XXIX. THE DETERMINATION OF THE MEMBRANE POTENTIALS OF PROTEIN SOLUTIONS AND THE VALENCE OF PROTEIN IONS.

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1. Introduction.

THE theoretical treatment of membrane potentials in very dilute solutions is mainly due to Donnan [1911], who considered a system in which a solution containing the sodium salt of a colloid such as Congo red is separated from a solution of sodium chloride by a membrane permeable by water and crystalloids but impermeable by the colloidal particles. After equilibrium has been reached, the concentration of sodium ions is greater in the solution inside the membrane, containing the colloid, and there is a potential difference between the colloidal solution and the salt solution or dialysate, determined by Nernst's formula

$$\frac{E}{1000} = \frac{-RT}{F} \cdot \ln \frac{[\text{Na}]'}{[\text{Na}]''} = \frac{-2.303 RT}{F} \log \frac{[\text{Na}]'}{[\text{Na}]''} \quad \dots\dots(1).$$

E = the potential difference or membrane potential in millivolts. F = 96,494 coulombs. R = 8.316 volt coulombs. T = the absolute temperature. $[\text{Na}]'$ = the concentration of sodium ions in the colloidal solution. $[\text{Na}]''$ = the concentration of sodium ions in the dialysate.

The sign of the potential is the same as the sign of the colloidal particle, negative in the case of Congo red and positive in the solutions of protein chlorides studied by Loeb [1922], who devised a method for the experimental investigation of membrane potentials.

An extension of the theory to more concentrated solutions was made by Donnan and Allmand [1914], who derived a formula in which the concentrations in equation (1) are replaced by effective concentrations or activities.

Brønsted [1929], Taylor, P. B. [1927] and Guggenheim [1929] have emphasised the difficulty of measuring the potentials between two different media and the activities of individual ions, but in colloidal solutions the molar fraction of water is so nearly equal on both sides of the membrane that the conditions for the application of the formula of Donnan and Allmand are more favourable, even if absolute accuracy is unattainable on account of liquid junction potentials.

In view of the number and variety of the practical applications of measurements of membrane potentials in colloidal systems, even approximate results may be of great value, and in certain cases it is possible to compare the results with data obtained by methods that are independent of liquid junctions, including measurements of osmotic pressure and determinations of the concentrations of ions on both sides of the membrane by chemical methods.

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Determinations of p_H values, membrane potentials, combined ions and other properties of proteins deduced from measurements in which approximate elimination of liquid junction potentials has been achieved by the use of saturated solutions of potassium chloride must be regarded as conventional rather than absolute values.

The chief application of membrane potentials described in this paper is the estimation of the electric charge or valence of the protein ions from the limiting value of the ratio of the membrane potential to the protein concentration. A preliminary note on the method has been included in a previous paper [Adair and Robinson, 1930, 2].

2. *Liquid junction potentials.*

Sørensen and Linderstrøm-Lang [1924] have found that in most of the solutions that are of biological interest the elimination of the liquid junction potentials by the use of saturated potassium chloride gives the same result as the Bjerrum extrapolation. Bjerrum and Unmack [1929] have made an extensive series of calculations of the liquid junction potentials with 3.5 *N* potassium chloride by the application of Henderson's formula. If the concentration of ions like sodium, with mobilities different from the potassium and chloride ions, be less than 0.2 *M*, the potentials rarely exceed 1 millivolt, and even if hydrogen and hydroxyl ions be present in concentrations up to 0.03 *M* the potentials are usually within 1.2 millivolts. The choice of solvents within this range is of importance from the experimental point of view in reducing variations in the potentials due to differences in the methods of forming the liquid junctions, so that reproducible results can be obtained by methods specially adapted for measurements on small volumes of solutions. In order to obtain reproducible results with markedly acid or alkaline solutions, it may be necessary to use flowing junctions [Lamb and Larson, 1920] or static junctions in wide tubes [Ferguson *et al.*, 1932].

In previous investigations on membrane potentials, liquid junctions in capillary tips have been used by Loeb [1922] and by Taylor [1924], in order to minimise the contamination of the solution with potassium chloride. Most investigators have used wider tubes, which give more reproducible liquid junctions. Kondo [1924] formed the junction at the end of a wide tube, Bjerrum made liquid junctions in glass taps, Northrop and Kunitz [1926] formed stirred junctions in wide tubes.

Different types of liquid junctions have been discussed by Guggenheim [1930]. He criticises the practice of stirring junctions, on the ground that it forms two junctions of indefinite type between the first solution and the mixture and the second solution. He advises the use of sharp junctions formed inside tubes and not at their tips. Maclagan [1929] compared junctions formed with tubes of different diameters and concluded that junctions formed inside tubes are more reproducible.

Stadie and Hawes [1928] showed that the liquid junction formed in the Clark hydrogen electrode gives reproducible results in measurements of the p_H values of mixtures containing haemoglobin and sodium salts. This method has been employed in this work in the determinations of membrane potentials from p_H values.

3. *The experimental determination of membrane potentials.*

In the method for the measurement of membrane potentials devised by Loeb [1922], a solution of the protein contained in a 50 ml. collodion membrane, with a manometer attached, was placed in a vessel of "outer fluid" or dialysate,

and the system was left for 20 hours to reach equilibrium. The membrane potential was determined by dipping saturated calomel-KCl electrodes into the dialysate and the protein solution, which was forced up into a funnel by pressure on the flexible collodion membrane. In most of Loeb's experiments the results obtained were verified within two millivolts by their agreement with values calculated by the application of Donnan's formulae to measurements of the activities of hydrogen ions on both sides of the membrane.

In the modifications of the methods devised by Bjerrum [1924] for chromium chloride, by Kondo [1924] for caseinogen chloride and by Henriques [1928; 1929] for laked blood corpuscles, the liquids were enclosed, and long glass tubes connected the inner and outer fluids with calomel electrodes. These designs facilitate the measurement of potentials at short intervals of time; moreover, the pressures may be adjusted at the values corresponding to osmotic equilibrium. According to Rinde [1926], measurements of potentials must be made at the equilibrium pressure, whereas Northrop and Kunitz [1926] consider that it is unnecessary to maintain the equilibrium pressure during the measurement. As will be stated below, our experience agrees with that of Northrop and Kunitz.

The method for the determination of membrane potentials to be described in this paper was developed for work on purified protein solutions, in order to obtain consistent results under conditions where the potentials may be small and the volumes of solution available for each determination may be restricted to 5 or even 1.5 ml. The types of apparatus used in this work are not adapted for making continuous records of the potentials, but they have the advantage that dead spaces, which may contain protein not in equilibrium with its dialysate, are practically eliminated, and the distances for diffusion are comparatively short (Fig. 1).

Fig. 1. Apparatus for measurement of membrane potential of small volumes of protein solutions with saturated calomel-KCl electrodes.

Collodion membrane containing protein shown by dotted lines. *R* is rubber connecting tube. Electrode tip, *A* to *B* 90 mm. tube of 5 mm. internal diameter; *B* to *E*, 55 mm. tube of 2 mm. internal diameter; *E* to *C*, 15 mm. Liquid junction at *D*, 7 mm. below orifice at *C*. *M* is mercury manometer bore 1.5 mm. attached to osmometer used for equilibration. *S* is for attachment of soda lime tube.

The establishment of equilibrium between the protein solution and the dialysate is the first consideration in studies of membrane potential. At ordinary temperatures, rapid equilibration may be necessary to prevent errors due to changes in the protein and to bacterial action. In the present research, the work throughout was carried out at 0°, and since bacterial action is inhibited and the stability of the proteins is greater at low temperatures, it has been possible to make more rigorous tests of equilibrium. Instead of leaving given volumes of protein solution and dialysate to reach equilibrium, as described by Loeb, the protein solutions were equilibrated by dialysis against relatively large volumes of solutions of well defined hydrogen ion concentration, in order to control the activities of the electrolytes in the protein solutions and to facilitate the thermodynamical treatment of the systems [Adair, 1929, 1, 2]. The standard buffer solutions remove traces of inorganic impurities which are not readily eliminated by a preliminary dialysis against distilled water; the rate of removal of traces of an acid or base combined with the protein is very greatly increased if the dialysate be a solution of electrolytes rather than pure water.

