and total phosphoric acid. Appreciable amounts of un-ionised ammonium hydroxide are present if the p_H is greater than 7.7 at 0°. Ammonium hydroxide in the dialysate is estimated from measurements of the total ammonia and of the p_H by the application of the law of mass action, and it is assumed that the corrected concentrations, defined by formula 2, are the same in the protein solutions and in the dialysates. The total phosphoric acid in the p_H range from 6 to 8 exists mainly in the form of the univalent ion H₂PO₄ and the bivalent HPO₄; the concentrations of free acid and of the trivalent ion can be neglected. The acid combined with protein can then be calculated without serious error by formula 4:

$$m_p A_p = 2 [\text{HPO}_4]_c' + [\text{H}_2\text{PO}_4]_c' - [\text{NH}_4]_c' = [\text{PO}_4]_c' (1 + x') - [\text{NH}_4]_c' \quad \dots\dots(4).$$

The symbol $[\text{PO}_4]_c'$ is used to represent the corrected concentration of total phosphate in the protein solution, in mols. per litre of solvent. x' = the fraction of

the total phosphate in the protein solution in the bivalent form; an estimate of x' can be obtained by the procedure below.

In the first place, x'' , the proportion of the bivalent ion in the dialysate composed of the pure salts $\text{NH}_4\text{H}_2\text{PO}_4$ and $(\text{NH}_4)_2\text{HPO}_4$, may be estimated by the application of the law of mass action to the measurements of hydrogen ion activities, or the concentrations of the ions HPO_4 and H_2PO_4 may be calculated

Table I. *Ion distributions and acid combined with haemoglobin.*

Sheep CO-haemoglobin, equilibrated with 0.02 *M* ammonium phosphate at 0°. Exp. 654. Osmotic pressure = 92.9 mm. Hg. Membrane potential = -0.57 mv. $p_{\text{H}''} = 7.008$, $p_{\text{H}'}$ (calc.) = 6.998. $C = 19.68$. $m_p = 0.003628$. $x' = 0.50$. $A_p = 4.96$. f'/f'' , the ratio of the activity coefficients, computed by formula $f' \cdot [\text{NH}_4]_c' = r_i f'' \cdot [\text{NH}_4]''$. In the dialysate $[\text{HPO}_4]'' = [\text{NH}_4]'' - [\text{PO}_4]''$, and $[\text{H}_2\text{PO}_4]'' = [\text{PO}_4]'' - [\text{HPO}_4]''$. $x'' = 0.505$.

Ion species	Dialysate concentration	Ideal distribution ratio r_i	Corrected concentration, ideal	Protein solution		f'/f''
				Concentration, observed	Corrected concentration, observed	
NH_4	0.03020	1/1.024	0.03093	0.02651	0.0327	0.945
PO_4 (mol)	0.02007	—	0.01935	0.02748	0.0339	0.571
HPO_4 (mol)	0.01013	1/1.05	0.00965	—	0.0169	—
H_2PO_4 (mol)	0.00994	1/1.024	0.00970	—	0.0170	—
PO_4 (equivalent)	0.03020	—	0.02900	—	0.0507	—

from analytical determinations of total ammonia and phosphoric acid, as shown in Table I, where the value of x'' is estimated by the formula

$$[\text{NH}_4]'' = (1 + x'') [\text{PO}_4]''.$$

The theoretical distribution ratio for each ion is then calculated by formula 5, a modification of the formula of Donnan and Allmand [1914] discussed by Adair and Adair [1934]:

$$r_i = \text{antilog} [-n_i E / 54.16] \quad \dots\dots(5),$$

r_i = ideal distribution ratio = a_i' / a_i'' , where a_i' and a_i'' denote the activities or effective concentrations of the diffusible ions in the protein solution and in the dialysate respectively; n_i = the valence of the ion (negative for anions). The ideal concentration of each ion in the protein solution is then calculated by formula 6,

$$[\text{NH}_4]_c' \text{ (ideal)} = r_i [\text{NH}_4]'' \quad \dots\dots(6).$$

The same notation is used in application of formula 6 to the other diffusible ions. x' is estimated from these "ideal" concentrations, as shown in Table I. Calculations of A_p based on these assumptions cannot be exact, but it is unlikely that the error is serious, since the titration curve obtained by this method agrees with results obtained by method A, as shown in Fig. 6.

In practical applications of formula 4, it is advisable to use solutions in which the protein concentration, C , exceeds 20 g. per 100 ml. solution, since an error of 0.00032 mol. per litre in the estimation of ammonia may cause an error of 0.01 p_{H} unit in the determination of the isoionic point of a solution in which $C_v = 26.8$ and $m_p = 0.004$, whereas in a solution where $C_v = 2.68$, the same error in the analyses causes a tenfold increase in the errors in the estimation of A_p and of the isoionic point.

Gasometric analyses showed that the protein solutions dialysed against ammonium phosphate buffers may contain from 0.0001 to 0.0002 mol. of CO_2 per litre, concentrations which are too low to cause serious errors in the estimation of

the isoionic point of solutions where C_v exceeds 20. In similar solutions dialysed against distilled water, the CO_2 content may be as much as 0.004 mol. per litre.

Method C is based on the hypothesis that if inorganic salts cause a difference between isoionic and isoelectric points, either by the combination of ions with the protein or by their effects on interionic forces, such differences must diminish as the salt concentration is reduced. A series of measurements of the isoelectric point was made and plotted against $\sqrt{\mu}$, the square root of the ionic strength of the dialysate, as shown in Fig. 7. If a straight line be drawn through the points and extrapolated to zero salt concentration, the p_H value obtained is not far removed from the isoionic point determined by method B.

4. *The comparison of different preparations of haemoglobin.*

In order to determine whether the proteins prepared from the bloods of different individuals are identical [Barcroft, 1928], a number of preparations of CO-haemoglobin were made from sheep's blood by method 1 and equilibrated with ammonium phosphate buffer mixtures. Seven preparations gave a value for the isoelectric point of $p_H 7.06 \pm 0.01$ at 0° . The values for the remaining preparations ranged from 7.0 to 7.14, with one exception, which gave the value 6.75; this material had been exposed to high temperatures during centrifuging.

Table II. *Comparison of preparations of sheep CO-haemoglobin, made by method 1.*

Osmotic pressures in mm. Hg and membrane potentials in mv. at 0° , extrapolated for protein concentration $C=21.29$, $C_v=26.8$ and $m_p=0.004$, and for dialysate $(\text{NH}_4)_2\text{HPO}_4=0.005 M + \text{NH}_4\text{H}_2\text{PO}_4=0.005 M$, $p_H=7.09$ at 0° . Isoelectric points for 0.01 M ammonium phosphates. Isoionic points estimated by method B, Section 3, except Exps. 594–601 and 613–622 by method C.

Exp. no.	Osmotic pressure	Membrane potential	Isoelectric point	Isoionic point
311	—	–0.40	7.05	7.50
331–337	105	–0.20	7.07	7.39
451–462	103	–0.40	7.05	7.41
467–477	102	–0.85	7.00	7.37
559–570	109	+0.50	7.14	—
577–588	109	+0.40	7.13	7.42
594–601	107	–0.35	7.05	7.56
613–622	101	–2.93	6.75	7.34
623	—	–0.20	7.07	—
654, 655	107	–0.20	7.06	7.63
641	107	–0.20	7.06	—
N. H.	106	+0.30	7.11	7.56
S.	107	+0.30	7.13	7.56

The experimental determinations of the isoelectric and isoionic points represent the total protein present in the preparations, which contain traces of other derivatives in addition to CO-haemoglobin. Oxyhaemoglobin is present in all solutions containing dissolved air, but the titration curves of Hastings, Sendroy *et al.* [1924] and measurements of cataphoresis [Svedberg, 1933] indicate that replacement of oxygen by carbon monoxide causes no change in the isoelectric or isoionic points. The isoionic point is affected by reduction, but the concentration of reduced haemoglobin is very small in the presence of carbon monoxide at 0° . Methaemoglobin or other derivatives which do not combine reversibly with oxygen and carbon monoxide are formed during the preparation of haemoglobin. The uncrystallised haemoglobin made by method 1 combined with 1.27 ± 0.03 ml. CO per g. protein; crystalline haemoglobin prepared by method 3, combined with

1.21 ± 0.04 ml. CO per g. protein. If the difference between these figures and the theoretical value of 1.34 ml. per g. be attributed to methaemoglobin, the proportion of this protein is about 5 % in the uncrystallised and 10 % in the crystallised preparations. If the protein be completely changed into methaemoglobin, the isoelectric point is increased from p_H 6.92 to 7.04 [Svedberg, 1933], whereas the isoionic point is increased by 0.15 ± 0.05 p_H unit [Hastings *et al.*, 1924]. The variations in isoelectric and isoionic points attributable to the formation of 10 % of methaemoglobin are 0.006 and 0.015 p_H unit respectively.

