MEMBRANE EQUILIBRIA AND THE ELECTRIC CHARGE OF RED BLOOD CELLS.

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The present work is an investigation into the electric charge of red blood cells from the point of view of the Donnan membrane equilibrium. It deals with the existence of an equilibrium state in the distribution of ions between the cell and a medium of simple composition, and the potential difference at the cell membrane arising from unequal distribution of H and Cl ions on opposite sides of the membrane; and the relation of this P.D. to that calculated from the rate of migration in cataphoresis.

The idea that a "concentration chain" might produce an electric P.D. of biological significance was considered by Höber[1] in connection with the first published observations on the cataphoresis of red blood cells. Ostwald[2] had pointed out the effect of a semipermeable membrane in limiting diffusion, but it was not until the development of the theory of membrane equilibrium by Donnan[3] that it became possible to deal with quantitative data. Warburg[4] and Van Slyke[5] have applied this theory to the red cell plasma system in papers which have appeared while the present work was in progress, and which deal with ion concentrations that apply to the physiological rôle of the

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[1] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607.

[2] Ostwald, W., *Z. physik. Chem.*, 1890, vi, 71.

[3] An interpretation of the theories of Donnan is given by Lewis, W. C. McC., A system of physical chemistry, London and New York, 3rd edition, 1920, ii, and by Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922.

[4] Warburg, E. J., *Biochem. J.*, 1922, xvi, 153.

[5] Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

cells. The numerous papers of Jacques Loeb appearing in the *Journal of General Physiology* on the application of the Donnan equilibrium to protein systems present the background of the present study.

Observations reported in a previous paper[6] had shown difference between the pH of the interior of red cells and that of the medium, so large that it seemed impossible to explain them by the Donnan equilibrium. It was found further that fresh red cells, when washed and suspended in saccharose solution showed a higher charge in cataphoresis than similarly washed cells which had been preserved 24 hours or longer in saccharose solution. This made it appear doubtful that the ionization of the cell protein was the sole source of the charge and it seemed possible that the cells owed their charge in part at least to a simple diffusion potential, due to diffusion outward of salt contained within the cell. Such a charge might be high when the cells were first placed in an electrolyte-free medium, and would fall to zero as soon as diffusion had brought the salts to the same concentration on both sides of the cell membrane. In view of the observed changes in pH of the medium, the most important salt in this connection appeared to be the phosphates of the cell. Accordingly the amount of phosphate which had diffused out of the cells was determined by the method of Tisdall[7] in the supernatant fluid from equal amounts (4 cc.) of washed sheep cells which had been allowed to stand for varying lengths of time in like quantities (40 cc.) of isotonic saccharose solution at about 10°C.

One experiment, which was repeated numerous times with the same general result, is given in Table I.

The amounts are so small that their exact determination is at the limit of accuracy of the method, but the relative error between determinations is probably small. It is apparent that the greater part of the total PO_4 which diffuses from the cells in 24 hours does so within the first 5 minutes or less. The subsequent rate of diffusion is so low that the condition approximates an equilibrium in the distribution of PO_4 ions between the cell and the medium. A potential due to diffusion of molecular phosphate must therefore be infinitesimal.

[6] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.

[7] Tisdall, F. F., *J. Biol. Chem.*, 1922, l, 329.

Measurements of the H concentration in the medium and in the interior of cells give evidence of the attainment of a like condition of equilibrium in the distribution of this ion. This is true both when the medium is originally electrolyte-free and when it contains salts, although the H distribution is markedly affected by the presence of salts.

Cells from fresh defibrinated sheep blood were washed twice with five volumes of isotonic NaCl solution and four times with similar volumes of saccharose solution; 4 cc. of strongly sedimented cells were added in the majority of experiments to 36 cc. of isotonic solution, in stoppered tubes. These were placed in the refrigerator at 5°–15°C. After varying intervals the supernatant fluid was separated by the centrifuge and removed as completely as possible without disturbing the cell sediment. The latter was then dissolved in 20 cc. of dis-

TABLE I.