Method 1. In the majority of the experiments to be described in this paper, the process of equilibration has been followed by observation of the osmotic pressure.

The collodion membranes used were prepared from a solution containing 4 g. of dry pyroxylin (B.D.H.) dissolved in a mixture of 50 ml. of absolute alcohol and 50 ml. of anhydrous ether, or by diluting one volume of "Necol" with one volume of alcohol and one volume of ether. The membranes were made by pouring three coats of collodion over the outside of a glass mould [Adair, 1925; Cole, 1933]; they were therefore less permeable but more rigid and probably more uniform than those used by Loeb, which were made on the inside of a tube with one coat of collodion. The permeability may be altered by varying the time allowed for drying the collodion. The membranes were mounted on rubber tubes or stoppers, and the permeability was tested by measuring the rate of flow of water through the membrane at room temperature under a pressure of 450 mm. Hg. For a cylindrical membrane of 10 cm. length and 1.5 cm. diameter, the rate of flow should range from 0.05 to 0.2 ml. of water per minute. Membranes of higher permeability may allow an appreciable leakage of a protein such as haemoglobin, whereas those which are less permeable entail an unduly prolonged period of equilibration.

The collodion membranes were attached to mercury manometers of the type illustrated in Fig. 1 for concentrated solutions, and of the type described by Adair [1925] for more dilute solutions. The dialysates were changed at intervals of one or two days until further changes caused no further alterations in the manometer readings. If the volume of the dialysate be about ten times as great as the volume of the protein solution, a close approximation to equilibrium is reached after three or four changes, in the case of well buffered solutions.

If the buffer solutions used are more dilute than 0.3 *M*, it is advisable to dialyse the protein solutions for two or three days against smaller volumes of buffer, changed at short intervals, before the membranes are attached to manometers, and if there be a large difference between the initial hydrogen ion concentration of the protein solution and the equilibrium value, it may be advisable to adjust the p_H of the protein solution or to add a slight excess of acid or base to the dialysate in the early stages of dialysis.

The apparatus and procedure may require modification for work on very dilute solutions (0.001 *M*), or for solutions in which the p_H values are altered by a short exposure to air.

The determinations of membrane potential were made after equilibrium had been reached and maintained for a few days. Closed saturated calomel-KCl electrodes with upcurved tips were used, of the design shown in Fig. 1. The electrodes were kept throughout at 0° and were connected by leads to a potentiometer in the laboratory outside. A mirror galvanometer of 450 ohms resistance was used, giving a deflection of about 40 mm. per millivolt, with electrodes dipped into solutions of high conductivity. A multiple range potentiometer was used for some experiments, but as a general rule the measurements were made with a simpler instrument, graduated in 0.2 millivolt.

The difference between the two calomel cells was first determined with both electrode tips placed in a tube of saturated KCl; the electrode tips were then washed with distilled water and dried with filter-paper, and the blank determination was repeated. The two measurements should not differ by more than 0.01 millivolt, but variations of 0.03 millivolt have been observed if the calomel cells have been shaken during the manipulations. If the blank determinations agreed, the electrodes were again washed with distilled water, and a few drops of KCl were run out to displace water which might have entered the tips. A small quantity of KCl was then forced out by pressure on the rubber tube connecting the electrode tip and the calomel cell, so that the level of KCl was about 7 mm. below the orifice; the electrodes were then dried with filter-paper. After both electrodes had been prepared, an osmometer was dismantled and the protein solution filling the dead space, *i.e.* that which had not been in contact with the membrane, was rejected. The bulk of the protein was removed for analysis, a depth of solution of 2 to 3 cm. being left in the membrane. The rubber mount was then cut off, and the membrane was placed in a small beaker containing dialysate. In cases where a positive charge on the protein was anticipated, the more positive of the two calomel cells was selected, and the orifice of the electrode tip, from which KCl had been removed, was filled with the protein solution; the other electrode was filled with dialysate. Both electrodes were then washed with dialysate and dried with filter-paper. The more positive electrode was then immersed in the protein solution remaining in the membrane and the other electrode in the dialysate.

In order to avoid the formation of air bubbles in filling the electrode tips, the rubber connecting tube was pressed until KCl rose to the level of the orifice. The protein solution could then be drawn into the tip from a capillary pipette by releasing the pressure on the rubber tube.

The first reading of the potential could generally be obtained between three and four minutes after the formation of the liquid junctions. The reproducibility of the junctions formed by this method and the variation of the reading with time are discussed in the following section.

Method 2. In this method, the protein solutions were contained in collodion membranes mounted on celluloid tubes of 1.5 cm. length. During equilibration, the membranes were closed with rubber stoppers and were inverted at intervals. The attainment of equilibrium was determined by measuring the potential difference between protein solution and dialysate on successive days and renewing the dialysate each day until further changing caused no alteration in the potential. Stoppered pyrex beakers were used for equilibration.

The osmotic method is more convenient for series of more than twelve membranes, as the manometer readings can be made more rapidly than a single estimation of membrane potential. The second method is useful for the measurement of small potentials, as a number of determinations can be obtained from each membrane, and the effects of certain sources of error, including changes in

the E.M.F. of the calomel cells and variations in the liquid junction potentials, can be minimised by taking the average of a number of observations after equilibrium has been reached. Observations on the rate of equilibration of haemoglobin solutions with 0.02 *M* phosphate buffers, obtained by this method, are given in Table I.

Table I. *Rate of equilibration of 22 % solutions of sheep carbon monoxide haemoglobin with 0.01 M NH₄H₂PO₄ + 0.01 M (NH₄)₂HPO₄ at 0°, shown by measurements of membrane potential on successive days.*

Permeabilities in ml. water per minute at a pressure of 450 mm. of mercury for membranes 4.5 cm. long and 1.4 cm. diameter. Times in days. Potentials in millivolts.

Membrane permeability	1	4	6	8	12	21	55 days
0.086	-1.35	-0.77	-0.66	-0.62	-0.54	-0.55	—
0.066	-1.20	-0.72	-0.64	-0.66	-0.46	-0.55	—
0.102	-1.15	-0.77	-0.62	-0.55	-0.51	-0.55	-0.59
0.028	-1.30	-0.77	-0.61	-0.50	-0.51	-0.50	-0.48
0.083*	+0.00	-0.35	-0.31	-0.32	-0.40	-0.29	-0.35
0.047*	+0.60	-0.30	-0.32	-0.27	-0.33	-0.27	-0.29

* Some methaemoglobin present.

4. *Preparation of protein solutions and methods of analysis.*

The proteins investigated included the haemoglobins of the sheep and the ox, horse-serum-albumin, total serum-protein, denatured edestin, acid haematin and kathaemoglobin. Some experiments were also carried out on Congo red.

The haemoglobin solutions were prepared by a slight modification of the method given by Adair [1928] which will be described in detail in a later communication. Horse-serum-albumin was recrystallised three times by the method of Adair and Robinson [1930, 1]. The total serum-protein was prepared by the dialysis of untreated serum with phosphate buffer of p_H 7.4. Denatured edestin was made by prolonged dialysis of crystalline edestin, prepared from hempseed, against 0.01 *M* HCl. Acid haematin was obtained by dialysis of haemoglobin against an acetate buffer of p_H 3.04. A soluble form of kathaemoglobin was prepared by mixing equal volumes of 0.9 *M* NaOH and a 22 % solution of haemoglobin (mainly in the form of methaemoglobin) which had been recrystallised by means of ammonium sulphate. After standing at room temperature for a few days, to ensure complete denaturation, the excess of alkali was removed by dialysis, and the alkaline haematin was converted into kathaemoglobin. In some experiments, carbon dioxide was passed through the solution before dialysis.

The Congo red was subjected to a preliminary dialysis against distilled water, in order to remove chlorides, before equilibration with sodium bicarbonate. The concentration of the solution obtained was estimated from dry weight determinations.