Unpublished experiments have shown that the denaturation of haemoglobin may yield products that are more acidic or more basic than native haemoglobin, and it is conceivable that the formation of soluble derivatives of a type intermediate between native and denatured protein might account for the variations in the isoionic points of haemoglobin shown in Table II and by other workers. The possibility that there are slight irreversible changes in the protein is indicated furthermore by the observation that a preparation kept at 0° for 16 months showed an increase in isoelectric point from 7.00 to 7.07, without appreciable change in the CO capacity. It is possible that the anomaly in the isoelectric behaviour of gelatin described by Pauli and Valkó [1933] can be accounted for by differences in individual preparations.

It is unlikely that the solutions used in this work contained appreciable amounts of completely denatured haemoglobin, since the protein was highly soluble at the isoelectric point and formed a wholly crystalline solid phase on addition of ammonium sulphate. The osmotic pressures, moreover, were all within 4 % of the mean value of 105.5 mm. at 0° for a solution with $m_p = 0.004$, without making corrections for the variations of pressure with the charge on the protein ions, which would reduce the differences observed.

Comparison of crystallised and uncrystallised haemoglobin prepared from the same blood, recorded in Table III, shows that the isoelectric point of the crystallised preparations is at a more alkaline p_H . No appreciable difference

Table III. *Isoelectric points of crystallised and uncrystallised haemoglobin of sheep.*

Dialysates, ammonium phosphates at 0°. Each number refers to blood of a different individual.

No.	Molarity of buffer	Total haemoglobin	Crystallised haemoglobin	Recrystallised haemoglobin	Mother-liquor
1	0.01	7.03	7.12	—	—
2	0.01	7.14	7.18	7.18	7.19
3	0.02	6.86	6.91	—	6.91

could be detected in measurements of nitrogen content, hydration, osmotic pressure, refraction or density. In the crystallised preparations made by method 3, described above, the amount of methaemoglobin formed, as estimated by the fall in CO capacity after crystallisation, is about 5 %; the variations in isoelectric point, ranging from 0.04 to 0.1, exceed the changes attributable to methaemoglobin. It is unlikely that the change is due to the existence of two different forms of haemoglobin, or to the removal of an acidic impurity by crystallisation, since preparations of concentrated mother-liquor appear to have the same isoelectric point as crystallised or recrystallised protein, as shown in Table III. If fractionation takes place, the acidic substances should be more concentrated in the mother-liquor than in the total protein or the crystalline fraction. From these experiments and from the observation that solutions of crystallised haemoglobin may yield traces of precipitate at 0°, it may be inferred

that the change in isoelectric points is due to the action of ammonium sulphate on the protein.

Dr K. O. Pedersen kindly examined two solutions, Exp. 2, total and crystallised haemoglobin in Table III (sent to Upsala in ice in a thermos flask) and found that the bulk of the protein, with a light absorption of wave-length 5–600 $m\mu$, was homogeneous [Pedersen, 1933]. A trace of a substance with a different mobility was detected, a larger amount of this being present in the crystallised than in the uncrystallised material. The isoelectric point was at p_H 7.09 for both preparations, measured by cataphoresis at 20° with KH_2PO_4 - Na_2HPO_4 buffers of 0.02 ionic strength. In view of Dr Pedersen's results, it seems that the haemoglobins made by methods 1 and 3 are much more nearly homogeneous than the preparations obtained by Geiger [1931], who reported the presence of relatively large amounts of two different fractions, isolated by the method of cataphoresis.

Measurements were made to determine whether ether has an injurious effect on the protein. The isoelectric points of preparations made with and without ether are not identical, but the mean difference found of 0.05 p_H unit is not excessive. It is probable that the preparations made with ether give a better approximation to a pure, unaltered protein, since the material obtained without the use of ether was kept at room temperature for a longer time, in efforts to remove stroma-protein by centrifuging. Measurements of the carbon dioxide dissociation curves of human haemoglobin showed no evidence of irreversible changes in the protein caused by the addition of ether during the process of laking [Adair, 1925, 1].

The comparisons of different preparations of sheep CO-haemoglobin have not been restricted to the measurements given in Tables II and III. The evidence as a whole has shown that although the absolute values of the isoelectric and isoionic points are affected by the methods of preparation, there are properties of the protein which are practically constant, including the changes in the membrane potentials and the valences with the p_H , the effects of salts on the isoelectric point and the effects of the protein on the activities of the diffusible ions. In the following sections, which are concerned with the properties which are more nearly constant, detailed comparisons of different preparations have not been recorded.

It is possible that the variations in the isoelectric and isoionic points of individual preparations of sheep haemoglobin would be less marked in the case of ox haemoglobin, since the globin moiety of this protein is more stable.

5. *Haemoglobin and ammonium phosphate.*

In the investigation of the equilibrium of sheep CO-haemoglobin and ammonium phosphate buffers, recorded in Tables IV, V, VI, VII and VIII, a single preparation of protein was used in each series of experiments, in order to obtain comparable results in studies of the effects of hydrogen ions and salts on the membrane potentials. If a curve be constructed from data obtained with more than one preparation, allowances must be made for differences between the preparations, referred to in Section 4. With the exception of the experiments with crystalline haemoglobin, given in Table VIII, the haemoglobin was prepared by method I. Estimations of the isoelectric points of the preparations are included in Table II.

The solutions of ammonium phosphates $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$, used in the dialysis fluids, were prepared from recrystallised salts or from an analysed sample of A.R. $(NH_4)_2HPO_4$, which gave theoretical values for ammonium and

phosphoric acid. Glass-distilled and boiled-out water was used and the solutions were stored at 0°. In estimations of the ionic strength and other calculations, it has been assumed provisionally that the salts are fully ionised in accordance with recent work on strong electrolytes [Debye and Hückel, 1923]. On this assumption, the ionic strength μ , defined as half the sum of the concentrations of the ions in the dialysate, multiplied by the squares of their valences, is equal to $[\text{NH}_4\text{H}_2\text{PO}_4]'' + 3[(\text{NH}_4)_2\text{HPO}_4]''$.

Table IV gives the experimental data required for the determination of the isoelectric point by interpolation (method 2, Section 2) as shown in Fig. 1, in

Table IV. *CO-haemoglobin and ammonium phosphate buffers of constant molarity.*

Exps. 451–462, preparation 1931, 4, isoelectric point 7.05. Exps. 467–477, preparation 1931, 5, isoelectric point 7.00. Dialysates 0.01 *M* ammonium phosphates. *C*=g. dry protein in 100 ml. solution. *p*=osmotic pressure in mm. Hg at 0°. $p_{\text{H}}''=p_{\text{H}}$ value of dialysate. *E*=membrane potential in mv. at 0°. $[\text{NH}_4]''$ =total ammonia in mols. per litre in dialysate. $[\text{NH}_4]'$ =total ammonia in protein solution. $[\text{PO}_4]''$ =total phosphoric acid in dialysate. $[\text{PO}_4]'$ =total phosphoric acid in protein solution.