Mg. $\times$ 10^{-3} *Phosphorus Lost by 4 Cc. of Cells.*

5 min.	1 hr.	3 hrs.	24 hrs.
9.0	9.5	10.4	11.2

tilled water. The water used for all solutions was distilled, with occasional exceptions, the same day it was used. The pH measurements were made with freshly palladinized electrodes in vessels similar to that of Clark,[8] using a separate electrode and vessel for each specimen of fluid. The vessels were not agitated. The battery of electrodes was placed in the refrigerator for 12 to 18 hours, removed one at a time, hydrogen admitted to restore atmospheric pressure, and the E.M.F. read 10 minutes after removal from the refrigerator. The temperature of the solution was taken immediately after the E.M.F. was read. The electrodes were allowed barely to touch the surface of the solutions; those containing dissolved cells showed, after the prolonged stay in the refrigerator, a narrow zone, at the surface, of completely reduced hemoglobin. The usual technique was em-

[8] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

ployed by changing the solution to maintain approximately the original concentration of CO_2 of the solution. Measurements by this method were more consistent than those in which electrode equilibrium was sought by rocking the vessel; the solutions containing much hemoglobin gave considerably higher pH values which could be reproduced within 0.01 pH. Control measurements in cell solution diluted 1:10, 1:5, and 1:1, the last made by freezing and thawing the cell sediment, gave in one experiment the following values: 1:10, pH 7.277; 1:5, pH 7.273; 1:1, pH 7.253. In several other experiments the 1:10 dilution gave values from 0.03 to 0.06 pH higher than the

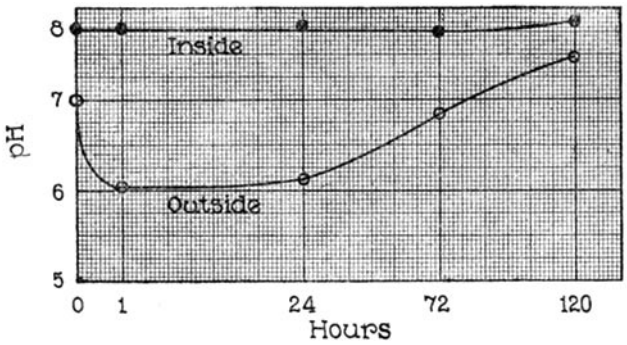

FIG. 1. Reactions of medium and cells suspended in 250 cc. of saccharose solution without salt.

undiluted cell fluid. The change in pH caused by reduction of the oxyhemoglobin to reduced hemoglobin by the hydrogen electrode has been considered too small to require correction for the purpose of this investigation. Frequent colorimetric controls were made of the pH of the outside fluids. Regardless of the method or technique employed for pH measurement, the relations between inside and outside pH were always of the same nature as those shown in the curves.

The buffer value of the cell interior shown by the figures for the effect of dilution in pH is thus very high, and we feel justified in concluding that the pH of the cell has not been altered to a significant degree by hydrolysis, on dissolving the cells in water. The pH of the 1:5 cell solution has been assumed to represent the actual pH within the cell.

In Fig. 1 are given the pH values of the inside (or 20 per cent) cell solution and of the outside solution (or suspending fluid) in an experiment in which 4 cc. of cells were suspended in 250 cc. of pure saccharose solution. The large volume of solution was used to keep the concentration of electrolyte, resulting from simple diffusion from the cells, as low as possible. Under these conditions the reaction which is reached in the outside fluid within the first hour persists without significant change for 24 hours. Other experiments show that this reaction is reached within a few minutes after adding the cells to the solution.

Careful attention has been paid to the apparent increase in acidity of the outside fluid, which reached in the majority of cases about pH 6, but in numerous experiments became as acid as pH 5.6 to 5.8. Hydrogen electrode measurements in solutions of so low conductivity and buffer value as these are difficult and uncertain; nevertheless saccharose solutions made with freshly distilled water gave repeatedly pH values of 6.8 to 7.0, and there could be no doubt of an actual increase in acidity of the solution after contact with the cells. It was given by both electrometric and colorimetric methods, and has been confirmed by Eggerth.[9] It was found that it was not due to a diffusion of CO_2 from the cells. Since it represents a movement of H from a region of low concentration (the cell interior) to one of high, with respect to this ion, it is evident that the H ion, in spite of its great mobility, is not primarily concerned in the process.