In all experiments on protein solutions, the concentrations were determined after equilibration by Kjeldahl nitrogen determinations or by the refractometric method, using factors based on nitrogen determinations [Adair and Robinson, 1930, 1].

The hydrogen ion concentration values of the dialysates at 0° were measured with the Clark hydrogen electrode and a saturated calomel-KCl electrode, standardised by measurements of a mixture of 0.01 *M* HCl in 0.09 *M* KCl. The conventional value $p_H = 2.04$ was assigned to this solution. In certain experiments

the p_H value of the protein solution was measured by the hydrogen electrode, but in most experiments it was calculated from the membrane potential and the p_H value of the dialysate.

The following methods were used for analysis of the dialysates: ammonia was estimated by nesslerisation or by the micro-Kjeldahl; phosphates by the method of Bell and Doisy [1920], carbon dioxide according to Van Slyke and Neill [1924], and chlorides by the method of Van Slyke [1923]. The technique for the estimation of sodium will be described in a subsequent communication.

In the analysis of the protein solutions, protein-free filtrates were obtained by heat-coagulation of diluted solutions at the isoelectric point in the presence of sodium sulphate.

In experiments with concentrated buffer solutions (0.05 M) complete analyses of the dialysate are not required in all cases, if the refractive indices of the stock buffer and the final dialysate be identical and no difference between their p_H values can be detected by colorimetric methods.

5. Sources of error in measurements of membrane potentials.

The sources of error in measurements of the membrane potentials of protein solutions may include impurities, alterations in the proteins during the process of preparation, deviations from a state of equilibrium between protein solution and dialysate due to the action of bacteria, imperfections in the membranes, changes in the dialysate and breakdown of the protein. During electrometric measurements there are additional sources of error, including alterations in the potentials of the calomel electrodes, contamination of the solutions with potassium chloride, imperfections in the method of forming the liquid junctions and, if the protein solution be not enclosed and maintained at the osmotic equilibrium pressure, there may be errors due to absorption of carbon dioxide, evaporation of water, the dilution of the protein solution and electrokinetic potentials and diffusion potentials which may be correlated with the flow of water across the membrane.

The method described in this work is restricted to solutions that are not greatly affected by carbon dioxide; during the measurements of the potentials, the solutions are exposed to the air. Experiments with buffered solutions including haemoglobin in equilibrium with 0.01 M phosphates, in which the solutions were left exposed to the air for several hours, indicated that the amount of carbon dioxide absorbed in the course of an hour was not sufficient to cause appreciable error. In the case of very dilute alkaline solutions, the risk of error due to carbon dioxide is much more serious, and it may be necessary to use an enclosed apparatus of the type figured by Bjerrum [1924].

In the apparatus shown in Fig. 1, the protein solutions are kept under the osmotic equilibrium pressure during dialysis, but the measurements of the potential are made with the protein solution at atmospheric pressure. Under these conditions, the dialysate flows across the membrane into the protein solution. The rate of dilution is not rapid with the type of membrane used in this work, and even if the dismantled membranes are left overnight, the final concentrations usually exceed two-thirds of the initial value. Since the first reading of the membrane potential is made a few minutes after the osmotic pressure is released, the degree of dilution is not appreciable. As a general rule, repetitions of the measurements made within the first hour agreed with the initial value, if the solutions were not contaminated with potassium chloride during the first series of readings. Agreement between the readings made after a time interval shows that the errors due to the fall in protein concentration and to

evaporation of water and absorption of carbon dioxide are not important, but it does not exclude errors due to diffusion potentials which may be associated with the movement of ions of different mobilities across the membrane and electrokinetic potentials due to the flow of fluid across a charged membrane. In neutral solutions collodion membranes are negatively charged, but the charge may be altered by adsorption of protein during equilibration.

Attempts were made to measure these effects in a system containing haemoglobin in equilibrium with 0.01 *M* phosphate buffer, using an apparatus of the enclosed type similar to that figured by Henriques [1928]. It was found that the potentials remained constant within 0.4 millivolt with arbitrary variations of pressure from 6.2 to 140 mm. of mercury.

Since the electrokinetic effects must depend upon the specific properties of the membrane, comparative experiments were set up with collodion, parchment and cellophane membranes. Discs of parchment or cellophane of about 9 cm. diameter were folded over one end of a glass tube 4 cm. long and 1.5 cm. diameter and held in position by rubber rings. The tubes prepared in this way were kept in buffer solution for one or two days; they were then filled, to a depth of about 2.2 cm., with haemoglobin solution which had been subjected to a preliminary dialysis against the same buffer solution. The tubes were placed in small beakers containing glass rods which prevented the parchment or cellophane membranes from touching the bottom of the beakers. The potentials were measured shortly after the tubes were filled with protein solution.

Table II. *Comparison of collodion, cellophane and parchment membranes.*

Exp. 618. Sheep carbon monoxide haemoglobin, 20.28 %. Osmotic pressure 93.6 mm. dialysate 0.01 *M* $\text{NH}_4\text{H}_2\text{PO}_4$ + 0.01 *M* $(\text{NH}_4)_2\text{HPO}_4$. p_{H} 7.046 at 0°.

Exp. A 7. Horse-serum-albumin, 15.72 %. Osmotic pressure 38.2 mm. dialysate 0.02 *M* CH_3COONa + 0.01 *M* CH_3COOH . p_{H} 4.99 at 0°.

Time in minutes. Potentials in millivolts.

618 Collodion		618 Parchment		618 Cellophane		A 7 Collodion		A 7 Parchment	
Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.
0	-1.81	0	-1.86	0	-1.78	0	-4.49	0	-4.25
2	-1.84	1	-1.91	1	-1.82	1	-4.59	2	-4.45
5.5	-1.89	3	-1.96	6	-1.91	5	-4.64	8	-4.70
9	-2.01	9	-2.04			10	-4.84	15	-4.95
15	-2.06	11	-2.06			15	-5.14	27	-5.30
21	-2.16	20	-2.10			18	-5.34	38	-5.35
31	-2.26	36	-2.06						

The results recorded in Table II show that there is a close agreement between the potentials given by different membranes, if comparisons are made at the same interval of time after the formation of the liquid junctions; no evidence is shown for the existence of errors due to specific properties of the membrane. The effects of time on the liquid junction potentials are discussed below.

Information as to the possibility of errors caused by electrokinetic potentials, diffusion potentials and imperfect equilibration may furthermore be obtained by a comparison of the observed membrane potentials with values calculated from electrometric measurements of hydrogen ion activity by a slightly modified form of the equation of Donnan and Allmand [1914].

$$E = 54.16 \left(\frac{273+t}{273} \right) (p_{\text{H inner}} - p_{\text{H outer}}) \quad \dots\dots(2).$$

t = temperature in ° C.

This formula has been verified by a number of investigators, including Loeb [1922] and Bjerrum [1924].

The investigations of Hastings *et al.* [1924] and of Stadie and Hawes [1928] indicated that fairly good results might be obtainable with the hydrogen electrode in solutions of carbon monoxide haemoglobin practically free from methaemoglobin. More concentrated solutions, containing 20 to 22 % of protein, used in this work, are very viscous, and the conditions are not ideal for the establishment of equilibrium with hydrogen electrodes at 0°, particularly in the case of haemoglobin. Though consistent results were obtained with most preparations, examples of which are given in Table III, in a long series of experiments the

Table III. *Direct and indirect measurements of membrane potentials of sheep carbon monoxide haemoglobin 20–22 % solutions.*

Dialysates 0.01 *M* ammonium phosphate buffers. *E* = membrane potential in millivolts.