Exp. no.	<i>C</i>	<i>p</i>	p_{H}''	<i>E</i>	$[\text{NH}_4]''$	$[\text{PO}_4]''$	$[\text{NH}_4]'$	$[\text{PO}_4]'$
451	20.6	142.6	6.30	+7.93	0.0133	0.00997	0.0089	0.02663
452	21.2	107.2	6.94	+1.03	0.0136	0.00969	0.0131	0.01739
454	21.0	100.7	7.10	−0.38	0.0150	0.00997	0.0155	0.01497
455	21.4	103.4	7.11	−0.39	0.0154	0.01000	0.0152	0.01505
456	21.6	98.9	7.26	−1.83	0.0161	0.00999	0.0152	0.01366
458	20.2	93.8	7.42	−2.70	—	0.01010	0.0182	0.01126
459	20.7	100.9	7.59	−3.67	0.0167	0.01000	0.0193	0.00930
460	20.7	98.5	7.53	−3.35	0.0176	0.01005	—	—
461	19.9	99.6	7.68	−4.57	0.0183	0.01010	0.0239	0.00861
462	19.3	152.7	8.44	−9.75	0.0215	0.01007	0.0331	0.00452
467	21.4	124.0	6.67	+3.17	0.0121	0.00984	0.0102	0.02180
469	21.5	109.7	6.94	+0.62	0.0141	0.00980	0.0141	0.01706
471	21.9	107.1	7.09	−0.88	0.0145	0.00982	0.0142	0.01510
473	21.8	105.6	7.26	−1.98	0.0152	0.00999	0.0147	0.01348
474	20.8	96.9	7.37	−2.45	0.0166	0.00996	0.0170	0.01190
475	21.8	105.4	7.40	−3.07	0.0160	0.00993	0.0181	0.01123
476	21.8	110.8	7.54	−3.93	0.0182	0.01006	0.0199	0.01008
477	21.7	166.9	8.23	−9.20	0.0224	0.00990	0.0301	0.00506

Fig. 1. Isoelectric point of sheep CO-haemoglobin, method 2, Section 2. Dialysates 0.01 *M* ammonium phosphates. Membrane potentials for 21.29 % protein solutions ($m_p=0.004$); extrapolated from data in Table IV. Circles, preparation 1931, 4; crosses, preparation 1931, 5.

systems in which the molarity of the dialysate is kept constant. The measurements of the concentration of ammonia and phosphoric acid included in this table were used in the calculations of the acid bound by the protein, the valence of the protein (formula 9) and the isoionic point (method B, Section 3), shown in Fig. 5. Values of the ratio of the activity coefficients f'/f''_{PO_4} , based on data in Table IV, are recorded in Table XV. This ratio represents a mean value for the ions HPO_4 and H_2PO_4 , computed from the formula

$$f'/f''_{\text{PO}_4} = [\text{PO}_4]_c' (\text{ideal}) / [\text{PO}_4]_c' (\text{observed}).$$

Similar values were obtained with other preparations. In the case of ammonium ions, the results for f'/f''_{NH_4} , computed from Table IV, are more irregular and smaller than values computed from Table VIII. It is probable that the difference is due to errors in the determinations of ammonia included in Table IV. In all cases the protein causes a much greater diminution in the activities of the anions than of the cations. Slight differences in the same direction have been observed by Hastings *et al.* [1927] and by Adair [1928], but their experiments were made at a higher salt concentration, under conditions less favourable for the demonstration of specific effects due to the protein.

The effects of variation in the hydrogen ion concentration with buffers of constant ionic strength are shown in Table V. The buffer value of the solutions of

Table V. *Effects of variations of p_H at constant ionic strength.*

Sheep CO-haemoglobin and ammonium phosphate buffers at 0°. Exps. 559–570, preparation 1932, 1; Exps. 577–588, preparation 1932, 4. Isoelectric points for both preparations are 7.34, 6.46 and 6.15 with dialysates of ionic strengths 0.01, 0.10 and 0.20 and phosphate concentrations of 0.04, 0.072 and 0.138 respectively.

Exp. no.	Ionic strength	Molarity PO_4	p_H''	C	p	E	E^*
559	0.01	0.008	6.40	19.8	147.1	+9.25	+9.10
560	"	0.006	6.90	20.7	121.8	+5.0	+5.05
561	"	0.005	7.13	20.8	109.8	+2.9	+2.80
562	"	0.004	7.42	20.5	94.7	-1.44	—
563	0.10	0.08	6.00	21.2	117.9	+0.63	—
564	"	0.06	6.58	20.7	101.5	-0.2	-0.12
565	"	0.05	6.89	20.9	102.7	-0.7	—
566	"	0.04	7.36	20.8	103.5	-1.25	-1.40
567	0.20	0.16	5.85	21.2	115.4	+0.08	+0.05
568	"	0.12	6.45	20.8	108.0	-0.18	-0.10
569	"	0.10	6.77	20.6	103.0	-0.40	—
570	"	0.08	7.27	21.1	108.6	-1.00	-0.80
577	0.01	0.008	6.33	20.8	169.5	+9.56	—
578	"	0.006	6.82	21.5	132.5	—	—
579	"	0.005	7.11	21.6	116.4	+2.47	—
580	"	0.004	7.47	21.7	106.0	-2.26	—
581	0.10	0.08	6.00	21.5	120.8	+0.75	—
582	"	0.06	6.57	21.5	111.5	-0.23	—
583	"	0.05	6.88	21.5	109.3	-0.65	—
584	"	0.04	7.37	20.5	102.3	-1.44	—
585	0.20	0.16	5.84	21.4	116.5	+0.4	—
586	"	0.12	6.43	21.1	109.4	-0.37	—
587	"	0.10	6.77	21.0	108.0	-0.60	—
588	"	0.08	7.28	21.0	108.4	-0.93	—

E^* . Supplementary experiments with stoppered membranes, at the same p_H and protein concentration.

ionic strength 0.01 is low, and the final p_H values of the dialysates and the standard solutions are not identical; analyses are given in Table VI. The pro-

Table VI. Concentrations of ions in protein solution and dialysate.

Exp. no.	$[\text{NH}_4]^+$	$[\text{PO}_4]^-$	$[\text{NH}_4]$	$[\text{PO}_4]^-$
577	0.0102	0.00871	0.00676	0.02400
579	0.0076	0.00496	0.00703	0.01044
580	0.0067	0.00393	0.00932	0.00560

portions of the total phosphate in the buffer solution in the bivalent form x'' can be computed by the formula $\mu = [\text{PO}_4]^- (1 + 2x'')$, from the values given in Table V.

Fig. 2. Isoelectric point of sheep CO-haemoglobin. Dialysates ammonium phosphate buffers of ionic strengths 0.01, 0.10 and 0.20. Membrane potentials for protein solutions of $m_p = 0.004$, extrapolated from data in Table V.

The membrane potentials recorded in Fig. 2 show that an increase in salt concentration displaces the isoelectric point towards the acid side, and reduces the slopes of the curves, in accordance with formula 8, a result which indicates that the membrane potential varies inversely as the ionic strength of the dialysate. The intersections of the curves shown in Fig. 2 are eliminated when the apparent valences are calculated as shown in Fig. 6.

In order to obtain values of the isoelectric point at a greater number of salt concentrations with each preparation of haemoglobin, series of experiments

were made by method 3, Section 2. The results for two preparations are given in Table VII. It will be seen that if the molarities exceed 0.01, there is little difference between the p_H values of the stock buffers and those of the final

Table VII. *Effects of increasing salt concentration on the isoelectric point.*

Sheep CO-haemoglobin, dialysates ammonium phosphates at 0°. Exps. 594–601, preparation 1932, 8; Exps. 613–622, preparation 1932, 10; isoelectric points estimated by method 3, Section 2, on the approximate assumption that the ionic strengths of the dialysates are equal to those of the standard buffer solutions, which contained equal proportions of $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$.