The gradual increase in alkalinity of the outside fluid which is observed after 24 hours is due probably to an alteration in the permeability of the cell membrane. If the volume of solution in which the cells are suspended is small, 40 cc. instead of 250 cc., the outside fluid shows the initial increase in acidity described, which is often sufficient to produce immediate agglutination of the cells, and undergoes after 2 to 4 hours a steady rise in alkalinity, reaching as a rule about pH 6.8. With this change in reaction, the stability of the cells increases, and they remain uniformly suspended after gentle agitation of the vessel containing them.

The inside reaction undergoes very slight change, if the cell suspension be kept at 5°C. However, if the cells are stored at room

[9] Eggerth, A. H., personal communication.

temperature, a distinct rise in alkalinity may be observed after a few hours. It seems possible that this is due to the formation of NH_4 within the cell.

If cells which have been washed in saccharose solution be suspended in isotonic solutions of NaCl or $CaCl_2$ the increase in acidity of the outside fluid does not occur, but the pH of this fluid rises and that of the inside falls within a few minutes, so that they approximate each

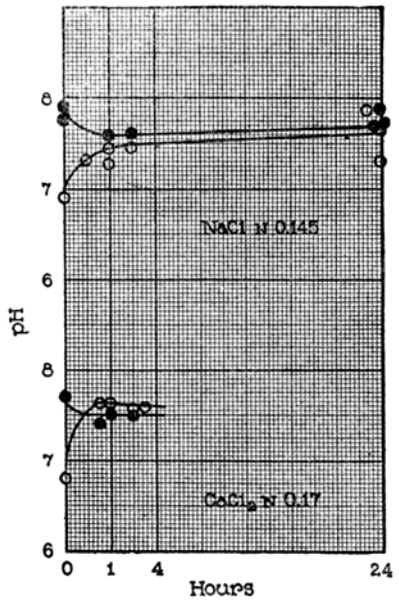

FIG. 2. Reactions of medium and cells suspended in isotonic NaCl and $CaCl_2$ solutions. Black circles, inside pH; white circles, outside pH.

other. This is shown in Fig. 2, which represents two experiments with each of the two salts. In the majority of experiments with NaCl the outside reaction remained slightly more acid than the inside, although in several experiments at the end of 24 hours in the refrigerator the two were identical. With $CaCl_2$ the outside pH rises higher and the inside pH falls lower than in the case of NaCl, so that the inside reaction is now the more acid. Experiments which for the sake of brevity are not detailed have shown that if the concentration of the salt in the suspending fluid is less than isotonic, the isotonicity

of the solution being maintained by saccharose, the changes in pH are intermediate between those just described and those that occur when the outside fluid contains no salt. The extent of change increases with the concentration of the salt.

The reaction attained in the outside fluid when acid is added is affected in a similar way by the presence of salts. In the experiment recorded in Fig. 3, the salts were present in one-quarter of their isotonic concentration, and the same amount of HCl was added before the addition of cells to each of the solutions, bringing them approximately to pH 3.3. The outside reaction remains more acid in the salt-free

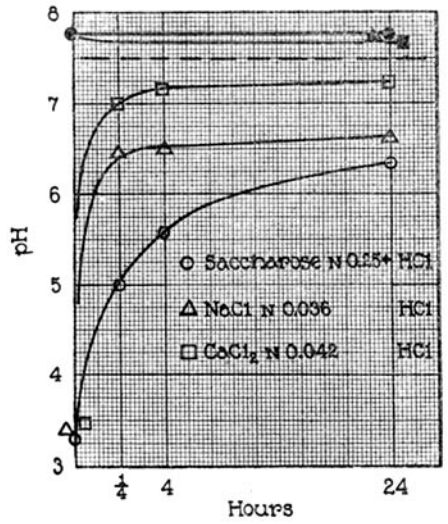

FIG. 3. Reactions of cells and medium to which HCl had been added bringing it to pH 3.3. The salts were present in one-quarter isotonic concentration. Upper curves (black circles) inside pH; lower curves (white circles) outside pH.

solution, and more acid in NaCl than in $CaCl_2$ solution. A close approximation to equilibrium between 4 and 24 hours is evident in the case of the salt solutions. The result is similar when the acid present is H_2CO_3 instead of HCl. Solutions of saccharose and of NaCl, KCl, and $CaCl_2$ were saturated with alveolar air, at about 15°C., bringing them all to the same pH (colorimetric) in each experiment. 4 cc. of packed cells were added to each of the solutions, which

had been covered with a layer of paraffin oil. The concentrations of the salts and the reactions after 80 minutes at 10°C., in two experiments are given in Table II.