Exp. no.	Dialysate p_H	Protein solution p_H	Protein solution p_H (calculated)	Direct <i>E</i>	Indirect <i>E</i>	Difference
451	6.295	6.431	6.441	+ 7.93	+ 7.3	– 0.6
452	6.939	6.942	6.958	+ 1.03	– 0.2	– 1.2
454	7.096	7.054	7.089	– 0.38	– 2.2	– 1.8
461	7.680	7.592	7.596	– 4.57	– 4.77	– 0.2
467	6.670	6.706	6.729	+ 3.17	+ 2.06	– 1.2
469	6.941	6.937	6.953	+ 0.62	– 0.3	– 0.9
475	7.398	7.321	7.341	– 3.07	– 4.15	– 1.1
477	8.229	8.087	8.059	– 9.20	– 7.7	+ 1.5

hydrogen electrode potentials drifted. In view of the difficulties experienced with haemoglobin, a comparison of membrane potentials, measured by direct and indirect methods, was made on solutions of serum-albumin (Table IV). In

Table IV. *Direct and indirect measurements of membrane potentials of horse-serum-albumin at 0°.*

Exps. A 15, 14, 16, 9, dialysate 0.005 *M* $\text{NH}_4\text{H}_2\text{PO}_4$ + 0.005 *M* $(\text{NH}_4)_2\text{HPO}_4$.

Exps. A 11, 10, dialysate 0.01 *M* $\text{NH}_4\text{H}_2\text{PO}_4$ + 0.01 *M* $(\text{NH}_4)_2\text{HPO}_4$.

Exps. A 7, 6, dialysate 0.02 *M* CH_3COONa + 0.01 *M* CH_3COOH .

Exps. A 5, 4, dialysate 0.02 *M* CH_3COONa + 0.025 *M* CH_3COOH .

Osmotic pressures in mm. mercury. E_1 and E_2 , initial and final readings of membrane potential in millivolts. E_3 and E_4 potentials calculated from initial and final hydrogen electrode readings.

Exp. no.	Protein g. per 100 ml.	Osmotic pressure	Dialysate p_H	Protein solution p_H	Direct		Indirect	
					E_1	E_2	E_3	E_4
A 15	1.95	7.1	7.056	7.005	– 2.75	– 2.75	– 2.75	– 2.56
A 14	3.36	13.8	7.055	6.963	– 4.47	– 4.54	– 5.05	– 5.05
A 16	5.38	27.3	7.054	6.911	– 6.92	– 7.12	– 7.80	– 7.55
A 9	15.92	171.2	7.041	6.640	– 19.0	– 20.3	– 21.6	– 20.2
A 11	15.15	133.6	6.987	6.742	– 11.78	– 12.58	– 12.26	– 11.16
A 10	19.09	211.7	7.001	6.709	– 14.59	– 15.59	– 15.8	– 15.8
A 7	15.45	38.2	4.985	4.900	– 4.5	– 5.6	– 4.9	– 4.8
A 6	20.06	56.4	4.979	4.867	– 5.38	– 6.68	– 6.1	– 5.9
A 5	15.87	64.6	4.588	4.607	+ 1.2	+ 0.2	+ 1.1	+ 1.0
A 4	19.99	91.2	4.582	4.603	+ 1.47	+ 0.33	+ 1.1	+ 1.7

dilute solutions of serum-albumin, there is a satisfactory agreement between the potentials determined by both methods. In solutions containing more than 15 % of the protein the experimental difficulties were greater on account of the effects at the liquid junctions, discussed below. The average discrepancy between

the initial readings of observed and calculated potentials was 0.6 millivolt, which corresponds to 0.01 p_H unit. The maximum discrepancy was 1.4 millivolts.

Traces of carbon dioxide, absorbed during dialysis, may cause small errors in indirect determinations of membrane potentials by means of hydrogen electrode measurements, because varying amounts of carbon dioxide may be removed by bubbling hydrogen through the liquids. The p_H changes due to loss of carbon dioxide are not identical in the protein solution and in the dialysate on account of the buffer action of the proteins. The agreement between the direct and indirect determinations, shown in Tables III and IV, is evidence against the existence of serious errors due to carbon dioxide.

The magnitude of the experimental errors due to liquid junction potentials depends upon the composition of the solutions under examination. The reproducibility of the liquid junctions formed in the electrodes illustrated in Fig. 1 was tested by measuring the potential difference between two electrodes dipping into the same solution. In the case of dilute buffer solutions the potentials agreed, but in concentrated (M) ammonium phosphate buffers there were variations of 0.5 millivolt. If the initial measurements were reproducible, the constancy of the potentials could be investigated by renewing the liquid junction in one of the electrodes at known intervals of time. In 0.01 M ammonium phosphate buffers, where it is possible to obtain initial values agreeing within 0.03 millivolt, the potential of the electrode left undisturbed in the solution remained constant within 0.03 millivolt for 30 minutes. In dilute solutions of proteins equilibrated with 0.01 to 0.05 M buffer solutions, the initial readings were reproducible but the variations with time might exceed 0.1 millivolt.

In solutions containing 20 % of haemoglobin, the results were reproducible if both junctions were formed within 30 seconds, but a freshly-formed junction was more positive than one left standing for a few minutes. The differences ranged from 0.2 to 0.5 millivolt, in cases where the first junction had been left standing for 15 minutes. In concentrated solutions of serum-albumin, the initial readings agreed, but the difference between a freshly formed and an older junction ranged from 1.0 to 1.3 millivolts.

Comparisons made between electrodes with wide and narrow tips showed little difference in the case of buffers and of haemoglobin solutions, but there were differences ranging from 0.2 to 0.6 millivolt in the case of serum-albumin.

It is possible that these variations with time are correlated with an alteration at the boundary between solution and saturated potassium chloride, which has been observed not only with this type of electrode but with liquid junctions formed by means of taps. At first the boundary is sharp, but within a few minutes it becomes irregular. The disturbance is probably due to convection currents, because the mixture formed by the diffusion of potassium chloride into the protein solution may be denser than the pure potassium chloride solution below it.

In a measurement of the membrane potential, the observed potential E can be regarded as the sum of E (KCl.Pr), the liquid junction potential at the boundary between saturated potassium chloride and protein solution, E_m , the potential at the membrane and E (D.KCl), the potential at the junction of the dialysate and the potassium chloride solution of the second electrode. The observations recorded in Table II show that the final values of E are more negative than the initial values, and the direction and magnitude of the changes are in accordance with those observed in studies of the liquid junction E (KCl.Pr), a result which might be expected if the potentials E_m and E (D.KCl) remained constant.

Observations of the potentials at different times after the formation of the liquid junctions are furthermore of value in detecting errors due to contamination of the solutions with potassium chloride, which may cause drifts in the potentials which are larger and more rapid than those due to liquid junction potentials. Rapid drifts may also be caused by local differences in the concentration of ions due to imperfect equilibration. It is advisable to reject experiments which show drifts in the potential which cannot be accounted for by liquid junction potentials. With dilute solutions of proteins, the potentials should remain constant within 0.1 millivolt.

In many problems, the accuracy of the ratio E/C , where C is the protein concentration, is more important than the accuracy of the potential E , and therefore an error of 0.5 millivolt on a 25 % solution is comparable with an error of 0.02 millivolt on a 1 % solution.

With the exception of the experiments in Tables II and IV, which exemplify the maximum variations with time, the potential values recorded graphically and in the Tables are those obtained between 3 and 4 minutes after the formation of the liquid junction in the protein solution. The practice of recording initial readings in measurements on inorganic solutions was adopted by Lewis *et al.* [1917] and by Clark [1928]. According to Maclagan [1929] and Guggenheim [1930], the final readings, obtained 10 or 15 minutes after the formation of the liquid junction, are more reproducible in the case of junctions formed with hydrochloric acid inside tubes.

In certain of our experiments, the final as well as the initial readings were reproducible, but it is more difficult to obtain satisfactory final readings on account of contamination of the solutions with potassium chloride. It is possible that in the case of protein solutions the initial readings represent a closer approximation to a sharp junction, and it is by no means certain that the final readings are of greater significance.

As the initial readings of membrane potentials that we have obtained for solutions of different protein concentration are reproducible, correction factors could be determined if a standard type of liquid junction for protein solutions were to be devised at a future date.

Henriques [1929], who used an electrometer, drew attention to polarisation as a possible source of error in measurements of membrane potentials by the use of a galvanometer and a potentiometer. In this work it was found that there was no difference between the potentials measured just before and immediately after attempts to polarise the system by closing the circuit with the potentiometer set at 5 or 10 millivolts above or below the equilibrium value, causing galvanometer deflections from 57 to 112 mm.