Exp. no.	Standard buffer solution			Dialysate p_H''	C	p	E	Isoelectric point
	Ionic strength	Molarity PO_4	p_H					
594	0.01	0.005	7.11	7.23	21.2	103.4	0.00	7.23
595	0.01	0.005	7.11	7.16	21.04	109.6	+1.46	7.27
596	0.02	0.010	7.09	7.10	20.5	101.6	-0.47	7.03
597	0.02	0.010	7.09	7.13	21.6	108.4	-0.23	7.10
600	0.04	0.020	7.02	7.03	21.4	106.7	-0.57	6.90
601	0.04	0.020	7.02	7.01	21.08	105.6	-0.69	6.85
614	0.01	0.005	7.11	7.20	20.3	92.5	-3.23	6.97
615	0.02	0.010	7.09	7.13	20.2	92.2	-2.92	6.76
616	0.02	0.010	7.09	7.12	20.4	93.7	-2.77	6.77
617	0.04	0.020	7.02	7.04	20.1	94.0	-2.22	6.51
619	0.07	0.035	6.95	6.96	20.3	94.9	-1.69	6.28
620	0.07	0.035	6.95	6.95	20.3	95.2	-1.72	6.26
621	0.10	0.050	6.89	6.89	20.3	—	-1.38	6.11
622	0.10	0.050	6.89	6.89	20.3	—	-1.32	6.14

dialysates. Although the absolute values of the isoelectric points for preparation 1932, 10 differ from normal values by 0.3 p_H unit, the changes caused by an increase in the concentration of phosphates are very similar to the results obtained with other preparations.

Measurements of the distribution of ions in solutions with increasing phosphate concentrations are given in Table VIII. Calculations of the ratio f'/f''_{PO_4} based on

Table VIII. *Membrane potentials and ion distributions at different salt concentrations.*

Sheep CO-haemoglobin and ammonium phosphates at 0°. Exps. 598, 599, crystallised protein preparation 1932, 8; Exps. 642, 645, 646, crystallised preparation 1932, 6 B. Remaining Exps., uncrystallised haemoglobin from 6 preparations. Exps. C 23 and C 24, from same blood as Exps. C 3 and C 6, Table X. f'/f''_{NH_4} usually exceeds 0.95.

Exp. no.	C	p	p_H''	E	$[NH_4]''$	$[PO_4]''$	$[NH_4]'$	$[PO_4]'$
598	21.02	104.8	7.08	+0.61	0.01500	0.01000	—	—
599	21.16	108.9	7.09	+0.97	0.01500	0.01000	—	—
645	21.40	110.2	7.08	+0.68	0.01510	0.00996	—	0.01730
642	21.67	108.1	7.02	-0.47	0.02993	0.02010	0.0269	0.02770
646	21.92	109.1	6.95	-0.69	0.05150	0.03440	0.0426	0.04370
NH 6	19.38	—	7.08	+0.63	0.01503	0.00999	0.0126	0.01559
NH 47	21.10	—	7.08	+0.69	0.01503	0.00999	0.0117	0.01601
C 23	20.20	98.4	7.08	+0.33	0.01485	0.00990	0.0116	0.01540
C 24	19.90	95.5	7.08	+0.30	0.01520	0.01010	0.0124	0.01610
640	21.94	113.1	7.05	-0.86	0.03057	0.01993	0.0255	0.02940
655	19.70	91.4	7.02	-0.61	0.03030	0.02000	0.0261	0.02750
641	21.90	110.9	7.04	-0.81	0.03053	0.01970	0.0278	0.02930
326	16.50	73.9	6.76	-0.10	0.22390	0.13830	0.1952	0.14500
611	20.30	124.0	6.30	—	1.48000	0.99100	1.1700	0.80730

these measurements for crystalline and for uncrystallised haemoglobin are almost identical. It may be noted that the phosphate determinations in Exps. 640 and 641 are less reliable than in Exp. 655.

6. Haemoglobin and sodium phosphates.

Preliminary measurements of the effects of sodium salts on the isoelectric points of two preparations of sheep CO-haemoglobin are given in Table IX. In

Table IX. *Effects of different cations and anions on the isoelectric point of sheep CO-haemoglobin at 0°.*

Molarities of anions in dialysates; HPO_4 , 0.005; H_2PO_4 , 0.005; Cl 0.05. Isoionic points, preparation 1933, 11=7.55; preparation 1934, 1=7.58; preparation 1934, 2=7.57.

Cation species	Cation molarity	Anion species	Isoelectric point		
			Preparation 1933, 11	Preparation 1934, 1	Preparation 1934, 2
NH_4	0.015	HPO_4 , H_2PO_4	—	7.15	7.13
Na	0.015	HPO_4 , H_2PO_4	7.05	7.10	—
Na	0.065	HPO_4 , H_2PO_4 , Cl	6.93	7.01	—
Na + K	0.015	HPO_4 , H_2PO_4	—	—	7.10

the second experiment (Jan. 1934), equilibration was carried out for a prolonged period during which slight changes took place in the protein. The final CO capacities were from 93 to 95.4 % of the theoretical values. From these results, the isoelectric point with 0.01 *M* sodium phosphates appears to be slightly on the acid side of that obtained with ammonium phosphates.

The addition of 0.05 mol. of sodium chloride to the sodium phosphate buffer, increasing the ionic strength from 0.02 to 0.07, alters the isoelectric point to a more acid reaction. The difference of 0.09 to 0.12 p_{H} unit is in the same direction as that observed when the ionic strengths of ammonium phosphate buffers are increased over the same range, but the change in isoelectric point with ammonium phosphates is about 0.46 p_{H} unit. There is little doubt that this difference between the effects of chlorides and phosphates on the isoelectric point is correlated with the observation that the diminution of the activities of the ions caused by haemoglobin is greater for phosphates than for chlorides, as shown by Adair [1928] and by the measurements recorded in Table X, which includes some

Table X. *Membrane equilibrium of sodium, chloride and phosphate ions.*

Sheep CO-haemoglobin at 0°. Exps. B, C, E, F, preparation 1933, 11, dialysate sodium phosphates. Exps. C 3, C 6, preparation 1934, 2 S, dialysate 0.005 *M* Na_2HPO_4 + 0.005 *M* KH_2PO_4 . Values for total base under heading Na. Concentrations of ions in mols. per l.

Exp. no.	B	C	E	F	C 6	C 3
$[\text{Cl}]''$	0	0	0.0499	0.0500	0	0
$[\text{PO}_4]''$	0.00996	0.00986	0.00998	0.0099	0.00975	0.0099
$[\text{Na}]''$	0.0149	0.0171	0.0618	0.0647	0.0147	0.0146
$[\text{Cl}]'$	0	0	0.0480	0.0460	0	0
$[\text{PO}_4]'$	0.01577	0.01168	0.0126	0.0113	0.0152	0.0146
$[\text{Na}]'$	0.01156	0.0154	0.0479	0.0521	0.0125	0.0126
C	22.4	23.6	23.9	22.23	20.04	17.75
p	115.6	125.8	134.4	115.8	94.9	76.0
p_{H}''	7.116	7.444	6.949	7.288	7.127	7.132
E	-0.65	-3.05	-0.07	-0.98	-0.08	-0.31
f'/f''_{Na}	1.04	0.97	0.99	1.03	0.96	0.97
f'/f''_{PO_4}	0.48	0.53	0.61	0.65	0.52	0.55
f'/f''_{Cl}	—	—	0.80	0.82	—	—

approximate estimates of the effects of the protein on the activity of the sodium ions, subject to an error of not less than 5 %.