The effect of salts is thus the same as when HCl is added. In the more concentrated $CaCl_2$ solution the original relation is reversed and the inside reaction becomes definitely more acid than the outside, as is the case when no acid is added, shown in Fig. 2. Determinations of combined CO_2 in the cells and in the outside fluid gave values which were so irregular as to be without significance.

In order to determine more exactly the distribution of H and Cl ions on the two sides of the cell membrane at equilibrium a separate series of measurements were made with cells which were washed once

TABLE II.

Effect of CO_2 on Reactions of Cells and Medium.

Suspending fluid.	pH outside.	pH inside.
Saccharose	6.27	7.54
KCl N 0.018	6.91	7.23
NaCl N 0.018	6.94	7.27
$CaCl_2$ N 0.021	7.11	7.08
Saccharose	5.65	7.78
NaCl N 0.145	7.43	7.40
$CaCl_2$ N 0.17	7.46	7.31

with saline solution and twice only with saccharose solution, and which were allowed to remain in contact with the outside fluid for 2 hours in the refrigerator. The Cl ratios were determined with AgCl electrodes, using a saturated solution of KCl as a reference solution. Electrodes of C.P. silver were coated with AgCl by electrolysis in an acid solution of N 0.145 NaCl and after thorough washing were allowed to remain in distilled water in the dark for 18 to 48 hours. The electrodes were placed in vessels such as were used for pH measurement. These are provided with a 4-way stop-cock at the lower end so that the two vessels may be connected with a short length of rubber tubing and a salt bridge established by connection with a reservoir of saturated KCl. The contact of the solution to be measured with the salt bridge was made by turning the 4-way cock, the bore of which

contained saturated KCl, until the periphery of this bore was separated from the vessel opening containing the unknown solution by about 2 mm., the stop-cock being greased, and the potential difference between the two electrodes measured with a potentiometer and a sensitive high resistance galvanometer. It was necessary to make the reading very rapidly, before the saturated KCl had opportunity to diffuse into the unknown less concentrated solution. All measurements were made in triplicate, with close agreement between the highest readings on each solution. In order to calculate the pCl of the outside solutions from these E.M.F. values, the E of the saturated KCl electrode was determined by measuring the E.M.F. of the cell: saturated KCl − N 0.1 KCl, and assuming an activity of 0.08 for the N 0.1 KCl solution. From this was obtained the value 0.0286 as the E.M.F. of the saturated KCl electrode against a solution normal with respect to Cl ion. The calculation of pCl from the E.M.F. between the unknown solution and the saturated KCl solution is then at 22° C.

$$\frac{\text{E.M.F. observed} - 0.0286}{58.5} = \text{pCl}$$

The packed cell sediment was suspended in saccharose solution to give a 40 per cent suspension; of this 10 cc. were added to 30 cc. of various concentrations of NaCl and $CaCl_2$ in saccharose solution. After 2 hours stay in the refrigerator, with occasional gentle agitation of the vessels, the supernatant fluid was separated completely after centrifugation and the cells dissolved by adding distilled water to 40 cc., giving a 10 per cent solution. The measurements with H_2 and AgCl electrodes were made on this dilute solution and on the supernatant fluid.

These measurements give the thermodynamic P.D. in millivolts between the two sides of the cell membrane, which is due to the difference in concentration of H or Cl, by subtracting the E.M.F. given by the more concentrated solution, with respect to H or Cl, from the E.M.F. given by the more dilute solution, when measured against a saturated calomel electrode in the case of the H electrode or against the saturated KCl electrode in the case of the Cl electrode measurements. In the latter case the value 58.5 must be subtracted from the

E.M.F. of the cell solution to correct for the 1:10 dilution of the cells. The calculation is as follows:

$Pd\,H_2$ – Saturated calomel chain: E.M.F. inside – E.M.F. outside = H P.D.
AgCl – Saturated KCl chain: E.M.F. outside – (E.M.F. inside – 58.5) = Cl P.D.