6. *Measurements of membrane potentials at different protein concentrations in solutions where the composition of the dialysate is kept constant.*

The observations recorded in Figs. 2 to 6 show that in all the protein systems investigated, which included proteins such as haemoglobin and serum-albumin with particles of uniform size and denatured proteins which may be mixtures of particles of different sizes, there is a straight line relationship between the membrane potential and the "corrected percentage concentration," C_v , defined by formula 3, under conditions in which the membrane potential is small.

The results of analyses of the protein solutions by volumetric or by refractometric methods are usually expressed in terms of C , the concentration of dry

protein in g. per 100 ml. of solution, and the values of C_v are calculated from these measurements.

$$C_v = C [100/(100 - V_1 C)] \quad \dots\dots(3).$$

C_v = g. of dry protein per 100 ml. of solvent. C = g. of dry protein per 100 ml. of solution. V_1 = volume occupied by 1 g. of dry protein.

The volume of V_1 cannot be less than 0.75 ml., the specific volume of the dry protein, and it may be more if the protein combines with water.

Fig. 2. Membrane potentials of CO-haemoglobin and acid haematin at different concentrations.

Dialysates of ionic strength 0.01, compositions given in mols. per litre:

p_H 3.04. CH_3COONa 0.0025, CH_3COOH 0.0975, NaCl 0.0975.

p_H 5.65. CH_3COONa 0.100, CH_3COOH 0.0105.

p_H 7.36. $(\text{NH}_4)_2\text{HPO}_4$ 0.03, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.01.

p_H 7.75. Na_2HPO_4 0.0321, KH_2PO_4 0.00357.

Fig. 3. Membrane potentials of haemoglobin (mixture of oxygenated and reduced) and kathaemoglobin from sheep blood.

Dialysate p_H 7.8, ionic strength 0.289, KCl 0.1, Na_2HPO_4 0.0613, KH_2PO_4 0.00533.

The hydration of haemoglobin, estimated from chloride distribution measurements, is 0.21 g. per g. of dry protein [Adair, 1928]. The hydration of the haemoglobin of the horse in the presence of ammonium sulphate has been investigated by Sørensen and Sørensen [1933]. The value 0.353 g. given in their paper is reduced to 0.306 g. if the calculation is based on the nitrogen determination of 16.69 %, made by Vickery and Leavenworth [1928], rather than the

Fig. 4. Membrane potentials of sheep CO-haemoglobin.

"Ideal" $n_p = 4.3$ curve calculated for a protein of valence 4.3 in ideal solutions. Dialysates of ionic strength approximately 0.01.

p_H 6.38. $(NH_4)_2HPO_4$ 0.00128, $NH_4H_2PO_4$ 0.00672.

p_H 6.61. $(NH_4)_2HPO_4$ 0.0017, $NH_4H_2PO_4$ 0.0053.

p_H 7.61. $(NH_4)_2HPO_4$ 0.0037, $NH_4H_2PO_4$ 0.0012.

At p_H 7.61 potentials are negative.

Fig. 5. Membrane potentials of serum-proteins.

Serum-albumin, p_H 7.06. Dialysate $(NH_4)_2HPO_4$ 0.005, $NH_4H_2PO_4$ 0.005. A 7.4 serum-albumin, Pr 7.4 total protein. Dialysate p_H 7.4. Na_2HPO_4 0.0533, KH_2PO_4 0.0133.

older value of 17.3 %. Adair and Robinson [1931] used a direct method, in which the dried haemoglobin of the sheep, exposed to the vapour of saturated ammonium sulphate solution, took up 0.2 g. of water per g. dried protein. Weber and Nachmannsohn [1929] estimated that the effective volume $V_1 = 1.0$ ml. in the case of serum-albumin. In the calculations given below, the volume $V_1 = 1.0$ has been used for serum-albumin and $V_1 = 0.965$ for haemoglobin and its derivatives. Although these values may require correction when more complete

Fig. 6. Membrane potentials of edestin. Dialysate 0.0104 *M* HCl. Curve 1, observed potentials. Curve 2, theoretical potentials for protein of constant valence 26.3. Curve 3, potentials calculated from chloride concentrations (Section 8).

data are available, the straight line relationships between the membrane potentials and C_v shown in Fig. 2 indicate that the corrections are small, although it is not impossible that a straight line might be obtained in a system where the error due to an underestimate of the hydration value is compensated by a reduction in the membrane potentials in concentrated solutions, due to the interionic forces described by Debye and Hückel [1923].

7. Relationships between membrane potentials and protein concentrations in ideal solutions.

Bjerrum [1924] showed that in an infinitely dilute solution of a colloid equilibrated with hydrochloric acid, the potential is proportional to the concentration of the colloid in mols. per litre of solution.

The calculations given below show that in ideal solutions the potentials are proportional to the concentrations in g. mols. per litre of solvent, under certain

conditions which can be defined by the application of the formula (4), due to Donnan and Guggenheim [1932], together with the additional calculations recorded in Table V.

$$(P' - P'') v_i \left(1 - \kappa_i \frac{P' + P''}{2} \right) + RT \ln \frac{N_i'}{N_i''} = \frac{-n_i FE}{1000} \quad \text{.....(4).}$$

R , T , F and E are defined as for formula (1). v_i = the volume of the ion in litres per g. mol. κ_i = the compressibility of the ion. n_i = the valence (negative for anions). N_i' = the molar fraction of the ion in the protein solution. P' = the hydrostatic pressure of the protein solution. N_i'' = the molar fraction of the ion in the dialysate. P'' = the hydrostatic pressure of the dialysate.

In all the formulae 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.

The effects of hydrostatic pressure, discussed by Hückel [1925] and represented by the first term in formula (4), are very small in colloidal solutions with low osmotic pressures. According to Kameyama [1925], a pressure of one atmosphere is equivalent to a correction of 0.06 % in the distribution of potassium chloride across a membrane, a correction which can be neglected in comparison with the experimental errors in the measurements of the molar fraction of the ions.

In aqueous solutions, the absolute values of the molar fractions are uncertain, on account of the polymerisation of water, but it is possible to calculate conventional values by formula (5), assuming that 1000 g. of water equal 55.51 mols. [Lewis and Randall, 1923].

$$N_i' = x_i' / (55.51 + S') \quad \text{.....(5).}$$

x_i' = the molality of the ion in g. mols. per 1000 g. of water in the protein solution. S' = the sum of the molalities of all solutes including the protein present in the solution.

A similar formula $N_i'' = x_i'' / (55.51 + S'')$ can be applied to the dialysates. The sum of the molalities S'' is less than S' , because the dialysate does not contain protein.

If, however, the difference between S' and S'' is of the order of 0.01 g. mol. per 1000 g. of water, the denominators in the formulae differ by less than 0.03 % and it follows that formula (4) is equivalent to the simpler formula:

$$x_i' \text{ (ideal)} = x_i'' r_i \quad \text{.....(6).}$$

r_i = the ideal distribution ratio, defined by formula (7).

$$r_i = \text{antilog} (-2.303 n_i FE / 1000 RT) \quad \text{.....(7).}$$

At 0°, $2.303 n_i FE / 1000 RT = n_i E / 54.16$.

The conditions under which formula (6) is valid can be determined from measurements of the osmotic pressures of the solutions with collodion membranes impermeable by proteins. Sørensen [1917] pointed out that the osmotic pressure of an ideal solution of a protein, in which the molar fraction of the protein is small, is determined by formula (8).

$$p \text{ (ideal)} = RT m_p \quad \text{.....(8).}$$

m_p is the corrected concentration of the protein, expressed in g. mols. per litre of dispersion medium. If p (ideal) be expressed in mm. mercury at 0°, then RT , expressed in mm. mercury and litres, is equal to $760 \times 22.4 = 17,033$ at 0°. In dilute aqueous solutions at 0°, there is little difference between the corrected concentration and the molality, and if the osmotic pressure be below 170 mm. mercury, the difference between S' and S'' cannot exceed 0.01.