The continuous line marked PO_4 0.01 in Fig. 3 is constructed from the results of experiments with 0.01 *M* ammonium phosphates (Nos. 467–477), by adding 0.13 p_{H} unit to the p_{H} values in Table IV, to facilitate comparisons with

Fig. 3. Effect of sodium salts on membrane potentials. Sheep CO-haemoglobin at 0° and $m_p=0.004$, preparation 1934, 1. Circles, dialysate 0.01 *M* sodium phosphates; squares 0.01 *M* sodium phosphates + 0.05 *M* sodium chloride. Continuous curve, results with ammonium phosphates with a constant correction applied to the p_{H} values.

the membrane potentials with sodium phosphates. The observed points are close to the calculated curve, and it seems that the general forms of the curves given by sodium and by ammonium phosphates are almost identical over the range investigated. The broken line, marked NaCl, shows that the addition of 0.05 mol. of sodium chloride to the dialysate diminishes the slope of the curves.

7. *Equilibrium of protein crystals, protein solution and dialysate.*

Sørensen [1917] showed that crystals of egg-albumin may contain an amount of combined ammonia or sulphuric acid approximately equal to the amounts combined with the protein solution. His method gives the difference between the ammonia and sulphuric acid, but it cannot give the absolute amounts of acids or bases present in the crystals. The method described below was devised in order to study the absolute amounts. A protein sparingly soluble in the region of the isoelectric point, such as horse CO-haemoglobin, was crystallised by dialysis against 0.01 *M* ammonium phosphate buffer for about 14 days at 0°. A 5 ml. density bottle was fitted to a 10 ml. tube of hard glass, and the vessel so obtained was filled with the crystal suspension and left to sediment at 0°. A sample of mother-liquor was obtained from the tube and filtered if necessary. After sufficient time had been allowed for sedimentation,

the density bottle, containing both crystals and mother-liquor, was stoppered and weighed, and the contents were washed out into a 100 ml. volumetric flask. If necessary, sodium hydroxide was added to facilitate dissolution of the protein crystals, care being taken that the alkalinity was not sufficient to cause the evolution of ammonia. Analyses were made for protein, ammonia and phosphoric acid content. The corrected concentrations have been calculated by formula 2, and the composition of the crystals estimated by graphical methods.

In an indirect analysis of this type, necessitated by the fact that the crystals cannot be separated from their mother-liquor without alteration in their composition, the experimental errors may be considerable, and the number of observations recorded in Table XI is insufficient for the establishment of definite formulae, but it is evident that of the three hypotheses that the crystals are composed of pure protein, pure protein salt, or protein *plus* acid *plus* ammonia,

Table XI. *Equilibrium of crystals, mother-liquor and dialysate. Horse CO-haemoglobin.*

The phase designated crystal is a mixture of crystals and mother-liquor.

Exp. no.	Phase	m_p	$[\text{NH}_4]_c$	$[\text{PO}_4]_c$
5B 5	Dialysate	—	0.0167	0.0101
"	Filtrate	0.00065	0.0189	0.0108
"	Crystal	0.00500	—	0.0179
SB 3	Dialysate	—	0.0173	0.0097
"	Filtrate	0.00131	0.0207	0.0117
"	Crystal	0.00229	0.0240	0.0142
"	Pressed crystals	0.00850	—	0.0283
SB 2	Dialysate	—	—	0.0099
"	Filtrate	0.00160	—	0.0177
"	Crystal	0.00225	—	0.0148
SB 1	Dialysate	—	0.0145	0.0096
"	Filtrate	0.00221	0.0167	0.0165
"	Crystals	0.00382	0.0225	0.0213
SB 4	Dialysate	—	—	0.0100
"	Filtrate	0.0024	0.0140	0.0109
"	Crystals	0.0061	0.0196	0.0250

as stated in formula 7, the third is correct. Cohn and Prentiss [1927] measured the solubility of haemoglobin in more concentrated buffers, of ionic strength 0.04 to 1.0, and state that their results suggest the identity of the "saturating body" in the systems studied.

The terms a and b appear to be of the same order of magnitude for both dissolved and crystalline protein, and both vary with the hydrogen ion concentration. It has been assumed that $w=800$ mol. of water per 67,000 g. dry protein. The higher value for hydration given by Sørensen and Sørensen [1933] would yield larger values for a and b . In the isoionic region (p_H 7.4) the term a is nearly 2 mols. of phosphoric acid per mol. of haemoglobin.

Evidence for the presence of water in the crystalline phase was obtained from measurements of density. The specific volume of anhydrous haemoglobin at 20°, determined by the method used by Svedberg [1930], is 0.75 ml., and the density 1.333. Direct measurements made in this work showed that the crystals remained in suspension in media of densities from 1.267 to 1.269 at 20°. Mixtures of half-saturated ammonium sulphate and sucrose were used for these experiments, rather than the solutions including calcium chloride usually employed to

determine the density of crystals by this method, in order to reduce the risks of alterations in the protein.

The value 1.259 is calculated on the assumption based on chloride distribution measurements [Adair, 1928] that 1 g. dry protein combines with 0.2145 g. water and that 1.2145 g. protein hydrate occupies the sum of the volumes 0.75 ml. + 0.215 ml. attributed to the dry protein and the water respectively. The difference between the values 1.268 ± 0.001 and 1.259 may be accounted for by the effect of the sucrose and ammonium sulphate on the vapour pressure of water and the hydration of the protein.

Svedberg's calculations of molecular weights of proteins from ultracentrifugal measurements are based on the specific volume of the dry protein; recalculations based on the density 1.259 give considerably higher values, but there is no fundamental discrepancy because the lower density refers to the protein hydrate. It may be noted that in the cases of haemoglobin and serum-albumin, estimations of the "molar friction" (or of the radius of the particles) from the sedimentation velocities and from the molecular weights [Svedberg, 1930] are in much closer agreement if the results are expressed in terms of the mass and density of the protein hydrate.

In order to minimise the risk of error due to imperfect equilibration, some experiments on the salt distribution in crystals were made by a modified method, in which pure crystals were first formed by dialysis against distilled water; dialysis was then continued against buffer solutions. It was found that the same excess of ammonia and phosphate was present in the crystalline phase as in the systems in which crystallisation was brought about by dialysis against buffer solution.

8. Relationship between osmotic pressure and isoionic and isoelectric points.

In solutions of haemoglobin equilibrated with distilled water, containing only slight traces of electrolytes such as ammonia and carbon dioxide, the isoelectric and isoionic points and the minimum of osmotic pressure should be almost identical. At the minimum of pressure published by Adair [1928], the uncorrected p_H of the dialysate, measured colorimetrically at room temperature, is 6.8 ± 0.2 .

In systems of this type, measurements of hydrogen ion concentration are more reliable if they are made on the protein solution rather than on the dialysate, which is an unbuffered solution of low conductivity. Table XII records measurements made on haemoglobin solutions at 0°, using the hydrogen electrode. The observations are incomplete, since measurements of the CO₂ content of the protein solution before electrometric measurements are required,

Table XII. *Osmotic pressures, p_H values, CO₂ content and isoionic point of haemoglobin.*

Sheep CO-haemoglobin, preparation 1931, 1. Dialysate distilled water at 0°. p = extrapolated osmotic pressures for $m_p = 0.0035$. CO₂ in millimols per l.

Exp. no.	p_H'	p	C	p observed	CO ₂	Isoionic point
554	6.83	163.0	16.08	132.3	—	—
551	7.24	112.2	18.65	108.7	2.8	7.37
479	7.27	112.0	20.70	124.0	—	—
553	7.32	103.7	18.58	100.3	1.9	7.40
552	7.34	98.5	18.50	94.5	2.8	7.45
550	7.35	94.5	18.90	93.2	—	—

as well as those made after equilibration with hydrogen, but the results indicate that the minimum pressure is not at p_H 6.8 but in the region of the isoionic point, p_H 7.4 ± 0.1 .