The values of H P.D. and Cl P.D. have been plotted as ordinates against the pCl of the outside fluid as abscissæ and are shown as experimental points along the two upper curves in Fig. 4.

If the Donnan equilibrium holds for the distribution of ions between the cell and the medium we should expect these two sets of values to be identical. Experimentally the Cl P.D. is consistently lower than the H P.D. It has been assumed that cells strongly sedimented in the centrifuge are actually concentrated; this was not the case since the cell mass did not show the transparency which develops when intervening fluid is entirely displaced from between the cells. Consequently the actual volume of the cells was less than 4 cc., and the actual Cl P.D. should be greater than that observed. The determinations of the H electrode E.M.F. of the cell solutions may be affected with a considerable experimental error, in spite of the reproducibility of results and are apparently too high. In hydrogen electrode measurements of hemoglobin solutions one is confronted with two sources of error: incomplete reduction, leading to low E.M.F., or the occurrence of chemical changes in the solution, leading possibly to an actual increase in alkalinity. It seemed most important to avoid incomplete reduction and the technique described was devised. It offers the possibility that the unexpectedly high cell pH values are due to the formation of NH_4. However, if H_2 be passed through a fresh hemoglobin solution continuously to a point of complete reduction, NH_4 formation must be insignificant, and the pH approximates but is slightly higher than that given by the technique of prolonged stay in a resting H_2 atmosphere. Furthermore the hemoglobin existed in the cells as oxyhemoglobin but was measured as reduced Hb; correction of the E.M.F. for the increase in alkalinity resulting from reduction would tend to make the H and Cl electrode values approximate, more closely than shown in Fig. 4.

Since the H is concentrated on that side of the cell membrane (outside) on which the Cl is dilute, in all the experiments of Fig. 4,

the P.D. has the same sign of charge in both H and Cl electrode measurements, and in either case the electrode placed in the outside fluid is positive in sign against the electrode which we imagine to be placed within the cell. Further, since the P.D. is determined by the ratio between the concentration inside the cell and that in the outside fluid,

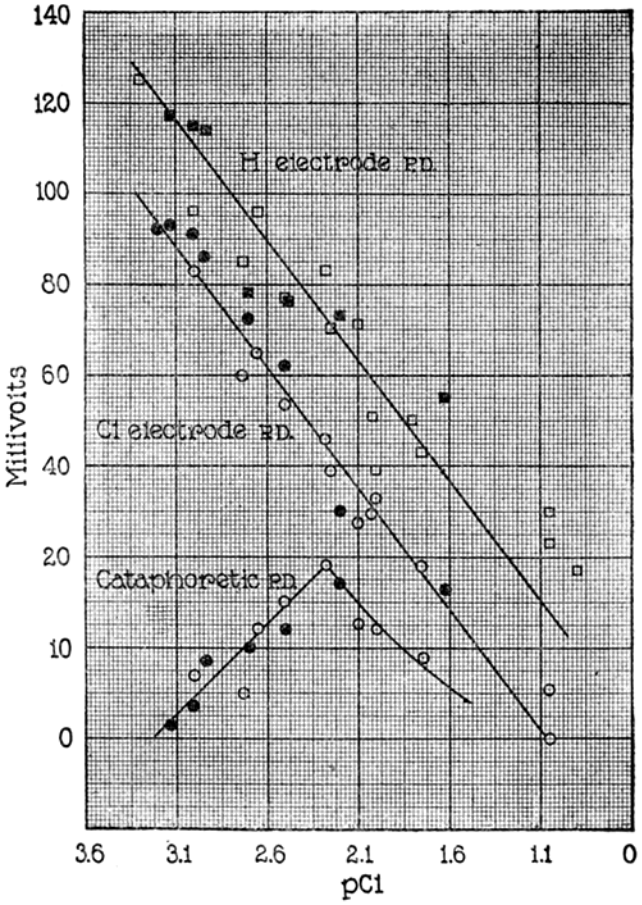

FIG. 4. H_2 electrode P.D., upper curve, the points represented by squares; Cl electrode P.D. middle curve, the points represented by circles. Cataphoretic P.D lower curve. Black squares and circles, $CaCl_2$; white squares and circles, NaCl. The ordinates give P.D. in millivolts, the abscissæ measured pCl values of the outside fluid.

the observed values satisfy the equation $\frac{C_{H_1}}{C_{H_2}} = \frac{C_{Cl_2}}{C_{Cl_1}}$ which is required by the Donnan equilibrium, within the limit of accuracy of the technique.