It is convenient to use the same units in calculations of osmotic pressures and of the distribution of ions, namely, the corrected concentrations, which are correlated with concentrations and molalities by formula (9).

$$m_i' = c_i' (100/100 - V_1 C) = x_i' / w' \quad \text{.....(9).}$$

m_i' = the corrected concentration of the ion in g. mols. per litre of solvent in the protein solution. c_i' = g. mols. per litre of protein solution. V_1 and C = effective volume and concentration of protein (formula (3)). w' = kg. of water per litre of solvent.

In the dialysate, the volume of solvent, defined above, is equal to the volume of the solution, and therefore $m_i'' = c_i'' = x_i'' / w''$, where w'' is the water content of the dialysate. The terms w'' and w' are almost identical in a system where the molar fraction of water in the protein solution is 99.98 % of its value in the dialysate, a value corresponding to an osmotic pressure of about 170 mm. mercury, and therefore formula (10) can be applied to solutions with low osmotic pressures.

$$m_i' \text{ (ideal)} = m_i'' r_i = c_i'' r_i \quad \text{.....(10).}$$

Further steps in the calculation of the relationship between the membrane potential and the protein concentration are shown in Table V.

Table V. *Relationship in ideal solutions between membrane potentials and the equivalent concentration of the protein, equilibrated with salts of different valence types.*

Type (1-1). Example $\text{Pr.Cl} + \text{HCl}$. Type (2-1). Example $\text{Pr.HPO}_4 + \text{Na}_2\text{HPO}_4$. Type (1-2). Example $\text{NaPr} + \text{Na}_2\text{HPO}_4$, potentials negative. Type (1-1.5). Example $\text{NaPr} + \text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$. $\Delta p_H = p_H$ difference between protein solution and dialysate.

E	Δp_H	$\frac{[\text{Cl}]_c'}{[\text{Cl}]''}$	$\frac{[\text{H}]_c'}{[\text{H}]''}$	u	Type (1-1) $\frac{m_p n_p}{J}$	Type (2-1) $\frac{m_p n_p}{J}$	Type (1-2) $\frac{m_p n_p}{J}$	Type (1-1.5) $\frac{m_p n_p}{J}$
1.18	0.022	1.051	0.951	0.05	0.050	0.051	0.049	0.049
2.35	0.043	1.105	0.905	0.10	0.100	0.105	0.095	0.096
4.72	0.087	1.221	0.819	0.20	0.201	0.224	0.184	0.188
7.05	0.130	1.350	0.741	0.30	0.305	0.360	0.267	0.276
9.40	0.174	1.492	0.670	0.40	0.411	0.518	0.348	0.363
11.75	0.215	1.649	0.606	0.50	0.521	0.704	0.427	0.450
16.45	0.304	2.014	0.497	0.70	0.759	1.695	0.589	0.631
23.54	0.434	2.718	0.368	1.00	1.175	2.340	0.861	0.940

In making these calculations, it is assumed that the experimental data given are the membrane potentials and the concentrations of the ions in the dialysate, but as all the terms given in the tables are functions of the membrane potentials, there are many alternative sets of data that could be utilised. The first column gives the membrane potentials in millivolts at 0°, the temperature at which the measurements were made in this work. For comparison with Loeb's measurements at 24°, the potentials should be multiplied by $(273 + 24)/273$. The terms in the other columns require no temperature corrections. The p_H difference between protein and dialysate is recorded in the second column. The third column gives the ideal distribution ratio for chloride ions, which may be calculated by formula (10) or by the exponential formula stated below:

$$r_i = e^{-n_i u} = [1 - n_i u + \frac{1}{2} n_i^2 u^2 - \frac{1}{6} n_i^3 u^3 + \dots] \quad \text{.....(11).}$$

$$u = E \times F / 1000 RT = E / 23.535 \text{ at } 0^\circ.$$

The fourth column gives the ideal distribution ratio for the hydrogen ions. Since the concentrations of the ions in the dialysate, symbolised $[H]''$ and $[Cl]''$, are known, their corrected concentrations in the protein solution, symbolised $[H]_c'$ and $[Cl]_c'$, can be calculated from the distribution ratios. The equivalent concentration of the protein chloride is then calculated as the difference between $[Cl]_c'$ and $[H]_c'$, and if m_p , the corrected concentration of the protein ions, is known, the valence, or more precisely the mean valence of the protein ions, defined as the ratio of the equivalent and the molar concentrations, can then be calculated by formula (12).

$$m_p n_p = [Cl]_c' - [H]_c' \quad \text{.....(12).}$$

If the exponential series in formula (11) is applied, the term $[Cl]_c'$ is equal to $[Cl]'' (1 + u + \frac{1}{2}u^2)$, and $[H]_c'$ equals $[H]'' (1 - u + \frac{1}{2}u^2)$ and if u be small, so that higher powers of u can be neglected, $m_p n_p$ is equal to $u ([Cl]'' + [H]'')$.

Formula (12) differs from the equations given by Bjerrum [1924] and by Adair [1928] in that c_p is replaced by m_p . Similar calculations show that if the dialysate contains a mixture of ions of different valences, $m_p n_p$ is determined by formula (13).

$$m_p n_p = -\sum m_i' n_i \quad \text{.....(13).}$$

$\sum m_i' n_i$ = the sum of the corrected concentrations of the ions in the protein solution, multiplied by their valences. The ideal values of m_i' are determined by formula (10). If u is small, formula (14) can be applied:

$$m_p n_p = uJ \quad \text{.....(14).}$$

J = the sum of the concentrations of the ions in g. mols. per litre of dialysate, multiplied by the squares of their valences. Both n_p and u are negative when the protein is acting as an anion.

In Table V, exact values of the ratio $m_p n_p / J$ have been calculated, not only for univalent ions but also for mixtures such as the phosphate buffers containing bivalent ions, by the application of formulae (13) and (10), using tables of values of e^u and e^{-u} to calculate the distribution ratios referred to in formula (11). The simpler formula (14) can be applied over the range where the difference between u and $m_p n_p / J$ is small.

8. *The determination of the valence of protein ions.*

Pauli and Valkó [1933] have discussed the difficulties inherent in estimations of the valence of protein ions and have given references to previous investigations bearing on this problem. The method of cataphoresis is of historical interest, because the existence of a charge on protein ions, which depends upon the hydrogen ion concentration, was first demonstrated by measurements of the mobilities of the proteins in an electric field [Hardy, 1899; 1905].

The calculation of the charge presents considerable difficulties, on account of the uncertain effects of the "atmosphere" of inorganic ions surrounding the colloidal particle, referred to by Tiselius [1930], who has made most accurate measurements of the mobilities of proteins, and by Abramson [1933].

All other methods depend upon estimates of the concentrations of the inorganic ions present in the free state in the protein solution and the application of a formula of the type stated below:

$$c_p n_p = \text{sum of equivalents anions} - \text{sum of equivalents cations} \quad \text{.....(15).}$$

The concentrations of the ions in the free state cannot be determined directly; the results obtained by chemical analyses represent the sum of free and combined ions; the activities of the ions measured by electrometric methods may be different from the true concentrations, unless the solutions are so dilute that it is possible to neglect the interionic forces described by Debye and Hückel [1923]. Similar difficulties arise in estimates based upon osmotic pressures, conductivities and membrane potentials of the solutions.

The method described in this work is based on measurements of membrane potential, but it differs in certain respects from the methods described by Bjerrum [1924], Rinde [1926] and other investigators. Firstly, the measurements are made on solutions with relatively small membrane potentials and secondly, the relationship between the potential and the protein concentration is determined under conditions where the composition of the dialysate is kept constant, as shown in Figs. 2 to 6.

In the theoretical interpretation of the curves, it must be remembered that the pure salt solutions of the dialysates do not follow the ideal solution laws. The formula of Donnan and Allmand, discussed in Section 1, for membrane potentials in non-ideal solutions, can be stated in the form $a_i' = a_i'' r_i$, where a_i' and a_i'' denote the activities of the ion in the protein solution and in the dialysate, or in the alternative form $x_i' f_i' = x_i'' f_i'' r_i$, where f_i' and f_i'' denote the activity coefficients of the ions; it follows that $m_i' (f_i'/f_i'') = m_i'' r_i$ (formula (16)) within the range of application of formulae (6) and (10).