In solutions containing salts, for which the isoionic point differs from the isoelectric point, the determination of the minimum osmotic pressure is difficult on account of the relatively small changes in pressure caused by alterations in the hydrogen ion concentration in the region of the isoelectric point. Experiments with 0.01 *M* phosphate buffers shown in Fig. 4 indicate a minimum value at

Fig. 4. Minimum osmotic pressure of sheep CO-haemoglobin, dialysates 0.01 *M* ammonium phosphates at 0°. Pressures extrapolated for $m_p = 0.004$, from data in Table IV. Circles, series 451-462; crosses 467-477; crosses enclosed in circles, mean values for both preparations.

Table XIII. *Osmotic pressures with ammonium phosphate buffers of different p_H values.*

Sheep CO-haemoglobin. Osmotic pressures for $m_p = 0.004$, extrapolated from data in Table V. Pressures for preparation 1932, 4 asterisked.

Ionic strength 0.01		Ionic strength 0.10		Ionic strength 0.20	
p_H	p	p_H	p	p_H	p
6.33	173.4*	6.00	118.2	5.84	115.2*
6.40	169.2	6.00	118.6*	5.85	116.7
6.82	130.0*	6.57	109.5*	6.43	115.0*
6.90	128.2	6.58	106.2	6.45	113.2
7.13	113.5	6.88	107.5*	6.77	110.4*
7.11	113.5*	6.89	105.8	6.77	109.1
7.42	100.6	7.36	107.3	7.27	110.2
7.46	102.2	7.37	109.1*	7.28	111.4*
7.48	102.6*				

p_H 7.27 ± 0.15 , a value intermediate between the isoelectric point 7.06 and the isoionic point, 7.4 ± 0.05 , in the preparations referred to in Table IV. Table XIII gives provisional estimates of the pressures for solutions containing 0.004 mol.

of protein per litre of solvent, calculated from data in Table V, on the assumption that pressures and concentrations are related by the empirical formula

$$p = RTm_p + k_1 RTm_p^2.$$

In solutions of 0.01 ionic strength, where the phosphate is approximately 0.004 *M* the minimum pressure seems to be near p_H 7.4. The minimum in more concentrated buffers appears to be at more acid reactions. It may be noted that in the more alkaline solutions, the change in pressure with p_H may be smaller than that calculated for the changes in the ion pressure difference by the formulae given by Adair [1929] and by Adair and Robinson [1930, 2].

9. Relationship between the buffer value β_H and the valence increment β_n .

The buffer value β_H is defined as the increase in the acid bound by one molecule (67,000 g.) of haemoglobin, divided by the increase in the p_H . Hastings, Van Slyke *et al.* [1924] showed that in solutions of reduced horse haemoglobin at 38°, containing 0.03, 0.05 and 0.145 equivalents of sodium ions, the buffer values are 10.0, 10.6 and 11.3 respectively. The buffer value of oxyhaemoglobin varied with the p_H . Human haemoglobin has a lower buffer value of 9.2 [Adair, 1925, 1]. In the case of sheep oxyhaemoglobin, the value of $\beta_H=8$ is estimated from CO₂ dissociation curves [Adair *et al.*, 1929] and $\beta_H=7.35-9.3$ from measurements made by means of the glass electrode by Terroux [1931]. In the present investigation similar measurements on sheep CO-haemoglobin made with the hydrogen electrode at 0°, shown in Fig. 6, give a buffer value of 7.7 in the region of the isoelectric point.

The estimation of buffer values from studies of membrane equilibria presents greater technical difficulties than the earlier methods, but for the purpose of this work, the method is of use as a means of comparing the buffer value and the valence increment β_n , defined as the change of valence per p_H unit; β_n cannot be determined by the earlier methods except in the special case of very dilute mixtures of protein and hydrochloric acid, referred to by Pauli and Valkó [1933].

The estimation of the valence n_p , from measurements of membrane potentials in buffer solutions, has been described by Adair and Adair [1934] who have given the formula

$$n_p = 0.0085 \mu M (E/C_v)_0 \dots\dots(8).$$

M = mol. wt.; μ = ionic strength; C_v = g. protein per 100 ml. solvent; $(E/C_v)_0$ = the limiting value of the ratio (E/C_v) when $C=0$.

In experiments in which this ratio has not been determined, approximate estimates of n_p have been made by formula 9:

$$m_p n_p = [\text{free anions}]_c' - [\text{free cations}]_c' \dots\dots(9).$$

The terms in square brackets represent the sums of the corrected equivalent concentrations of free anions and free cations, estimated on the approximate assumption that concentrations of ions uncombined with proteins are the same as the "ideal" concentrations, defined by formula 6. The values of n_p estimated by formula 9 are used in approximate calculations of the isoelectric point I by the empirical formula $n_p = \beta_n (I - p_H)$. A similar formula $I_H = p_H + A_p/\beta_H$ is of service in estimates of the isoionic point I_H . The range over which β_H and β_n are approximately constant is shown in Figs. 5 and 6.

Table XIV gives a series of determinations of β_H and β_n for sheep CO-haemoglobin equilibrated with ammonium phosphate buffers of constant ionic strength, or with a constant molarity. The values of β_n recorded are based on approximate estimates of n_p made by application of formula 9, from the measurements of

membrane potential, some of which are given in Tables IV, V and X. In systems in which E is below 2 millivolts, formula 9 gives the same results as formula 8,

Fig. 5. Acid bound and valence of sheep CO-haemoglobin, dialysates 0.01 M ammonium phosphates at 0°; calculated from Exps. 452-462, Table IV. Isoionic point at $A_p = 0$.

Fig. 6. Valence of sheep CO-haemoglobin, calculated from data in Table V. Dialysates ammonium phosphate buffers at 0°. Broken line, acid bound by haemoglobin; crosses, A_p in mixtures containing Na, Cl and HCO_3 ; squares, A_p in ammonium phosphates, calculated from data in Tables V and VI. (Crosses, calculated by method A; squares, method B, section 3).

but if E exceeds 5 millivolts, there are appreciable differences, as shown by the curves given by Adair and Adair [1934]. In the experiments with buffers of ionic strength 0.01, where the potentials with 20 % solutions may exceed

9 millivolts, the value of β_n estimated by formula 9 is 3.6, as shown in Table XIV, whereas that estimated by formula 8 is approximately 4.6. Although a number of the values in Table XIV may be subject to correction, there seems to be little doubt that β_n is quite markedly less than β_H . This difference, discussed in Section 11, is of importance in the theoretical interpretation of the mobilities of proteins; Abramson [1933], in the absence of direct measurements, assumes that β_H and β_n are identical.

Table XIV. *Buffer values and valence increments of haemoglobin.*

Sheep CO-haemoglobin, dialysates phosphate buffers at 0°. Mean values of β_H and β_n over the same p_H range. Concentrations of proteins and salts given in Tables IV, V, VI and X, except series 331–337, which is comparable with series 451–462. Asterisked figures show that β_n and β_H both vary with the p_H , and that the difference between them is greater in more acid solutions.

Series	Notes	Isoelectric point	p_H range	β_n	β_H
577–580	Ionic strength = 0.01	7.42	6.5–7.5	3.6	7.74
581–584	„ = 0.10	6.47	6.0–7.3	3.6	—
585–588	„ = 0.20	6.18	5.8–7.3	3.8	—
331–337	Molarity = 0.01	7.06	6.7–7.5	4.2	6.9
451–462	„ „	7.05	6.7–7.5	3.7	7.8
467–477	„ „	7.00	6.7–7.5	3.9	7.8
467–477	„ „	7.00	6.7–7.0*	3.3*	7.8*
467–477	„ „	7.00	7.0–8.0*	4.6*	8.4*
Na-PO ₄	„ „	7.05	7.0–7.6	3.7	8.6
Na-Cl-PO ₄	„ PO ₄ 0.01 Cl 0.05	6.93	6.9–7.2	4.5	—

10. *Effects of salts on the isoelectric points of proteins.*

The results recorded in Fig. 7 indicate that as the salt concentration is increased, the p_H of the isoelectric point of haemoglobin at 0° becomes more acid. Michaelis and Davidsohn [1912] measured the cataphoresis of haemoglobin at

Fig. 7. Variation in isoelectric point of sheep CO-haemoglobin caused by ammonium phosphates. Circles, crystalline haemoglobin, calculated from data in Table VIII; squares, preparation 1932, 4, Table V. C = isoionic point by extrapolation, B = isoionic point determined from chemical analyses.

room temperature and concluded that the isoelectric point is independent of salt concentration. In view of this discrepancy, the evidence obtained by different methods as to the constancy of the isoelectric point may be briefly summarised.