The effect of the electrolyte upon the reactions of the cell and the medium shown in Figs. 1, 2, and 3 is thus a necessary consequence of the Donnan equilibrium. The points for NaCl in Fig. 4 fall as close to each curve as those for $CaCl_2$, so that the effect of the Ca appears to be negligible as showing any differences from Na. In the curves of Figs. 1, 2, and 3 the greater effect of $CaCl_2$ in causing changes in reaction is probably due, therefore, to the greater concentration of Cl in solutions of this salt than in the approximately equimolar solutions of NaCl.

The P.D. arising from differences in concentration of H and Cl on opposite sides of the cell membrane may be compared in Fig. 4 with the P.D. of the cell determined by cataphoresis. The measurements were made with the macroscopic apparatus and technique described in a previous paper [10] at the same time and on the same specimens of cells which were used for the H and Cl electrode determinations. The calculation of P.D. from the observed rate of movement was made from the Helmholtz-Lamb equation, as used by Northrop [11] assuming a relative viscosity of 1.1 for the isotonic saccharose solution compared with pure water. Criticism has recently been made of such evaluation of cataphoresis data by Winslow[12] and his coauthors. The P.D. values so calculated are, however, as nearly accurate as seems at present possible and may be compared with other P.D. values similarly calculated.

The cataphoretic P.D. of the cells is shown in the lower curve of Fig. 4. The P.D. is of the opposite sign, and is first increased by the same low concentrations of Cl which decrease the H and Cl electrode potential. Such a charging effect low of concentrations of NaCl and $CaCl_2$ is evident in the experimental points given by Loeb, in cata-

[10] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[12] Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177.

phoresis of gelatin-coated and casein particles[13] at reactions on the alkaline side of the isoelectric point, and is very prominent in the cataphoresis of collodion particles.[14] After a maximum is reached, the cataphoretic P.D. of red cells falls with increasing Cl concentration, as does the H and Cl electrode potential, but remains consistently lower.

The marked valency effect of Ca in higher concentrations of $CaCl_2$ than those of Fig. 3 and of La in very low concentration upon the

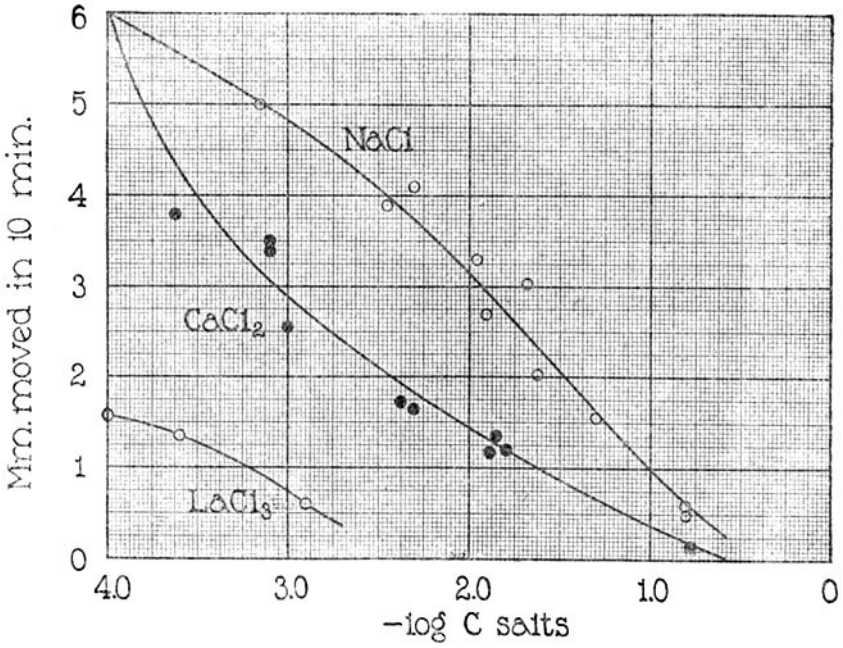

FIG. 5. The rate of movement of red cells under a potential gradient of 3.9 volts per cm. in the presence of NaCl, $CaCl_2$, and $LaCl_3$, at pH 7.