According to the theory of Debye and Hückel [1923], the activity coefficient is a function of the ionic strength of the solution, which is equal to $\frac{1}{2}J$. The calculations recorded in Table V show that in systems with large membrane potentials, there is a marked excess of ions inside the membrane. The difference between the values of J' and J'' in the protein solution and in the dialysate must affect the activity coefficients. If the membrane potential be less than 2 millivolts, the excess of ions inside the membrane is relatively small, and unless there are specific effects due to the protein, the ratio f_i'/f_i'' should approach unity. If this ratio be equal to unity at very low protein concentrations, formula (16) is the same as formula (10), and it follows that the straight line relationship shown by formula (14) and by Table V can be applied to the non-ideal as well as the ideal solution. The valence of the protein ions is then determined by formula (17).

$$n_p = \left(\frac{uJ}{m_p} \right)_0 = 0.00425 M \cdot J \left(\frac{E}{C_v} \right)_0 \dots\dots(17).$$

0.00425 is calculated for a temperature of 0° using the constants in formula (11). M = the molecular weight of the protein. $(E/C_v)_0$ = the value of the ratio (E/C_v) at $C_v = 0$, estimated by extrapolation from a series of measurements in which the composition of the dialysate is kept constant. $C_v = m_p \cdot M/10$.

An experimental test of the formula (17) has been made by determining the relationships between E and C_v for a number of different proteins and a number of different solvents, covering a p_H range from 2.0 to 7.8 and ionic strengths from 0.01 to 0.287. In all the systems investigated, there is a straight line relationship between E and C_v over a range from 0 to 2 millivolts, as shown in Figs. 2 to 6, a result in accordance with the assumptions made in the derivation of formula (17).

Deviations from the ideal relationships are observed only in solutions where the membrane potentials are large as in the case of haemoglobin phosphate (Fig. 4) and edestin chloride (Fig. 6), but under these conditions there is a marked excess of ions inside the membrane which must alter the activity coefficients.

A part of the difference between the ideal and the observed curves for haemoglobin phosphate can be accounted for by the difference between the p_H values of the protein solution and the dialysate and the effects of the p_H on the valence, a subject which will be discussed in a subsequent communication.

Although the results recorded in Figs. 2 to 6 are all consistent with formula (17) and show that n_p is constant in solutions with low membrane potentials, they do not prove that the absolute value of n_p is the same as that calculated by formula (17). Evidence bearing on the absolute value of n_p is given below, but the results cannot be regarded as decisive because all alternative methods are subject to uncertainty.

Congo red and 0.02 M NaHCO₃. The determination of the valence of Congo red from measurements of membrane potential is of interest because in the case of this colloid, independent evidence concerning the degree of ionisation has been obtained by osmotic methods. Bayliss [1911] and Donnan and Harris [1911] obtained osmotic pressures about one-third of those calculated for a fully ionised disodium salt Na₂R, and Donnan and Harris showed that the observed pressures could be accounted for if Congo red ionised according to the equation

where n is the number of molecules present in the colloidal micelle of Congo red. The electrical conductivities indicate greater ionisation, but this difference may be accounted for by McBain's theory that the ionic micelles have a relatively high mobility [McBain *et al.*, 1919].

The data recorded in Table VI show that the equivalent concentration $m_p n_p$, deduced from the membrane potentials, is from 47 to 53 % of the total sodium equivalent to Congo red, a result in accordance with the osmotic measurements of Donnan and Harris.

Table VI. *Membrane equilibrium of Congo red at 0°. Dialysate 0.02 M Na₂CO₃ + NaHCO₃. p_H 9.*

All concentrations expressed in millimols. per litre, except that of Congo red, given in milli-equivalents.

$[\text{CO}_2]'' = \text{sum of } [\text{HCO}_3]'' + [\text{CO}_3]''$. Proportions of ions determined by formula $[\text{Na}]'' = (1 + x'')[\text{CO}_2]''$, where $x'' = \text{fraction of total CO}_2 \text{ present as carbonate in the dialysate}$. $[\text{CO}_2]'$ = total CO₂ in the colloidal solution. $[\text{CO}_2]_c'$ (calc.) computed from values of $[\text{CO}_2]''$, x'' and E by application of formula $m_i' = m_i'' r_i$. $-m_p n_p$ = corrected equivalent concentration of Congo red computed from formula $-m_p n_p = [\text{Na}]_i'$ (ideal) $- (1 + x') [\text{CO}_2]$ (ideal). $x' = \text{fraction of total CO}_2 \text{ present as carbonate}$. Values for [Congo red] represent total sodium equivalent to Congo red, determined by analyses of $[\text{Na}]'$ and $[\text{CO}_2]'$ and the calculation of x' . Observed values of [Congo red] $[\text{Na}]'$ and $[\text{CO}_2]'$ uncorrected because of the low concentrations of Congo red (0.51 to 1.79 g. per 100 ml.).

Dialysate		Inner fluid					
$[\text{Na}]''$	$[\text{CO}_2]''$	[Congo red]	$[\text{Na}]'$	$[\text{CO}_2]'$	$[\text{CO}_2]_c'$ calc.	$-m_p n_p$	E
20.2	18.9	14.4	30.8	15.5	15.6	7.7	- 4.21
20.1	18.9	22.0	37.1	14.4	14.5	10.5	- 5.87
19.9	19.0	24.0	38.4	13.9	14.3	11.3	- 6.42
19.9	19.0	25.9	40.1	13.8	13.5	13.1	- 6.92
20.2	18.9	32.8	45.8	12.4	12.6	16.4	- 9.04
20.2	19.2	50.3	61.3	10.7	10.8	23.7	- 12.92
20.2	19.2	51.0	61.7	10.4	10.6	24.8	- 13.48

Edestin chloride and 0.0104 M HCl. The ratio $(E/C_v)_0$, calculated from the experiments on edestin recorded in Fig. 6, is equal to 17.5, and making the provisional assumption that in a solution of edestin denatured by hydrochloric

acid, the mean molecular weight is 17,000, the mean valence is 26.3. An approximate confirmation of this value is afforded by the determinations of the chloride concentrations on both sides of the membrane by chemical analyses. The curve marked 3 in Fig. 6 gives the potentials calculated from these analyses by the application of formulae (7) and (10) on the assumption that the protein chloride is fully ionised. Even in the most concentrated solutions, the observed potentials are about 70 % of the calculated values.

A rough estimate of the valence of edestin as 24.0 has been made on the assumptions that the chloride is fully ionised and that the activity coefficient of the hydrogen ions is the same as that of the chloride ions [Adair, 1928]. The electrometric measurements of Hitchcock [1923] on an edestin preparation not exposed to prolonged dialysis with acid give the slightly smaller value of 21. The criticism published by Stadie and Sunderman [1931] of the value 24.0 is based on a misunderstanding. They overlook the fact that in solutions of protein chlorides equilibrated with free hydrochloric acid, it is necessary to deduct the free hydrochloric acid from the total chloride in order to estimate the chloride equivalent to the protein. In their criticism of Adair's conclusion that an increase in the concentration of edestin diminishes the activity coefficient of the chloride ions, they point out that the electrometric measurements involved liquid junction potentials. It is unlikely that the change in the activity coefficient can be accounted for by the liquid junction potentials, because the protein diminishes not only the activity coefficients but also the osmotic coefficient g' defined by the formula $\pi = RTg'([H] + [Cl] + [Pr])$, where π is the total osmotic pressure. In solutions containing 0.246, 0.60 and 2.16 g. of protein per 100 ml. the values of g' were 0.94, 0.85 and 0.73 respectively.