The point of maximum precipitation of globulin is shifted to the acid side by the addition of neutral salts, as shown by Hardy [1905], and it may be noted that Przyłęcki [1931] regards an effect of salts on precipitation as a proof that salts change the isoelectric point, but in view of the more critical work of Hardy and of Cohn [1922], who discusses the work of Michaelis and Szent-Györgyi [1920] and of Hopkins and Savory [1911], it appears that the point of maximum precipitation depends upon a number of factors. It may be noted that in the case of haemoglobin there is a marked discrepancy between the isoelectric point and the minimum solubility, in that the isoelectric point varies by approximately $0.45 p_H$ unit over a range of 25° [Stadie and Martin, 1924], whereas the minimum solubility of horse haemoglobin at 25° recorded by Green [1931] is the same as the value of 6.6 at 0° given by Cohn and Prentiss [1927]. Green concluded that salts alter the apparent dissociation constants and presumably the isoelectric point of haemoglobin. A minimum solubility can be obtained where haemoglobin is not isoelectric, as shown by Sørensen and Sørensen [1933], who published a curve with two minima, at p_H 5.4 and at p_H 6.6.

The point of minimum swelling of gelatin may be altered by the addition of salts [Kuntzel, 1929], but here again it can be shown that the evidence is indecisive. Swelling is correlated with osmotic pressure, which in the case of a protein, can be regarded as the sum of two terms, namely p_i , the pressure due to the diffusible ions, and p_p , the partial pressure of the protein [Adair, 1928; 1929]. The term p_i is zero at the isoelectric point and, if p_p is unaffected by hydrogen ions, minimum osmotic pressure and swelling should correspond with the isoelectric point, but if hydrogen ions alter the state of aggregation of the protein, or the forces between the particles, the observed minimum is a function of two variables and not necessarily equal to the isoelectric point.

Although the measurements of Michaelis and his colleagues showed no evidence for an effect of salts on the isoelectric point, the more accurate apparatus devised by Tiselius [1930] gave an isoelectric point of p_H 4.55 for egg-albumin in acetate buffers of 0.02 ionic strength, containing 0.02 mol. of sodium acetate per litre, whereas the isoionic point is at p_H 4.76. Abramson [1933] supports the view that the isoelectric point can be changed by the addition of salts. The measurement made by Tiselius is of greater importance as evidence for a change of isoelectric point than are the results obtained from observations of maximum precipitation and swelling, as the movement of protein particles in an electric field should be more directly correlated with the electric charge on the particles, but the evidence cannot be regarded as an unqualified proof, in view of the observations of Loeb [1924] and of Freundlich [1930] that the cataphoresis of air bubbles is greatly affected by the addition of electrolytes, which suggests that suspended particles are surrounded by a film of water, containing an excess of one species of ion, usually the anion, which moves with the particle. It follows that the addition of electrolytes may cause an uncharged particle to move in an electric field. The theory that a sheath of solution moves with the particle is of interest in connection with the differences between the results of Michaelis and those obtained in the present work. In measurements of membrane potentials, the diffusible ions surrounding the protein are in a state of thermodynamic equilibrium, unaffected by an external electric field.

Loeb [1924] made a series of comparisons of the cataphoric potential ζ , and of the membrane potential, and concluded that salts with tri- or quadri-valent ions may reverse ζ , but only bring the membrane potentials to zero. In the present investigation, where the membrane potential measurements have been more closely spaced, it is evident that there is a limited range of p_H values,

shown in Fig. 2, where an increase in phosphate concentration can alter the sign of the membrane potential.

The evidence for a variation in isoelectric point with salt concentration, obtained by measurements of membrane potentials, is of interest as it seems probable that the electric charge or valence of protein ions is correlated with the membrane potentials by the simple formula 8, discussed by Adair and Adair [1934]. It is possible, however, that the results may require correction, as they are based on the approximate assumption that a saturated solution of potassium chloride annuls the liquid junction potentials. The curves in Fig. 2 show that the addition of a small constant correction of 0.3 mv. to the observed potentials tends to diminish the distance between the isoelectric points at different ionic strengths.

The hypothesis that the changes in the isoelectric point caused by salts are entirely due to the liquid junction potentials may be advanced in order to reconcile the results with the conclusion of Michaelis, but it is necessary to postulate the existence of specific effects of haemoglobin on the liquid junction potentials ranging from 1.5 to 3 mv. at different salt concentrations. In the absence of independent evidence, it seems difficult to accept this hypothesis. No evidence of the existence of marked differences between the liquid junction potentials, due to the unequal concentrations of diffusible ions in the protein solution and the dialysate, was obtained in the comparison of the membrane potentials of haemoglobin with ammonium phosphate and with sodium phosphate buffers, shown in Fig. 3. In systems where the diffusion potentials play an important part in the observed potentials, the replacement of sodium by an ion of higher mobility, such as potassium or ammonium, may alter the potentials by several millivolts [Bjerrum and Unmack, 1929]. The experiments show that there is not much difference between the values of dE/dp_H measured with sodium and with ammonium phosphates.

11. *Chemical significance of the difference between isoelectric and isoionic points.*

The curves in Figs. 5, 6 and 7 show that in the presence of salts, the isoelectric point of haemoglobin is on the acid side of the isoionic point, at which the protein appears to have a negative charge. The values of the isoelectric points in 0.01 *M* ammonium phosphate buffers, obtained with different preparations of haemoglobin, are not identical, but they all agree in showing a difference of about 0.4 p_H unit from the isoionic points of the preparations in the same buffer mixture. The results may require corrections, referred to in Section 10, but it seems probable that the effects of salts on the isoelectric point are mainly due either to the formation of a compound between the protein and the negatively charged phosphate ions, or to the formation round the particle of a layer, containing an excess of phosphate ions. On both of these hypotheses, the protein may diminish not only the activity coefficient of the phosphate ions, a conclusion in accordance with the data in Table XV, but also the mean activity coefficient of the ammonium and phosphate ions, symbolised $f_{\pm}$, which can be investigated by chemical methods, without using cells with liquid junctions. In the case of Exp. 654, given in Table I, where the products of the corrected concentrations of the ions NH_4^+ and $H_2PO_4^-$ in the protein solution and in the dialysate are 0.000556 and 0.0003 respectively, the protein diminishes the mean activity coefficient of the ions by 26.5 %.

Calculations based on the data for 0.01 *M* buffers, given in Table IV, show that the protein may cause even greater diminutions in $f_{\pm}$. Similar effects are

obtained for sodium phosphates, but the effect of the protein on the mean activity coefficient of sodium chloride is smaller (approximately 10 %, in the case of Exps. E and F in Table X), in accordance with the relatively small change in the isoelectric point caused by sodium chloride. Stadie and Sunderman [1931] measured the freezing-points of mixtures of haemoglobin and salts and concluded that the protein causes no change in the mean activity coefficient, but their observations were not made under the most favourable conditions, namely a high concentration of protein and a low concentration of salts.