cataphoretic P.D. is shown in Fig. 5, in which the actual rates of movement in mm. per 10 minutes are plotted against the negative log of the molar concentration of the salts calculated from their dilution. The rates shown were extrapolated on a curve drawn between 0 at pH 4.7[10] and the value observed at the pH of the suspending fluid, in

[13] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.
[14] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

order to give the rate of movement at pH 7. The curve for extrapolation was drawn conformable to that for the migration of cells in the absence of added salt. The error involved is not of significance as the actual pH values fell between pH 7.1 and 6.2. Only the discharging effect of the salts is shown in Fig. 5 since the left-hand extremity of the curve represents the maximal rate of movement observed in previous experiments at pH 7. The data for this curve were obtained before the charging effects of low concentrations of salts had been observed, and the pCl of the medium was not measured.

It is evident that the cataphoretic P.D. of red cells is lower than the thermodynamic P.D., and is affected differently by the presence of salts. The valency influence of the cation of the salt is not apparent in the charging effect of low concentrations of NaCl and $CaCl_2$, although the experimental inaccuracies may render this point somewhat uncertain; the valency influence is well marked with high concentrations of NaCl and $CaCl_2$ and with $LaCl_3$. This is the usual effect. No influence of the cation is observed in the reduction of the thermodynamic potential; the effect of salts is shown only in the concentration of the Cl. Differences between the two types of potential appear to prevail generally; they were observed by Loeb in extensive observations on various types of suspended particles.[15,16]

DISCUSSION.

It might be supposed that it is the impermeability of the cell membrane to the protein ions within the cell which would be responsible for a Donnan equilibrium between the cell and the medium. That is apparently not the cause of the unequal distribution of H and Cl reported here. At the cell reactions observed, the hemoglobin must exist as K Hb and Na Hb, and if the protein ions were responsible for the Donnan equilibrium, we should expect a membrane hydrolysis to occur, with the development of a more acid reaction within the cell and a more alkaline reaction in the medium. Exactly the opposite change occurs, and we note further that the cation of salts present in the suspending medium has no apparent effect on the

[15] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 505.
[16] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 307.

thermodynamic P.D. We are led, therefore, to the conclusion that it is the cations, K and especially Na in the case of sheep blood cells, which are responsible for the equilibrium. Gürber[17] and Doisy and Eaton[18] have shown by direct analyses that K and Na do not pass through the cell membrane, and Van Slyke[5] and his collaborators have considered this fact fundamental in interpreting the electrolyte and water distribution in the blood on the basis of the Donnan equilibrium. The concentration of these cations within the cell is considerably greater than that of protein ions, and Michaelis[3] has considered the probability of potential differences across the cell membrane due to impermeability to inorganic cations. The mechanism of the observed changes in reaction appears to be as follows: in a salt-free medium, the permeating Cl tends to diffuse from the cell because of the great difference in concentration on the two sides of the cell membrane, and since K and Na do not permeate, the Cl carries with it H until the pressure of this ion on the outer side of the membrane equals that of the Cl from the inside. The interior reaction of the cell does not change appreciably because of the high buffer value of the cell.

It is suggested that this is the mechanism of the production of HCl by the cells of the gastric mucosa. To produce the high acidity of the gastric secretion by this mechanism would require the maintenance of a very high Cl concentration within the cell, as well as a means for the removal of the excess of OH ion within the cells.

Increasing concentration of Cl in the outside fluid may be regarded as opposing the diffusion pressure of Cl from the inside, and if the outside C_{Cl} is the greater, Cl will enter the cell taking with it H and increasing the acidity of the cell. This is the result observed with isotonic NaCl and $CaCl_2$ solutions in Fig. 2 and in Table II. If Fig. 2 be compared with Fig. 3, it is seen that a solution of low C_H but high C_{CL} produces a greater increase in acidity within the cell than one of of high C_H but low C_{Cl}. It is well known that the presence of neutral salt makes possible the appreciation of sour taste in a solution of less acidity than in one in which neutral salt is absent.

[17] Gürber, A., *Jahres.-ber. fortschr. Thier-chem.*, 1895, xxv, 165. Quoted by Van Slyke.[5]

[18] Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377.