Serum-albumin. The potentials of serum-albumin recorded in Table VIII are relatively large, and instead of determining the ratio $(E/C_v)_0$ by extrapolation, the "apparent valence" at each protein concentration was calculated, as shown in Table VIII. The limiting value for $C_v = 0$, determined by extrapolation, is -17.1. The apparent valences diminish as the protein concentration increases, but in all cases they exceed the values of y , the number of ammonium ions equivalent to the protein determined by chemical methods. The values of y are subject to error on account of the technical difficulties of the analyses, but the difference between n_p and y exceeds the experimental errors.

In a pure aqueous solution of an ammonium proteinate, the value of n_p cannot be greater than y , and it may be less if the proteinate be not fully ionised. In the presence of neutral salts, it is theoretically possible for n_p to exceed y , if the protein exists as a zwitterion, with many positive and negative charges, and if the anions of the neutral salt are capable of combining with, or neutralising some of the positive charges of the zwitterion. In the case of a zwitterion, n_p represents the difference between the numbers of positive and negative charges, and it is possible to increase n_p without altering the amount of acid or base combined, as determined by titration curves and by analyses of the type recorded in Table VII. This hypothesis is open to objection on chemical grounds, because the salts of ammonia and its simpler derivatives appear to belong to the class of strong electrolytes in aqueous solutions, and therefore a compound of the type RNH_3Cl should be fully ionised [Sidgwick, 1927]. The evidence is not decisive, because a protein may have different types of positive groups.

If unionised phosphate be present associated with the protein, the observed concentration of the phosphates (m_i') should exceed the ideal value.

$$m_i' \text{ (observed)} = m_i'' r_i + b m_p \quad \dots\dots(18).$$

The term b represents the apparent combination of ions with the protein, the sum of b_1 , the number of ions combined with one molecule of the protein and of b_2 , which represents the excess of ions in the neighbourhood of the protein which may be attracted by the interionic forces. Formula (18) differs from an equation published by Northrop and Kunitz [1926] in that a correction for the hydration of the protein is made in the calculation of m_i' by formula (18), and that b is regarded as the sum of two terms and is expressed in corrected concentrations rather than molalities.

The observations recorded in Table VII showed differences between the ideal and the observed concentrations of ammonium ions scarcely greater than the experimental errors. Theoretically, the ammonium proteinate tends to diminish the activity coefficients, but the relatively low concentration of the bivalent ion HPO_4 in the protein solution acts in the opposite direction. The observed concentrations of total phosphate are greater than the ideal concentrations, and the values of b recorded in Table VIII show an excess of about four phosphate molecules per molecule of albumin in dilute solutions, which is sufficient to account for the difference between y and n_p , on the assumption that the phosphate is unionised. The estimate of b , the apparent combination of phosphate ions with the protein, is affected by errors in the electrometric measurements as emphasised by Stadie and Sunderman [1931], but the chemical measurements, given in Table VII, show that the product of the corrected con-

Table VII. *Membrane equilibrium at 0° of serum-albumin and 0.005 M $\text{NH}_4\text{H}_2\text{PO}_4 + 0.005 \text{ M } (\text{NH}_4)_2\text{HPO}_4$.*

Mean p_{H} of dialysates after equilibration = 7.06. All concentrations in millimols. per litre solvent. m_p = corrected concentration of albumin, assuming mol. wt. 72,000. $[\text{PO}_4]'$ = total phosphate in dialysate = $\text{HPO}_4 + \text{H}_2\text{PO}_4$. x' = fraction of total phosphate in protein solution in bivalent form, computed from $[\text{NH}_4]'$, $[\text{PO}_4]'$ and E .

m_p	$[\text{NH}_4]'$	$[\text{NH}_4]'$ ideal	$[\text{NH}_4]'$ observed	$[\text{PO}_4]'$	$[\text{PO}_4]'$ ideal	$[\text{PO}_4]'$ observed	x'
0.287	15.11	17.00	17.50	10.07	8.47	9.71	0.471
0.482	14.95	18.08	18.50	9.73	7.31	9.61	0.489
0.792	14.80	19.85	20.6	10.15	6.70	9.73	0.386
0.882	15.15	21.11	20.3	10.17	6.30	9.15	0.408
1.645	14.75	26.20	27.9	10.00	4.46	8.86	0.339
3.320	15.00	41.20	37.4	10.00	2.48	9.15	0.267
2.480*	29.90	49.20	48.6	20.40	10.12	19.78	0.346
3.280*	29.97	55.70	48.9	20.00	8.28	19.80	0.350

* 0.02 M phosphate buffer.

Table VIII. *Observed membrane potentials of serum-albumin and ammonium phosphate at 0°.*

n_p , the apparent valence, computed from data in Table VI by formula $-m_p n_p = [\text{NH}_4]'$ (ideal) - $(1+x')[\text{PO}_4]'$ (ideal). y = base per mol. protein, computed from formula $m_p y = [\text{NH}_4]'$ (observed) - $(1+x')[\text{PO}_4]'$ (observed). b = excess PO_4 per mol. protein, defined by formula (18). p = osmotic pressure in mm. mercury. p_{H} represents the protein solution.

m_p	E	p	p_{H}	n_p apparent	y	b
0.287	- 2.75	7.1	7.00	- 15.8	11.2	4.3
0.482	- 4.47	13.8	6.96	- 14.9	8.7	4.8
0.792	- 6.92	27.3	6.91	- 13.4	9.0	3.8
0.882	- 7.78	32.5	6.88	- 13.9	8.4	3.2
1.645	- 13.56	90.7	6.80	- 12.3	9.9	2.6
3.320	- 23.80	251.8	6.64	- 11.5	8.5	2.0
2.480	- 11.78	133.6	6.74	- 14.3	8.9	3.9
3.280	- 14.59	211.7	6.71	- 13.5	6.8	3.5

centrations of the ions is considerably greater in the protein solutions than in the dialysates, and that the protein must affect the mean activities of the ions of ammonium phosphates, although such measurements do not prove that the phosphates specifically are affected by the protein.

In addition to the measurements on ammonium phosphates, a number of experiments were made with a buffer containing 0.0533 mol. of Na_2HPO_4 plus 0.0133 mol. of KH_2PO_4 , p_{H} 7.4 at 0° , which showed that the provisional estimate of $(E/C_v)_0 = 0.32$, given by Adair and Robinson [1930, 2], is too large. The value of $n_p = -25.4$ has been calculated from a more recent determination $(E/C_v)_0 = 0.24$. This value of 25.4 is larger than that obtained from 0.01 M ammonium phosphates at p_{H} 7.06 (17.1), but the difference is accounted for by the composition of the buffers.

Although the results obtained with serum-albumin and phosphate mixtures are consistent with formula (17) if the additional assumption is made that the phosphate is not completely ionised, they cannot be regarded as a proof of the formula on account of the lack of independent evidence.

SUMMARY.

The sources of error in measurements of the membrane potentials of protein solutions have been investigated, and a method has been developed which can be applied to systems where the potentials are small and volumes of the solution available are restricted to 5 ml. or 1.5 ml.

The haemoglobin of sheep and ox, serum-albumin, acid haematin, kathaemoglobin and edestin have been equilibrated at 0° with dialysates of well-defined hydrogen ion concentration, with p_{H} values ranging from 2.0 to 7.8 and salt concentrations from 0.005 to 0.16 M , and it has been found that the observed relationship between the membrane potential E and the concentration C_v , expressed in g. protein per 100 ml. solvent, is a close approximation to a straight line when E is less than 2.0 millivolts.

The hypothesis is advanced that, from such measurements, it is possible to calculate the mean valence of the protein ions, symbolised n_p , by the formula $n_p = 0.00425 MJ (E/C_v)_0$, where M is the molecular weight of the protein and J the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valences. $(E/C_v)_0$ is the limiting value of the ratio at $C_v = 0$, determined by extrapolation. The hypothesis is consistent with measurements of the relationships between membrane potentials and protein concentrations and with additional evidence obtained from chemical analyses and osmotic pressure determinations on dilute solutions of edestin and on Congo red.

In the case of serum-albumin, the results obtained can be reconciled with the investigations of the distribution of ions on the assumption that the protein exists as a zwitterion and that some of the positive charges are neutralised by phosphate ions.

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