The discrimination between the effects of interionic attraction and of chemical combination is a difficult problem. The suggestion that proteins form compounds with heavy metals and with alkaline earths has been made by a number of investigators, referred to by Pauli and Valkó [1933], who have given an extensive list of references to papers bearing on the alternative hypothesis that proteins attract ions. The existence of compounds between proteins and the simpler ions Na^+ , Cl^- and HCO_3^- has been postulated to account for the effects of salts on the solubility of proteins [Kondo and Hayashi, 1928], but in view of the lack of discrimination between combination and attraction, and the absence of quantitative data as to the composition of the supposed compounds, their existence is generally discredited.

Evidence in favour of the existence of interionic attractions and repulsions has been obtained in measurements of the osmotic pressure of haemoglobin [Adair, 1928] and of the solubility of haemoglobin [Cohn and Prentiss, 1927]. In the present investigation, certain consequences of the interionic attraction theory have been compared with the data recorded in Table XV.

Table XV. *Effect of variation in the valence of haemoglobin ions on the activities of phosphate ions.*

Sheep CO-haemoglobin, dialysates 0.01 *M* ammonium phosphates. Concentrations of proteins and of salts given in Table IV. Exps. 467, 469, 475 and 477, $p_{\text{H}'}$ measured with hydrogen electrode, remaining values calculated by Formula 1. b = apparent combination in equivalents of phosphate per mol. haemoglobin. Corrected concentrations of ions in mols. per l. solvent.

Exp. no.	p_{H}''	p_{H}'	$[\text{PO}_4]_c'$ ideal	$[\text{PO}_4]_c'$ observed	b	x'	f'/f''_{PO_4}	n_p
467	6.67	6.71	0.01164	0.02760	4.9	0.26	0.42	+1.0
469	6.94	6.94	0.01018	0.02177	4.1	0.45	0.47	+0.2
471	7.09	7.07	0.00930	0.01913	3.6	0.47	0.49	-0.4
473	7.26	7.22	0.00880	0.01710	3.0	0.50	0.51	-0.8
474	7.37	7.32	0.00838	0.01490	2.7	0.63	0.56	-1.1
475	7.40	7.32	0.00806	0.01429	2.4	0.64	0.56	-1.3
476	7.54	7.47	0.00748	0.01275	2.2	0.76	0.59	-2.0
477	8.23	8.09	0.00465	0.00640	0.8	0.92	0.73	-5.0

In the first place, an increase in the valence of the protein, positive or negative, increases the ionic strength of the solution, and according to the theory of Debye and Hückel [1923] the activity coefficient of the ions is reduced. The experiments recorded in Table XV show that over the range of valences from +1 to -5, protein causes the smallest change in f'/f''_{PO_4} not at $n_p=0$ but at $n_p=-5$. In the second place, bivalent ions are affected more strongly than univalent ions, according to the theory, but an increase in x' , the fraction of the total phosphate in the bivalent form, is correlated with an increase rather than a diminution in f'/f''_{PO_4} . In the third place, evidence for and against the existence of compounds can be obtained by examining the equilibrium of crystals and mother-liquor, recorded in Table XI; it will be noted that there is an excess of

phosphate in the solid phase, comparable with that found for protein in solution, and in addition, there is an excess of ammonia. The presence of ammonia and phosphate in the solid phase might be anticipated on the assumption that chemical compounds are formed. The results of the three tests are in favour of combination rather than attraction.

The term b in Table XV represents the "apparent combination" or the sum of the effects of attraction and combination in equivalents of phosphoric acid per mol. of haemoglobin; it may be observed that the increase in b with increasing acidity accounts quantitatively for the difference between β_H and β_n referred to above.

In planning further tests of the theory, it must be remembered that at the isoionic point, the addition of salts gives a negative charge to the protein, as shown by the experiments made in this work, although the measurements of Hastings, Van Slyke *et al.* [1924] and of Sørensen *et al.* [1927] show that there is no change in p_H on the addition of salts to isoionic protein.

These conditions are satisfied by the reaction in formula 10, suggested by Adair and Adair [1934],

The symbol $+Pr^-$ represents a pair of groups with a positive and negative charge, existing on the protein molecule in an aqueous solution. The presence of such groups is highly probable, since Bjerrum [1924] has found that in neutral solutions amino-acids are represented by the zwitterion formula $+H_3N.R.COO^-$ rather than by $NH_2R.COOH$. In proteins of molecular weight 67,000, the number of groups capable of reacting with acids or bases may exceed 200, but for the sake of simplicity, only the pair represented by the formula $+Pr^-$ may be considered. In pure water, this group is both isoionic and isoelectric. The protein can acquire a negative charge by the loss of a hydrogen ion, which takes place when alkalis are added, but this reaction cannot account for the negative charge caused by the addition of neutral salts at the isoionic point, where the protein neither gains nor loses hydrogen ions. In the reaction suggested in formula 10, the net negative charge is attributed to the loss of a positive charge by the zwitterion, due to the formation of un-ionised protein chloride $[Cl\ Pr]^-$. On crystallisation, the double salt $Cl.Pr.Na$ is obtained. On the addition of a sufficient amount of acid, a negative group of the type $R.COO^-$ is neutralised by a hydrogen ion, and the isoelectric protein $Cl.Pr.H$ is obtained.

The crystals of isoelectric protein should contain less base, in accordance with the experiments described above.

In the case of proteins other than sheep CO-haemoglobin, very little evidence is available, since comparatively few measurements of isoionic and isoelectric points have been made on the same preparation, with the exception of the work of Tiselius [1930], referred to above, on egg-albumin. Indirect evidence for a difference between isoelectric and isoionic points has been obtained in the case of serum [Hastings *et al.*, 1927], haemoglobin [Adair, 1928] and serum-albumin, which were shown to cause a marked diminution of the activities of chlorides and other anions, and relatively little change in the activities of cations [Adair and Adair, 1934]. On the other hand, Hitchcock [1931] found no difference between the isoionic and the isoelectric points of gelatin in acetate buffers.

SUMMARY.

The possibility of determining the isoelectric point from measurements of membrane potentials has been investigated. It seems that the most accurate results are obtained under conditions where concentrated solutions of protein are equilibrated with dilute buffer solutions at 0° .

In order to facilitate comparisons of the isoelectric point with the isoionic point, at which the protein neither gives off nor takes up hydrogen ions, methods have been developed for the estimation of the isoionic point from the distribution of salts across membranes.

A study has been made of methods for the preparation of sheep CO-haemoglobin, a protein which is highly soluble at the isoelectric point. Slight alterations in the protein may take place on crystallisation, and different preparations are not always identical, but comparative results can be obtained on a given preparation.

At 0° , the isoionic point of crystallised haemoglobin was at p_H 7.6. Uncrystallised protein gave lower values of 7.45 ± 0.1 . In ammonium phosphate buffers, of ionic strengths 0.02, 0.04 and 0.07, the isoelectric points of the crystallised protein were 7.16, 6.91 and 6.70 respectively. Seven preparations of uncrystallised material, with buffers of ionic strength 0.02, gave an isoelectric point 7.06 ± 0.01 . The isoelectric points become more acid as the salt concentration is increased; the effects of increasing concentrations of chlorides are less than those of phosphates.

There are slight but measurable differences between the results obtained with sodium and with ammonium phosphate buffers.

In sodium as in ammonium phosphate buffers, the increase in the valence of the protein correlated with increasing hydrogen ion concentration is smaller than the increase in the acid bound by the protein.

The minimum of osmotic pressure appears to be at p_H values between the isoionic and isoelectric points.

The distribution of ammonia and of phosphoric acid between protein crystals, protein solution and dialysate has been investigated.

Experiments have been made to test the hypotheses that the proteins combine with ions, or that they attract the ions in their neighbourhood, and it is suggested that the effects of salts on the isoelectric point, and the effects of proteins on the activities of diffusible ions may be due to a reaction of the type ${}^+\text{Pr}^- + \text{Na}^+ + \text{Cl}^- = [\text{Cl } \text{Pr}]^- + \text{Na}^+$, where ${}^+\text{Pr}^-$ represents a zwitterion.

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