If the HPO_4'' ions readily permeate the cell membrane we should expect the ratio $\sqrt{\frac{C_{HPO_4''} \text{ inside}}{C_{HPO_4''} \text{ outside}}}$ at equilibrium to equal $\frac{C_{Cl} \text{ inside}}{C_{Cl} \text{ outside}}$. Although we have found by analyses that the total amount of inorganic phosphate lost by the cell to the medium increases slightly with increasing salt concentration of the medium, the ratio of $\frac{\text{inorganic phosphate inside}}{\text{inorganic phosphate outside}}$ is not that to be expected, since in several experiments it was approximately $\frac{100}{1}$ in cases where the Cl ratio $\frac{\text{inside}}{\text{outside}}$ was very nearly 1. It seems probable, therefore, that the distribution of HPO_4'' is not determined by the same mechanism as that of Cl, and a relative impermeability of the cell membrane to HPO_4'' under the conditions of these experiments, is suggested. The determination of HPO_4'' concentration within the cell is subject to far greater difficulty than is that of Cl. The discrepancy observed may also be due to the fact that only a small proportion of the total phosphate within the cell is in the form of ions.

The impermeability of the cell membrane to inorganic cations is not absolute, since resistant specimens of cells may show after 3 to 5 days suspension in a large volume of saccharose solution such a loss of their electrolyte content, without losing hemoglobin, that they do not undergo hemolysis in distilled water until fifteen times their volume of water is added. When examined microscopically in suspension in five times their volume of distilled water such cells appear wholly normal. The reactions of cell and medium at such a time are those shown in Fig. 1. On the other hand, in solutions of relatively high salt concentration the membrane shows a considerable permeability to hemoglobin, although the Donnan equilibrium applies in this case almost as well as where the membrane is entirely impermeable to hemoglobin.

In the case of gelatin particles studied by Loeb,[19] at acid reactions, at which the non-permeating ion is the gelatin cation, the cataphoretic

[19] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 215.

charge is positive in sign, and we should expect that result in the case of red cells, since the non-permeating cations appear to determine the Donnan equilibrium. With both red cells, at the H and Cl concentrations considered here, and acid gelatin particles, the observed thermodynamic potential would be negative in sign if one H (or Cl) electrode were placed within the particle, and one H (or Cl) electrode in the fluid medium; the true membrane potential in this case should be positive in sign, since it is this potential which maintains equilibrium by opposing the thermodynamic or diffusion-pressure potential (Michaelis[3]). This sign of charge corresponds with the observed cataphoretic charge of acid gelatin particles. The cataphoretic charge of red cells in all the experiments of Figs. 4 and 5 is beyond question negative in sign. We should expect it to be positive if both thermodynamic and cataphoretic P.D. have the same origin, as suggested by Wilson.[20] Loeb has considered the valency effect of discharging ions to be strong evidence that the cataphoretic P.D. is determined by the Donnan equilibrium; [16] if this is the case with red blood cells, the cataphoretic P.D. arises conceivably at a different membrane or phase-boundary from that concerned in the thermodynamic P.D. The boundary at which the cataphoretic P.D. is produced in such a case is probably that between the outside of the cell membrane and the medium, since the ionization of the protein of the membrane would be expected to manifest itself here, as it undoubtedly does when the cell assumes a positive charge in reactions more acid than pH 4.7.[10]

SUMMARY.

It has been shown, within the probable limit of error of the methods of measurement employed, that the Donnan equilibrium determines the distribution of H and Cl ions between the cell and the surrounding fluid. This equilibrium is a consequence of the impermeability of the cell membrane to the inorganic cations of the cell. The mechanism responsible for this equilibrium is suggested as that concerned in the secretion of HCl by the cells of the gastric mucosa. If the salt concentration of the medium is low there may result from the Donnan equilibrium a thermodynamic P.D. of considerable magnitude. In

[20] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

the presence of low concentrations of electrolytes, this P.D. is to be regarded as positive in sign at reactions of the medium at which the cataphoretic charge of the cell is negative in sign. The explanation of this discrepancy in sign of charge may lie in the existence at an outer phase-boundary of a second Donnan equilibrium the nature of which is determined by the ionization of the protein of the cell membrane.