THE ISOELECTRIC POINT OF RED BLOOD CELLS AND ITS RELATION TO AGGLUTINATION.

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INTRODUCTION.

A number of investigations have been made into the electrical charges carried by bacteria and other cells in watery suspension. The movement observed in cataphoresis is affected by a number of factors, the analysis of which is so difficult that we have at the present time very little information as to the relation between agglutination and the electrical charge of the cells. Cellular suspensions behave in many ways like colloidal solutions, and it is generally understood now that colloidal protein solutions are amphoteric electrolytes. Their colloidal particles carry a negative charge in alkaline reactions and a positive charge in acid reactions; at a definite hydrogen ion concentration there exists no difference in electrical potential between the particles and the medium, so that the particles appear uncharged. This is known as the isoelectric point. At this point a number of physical properties such as solubility, viscosity, and conductivity pass through a minimum. Further, as shown by Loeb,[1] the isoelectric point is a turning point for the chemical change that determines the nature of the ionization. It is to this ionization that the electrical charge is due, so that when ionized as an acid and combined with the cation on the alkaline side of the isoelectric point the protein particle behaves as an anion and moves in the electric field to the anode; when ionized as a base and combined with anion, at reactions more acid than the isoelectric point, the protein becomes part of a complex

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[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

cation and moves to the cathode. Chemically, therefore, the protein exists most nearly "pure," least combined with acid or alkali, at the isoelectric point. It is the purpose of the present paper to show the applicability of this conception of the physical and chemical significance of the isoelectric point to the agglutination of red cell suspensions.

The literature pertaining to this subject may be reviewed briefly. The movement of red blood cells in the electric field has been observed by Lillie,[2] by Girard-Mangin and Henri,[3] and by others since. Höber[4] found that the original negative charge became positive on saturation of the solution with CO_2 in the presence of very small amounts of electrolyte, but found that the charge was not reversed if large amounts of salt were present. This effect he referred to the acid character of the CO_2 introduced, and ascribed the reversal of charge to the permeability of the cell wall to anions at the increased hydrogen ion concentration. He noted that ferric and aluminium salts conferred a positive charge upon the cells in all concentrations of NaCl in the solution. Mines[5] found the red blood cells of *Scyllium* negatively charged in the serum. On the addition of strong cerium chloride solution to a suspension of cells the charge becomes uniformly positive and no agglutination occurs; with dilute cerium solution the cells promptly agglutinate. This Mines explains by assuming that in dilute cerium solution only certain of the cells are affected; these cells encounter others still negatively charged and mutual neutralization results, with precipitation of the neutralized cells.

Electric transport of bacteria has been carried out by many investigators. Sears and Jameson,[6] pupils of Zinsser, studied the migration of bacteria at varying degrees of acidity and found that addition of alkali increased their rate of movement while acid lessened it. The fact that protein solutions flocculate most readily near their isoelectric point has suggested that this may be the mechanism of bacterial agglutination, but Beniasch[7] in studying acid agglutination of bacteria

[2] Lillie, R. S., *Am. J. Physiol.*, 1903, viii, 273.

[3] Girard-Mangin, and Henri, V., *Compt. rend. Soc. Biol.*, 1904, lvi, 866.

[4] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607; 1904, cii, 196.

[5] Mines, G. R., *Kolloid Chem.*, 1911–12, iii, 191, 236.

[6] Sears, H. J.,and Jameson, E., Cataphoresis of Bacteria, San Francisco, 1912.

[7] Beniasch, M., *Z. Immunitätsforsch., Orig.*, 1911, xii, 268.

found that alterations in the hydrogen ion concentration produced no change in the electrical properties of the bacteria; their original negative charge was not neutralized at the optimal point for agglutination and was not reversed by a more acid reaction. Arkwright[8] found, however, two optimal zones for acid agglutination of typhoid bacilli; in the more acid zone (pH 3.0) agglutination of the bacterial bodies is associated with absence of electrical charge. In the less acid zone (pH 4.7 to 3.5) a soluble bacterial protein precipitates and no change in charge is noted.

As to the influence of specific sensitization very little is known. Neisser and Friedemann[9] observed that agglutinin bacteria flocculate in the electric field as a direct result apparently of the electric flow. Michaelis and Davidsohn[10] conclude that specific typhoid agglutination and precipitation are independent of the H concentration, although from their tables for agglutination it appears that there is an optimum for this phenomenon lying between pH 4.6 and 3.7 very close to that of the native bacteria, which is at pH 4.4.

EXPERIMENTAL.

The isoelectric point was determined in the present work by the method of cataphoresis, using a model of U-tube evolved after experiments with many types of apparatus. It is illustrated in Fig. 1. The non-polarizable electrodes consist of zinc rods inserted through perforated rubber stoppers into the long ends of large stop-cock tubes. The lower portion of these tubes and the cock itself are filled with 15 per cent zinc sulfate solution. The upper part of these tubes contains a buffer solution of acetic acid acetate or phosphate mixture. This solution is made up to 10 per cent of saccharose, in order to have a specific gravity less than that of the zinc sulfate and greater than that of isotonic (M/4) saccharose solution alone, to minimize diffusion when the different fluids are in contact. The U-tube itself has an inside diameter of 6 mm. and a total length of 30 cm. It is made in two equal portions, so that after filling the side arms including the

[8] Arkwright, J. A., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 396.

[9] Neisser, M., and Friedemann, U., *Münch. Med. Woch.*, 1904, li, 465, 827.

[10] Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

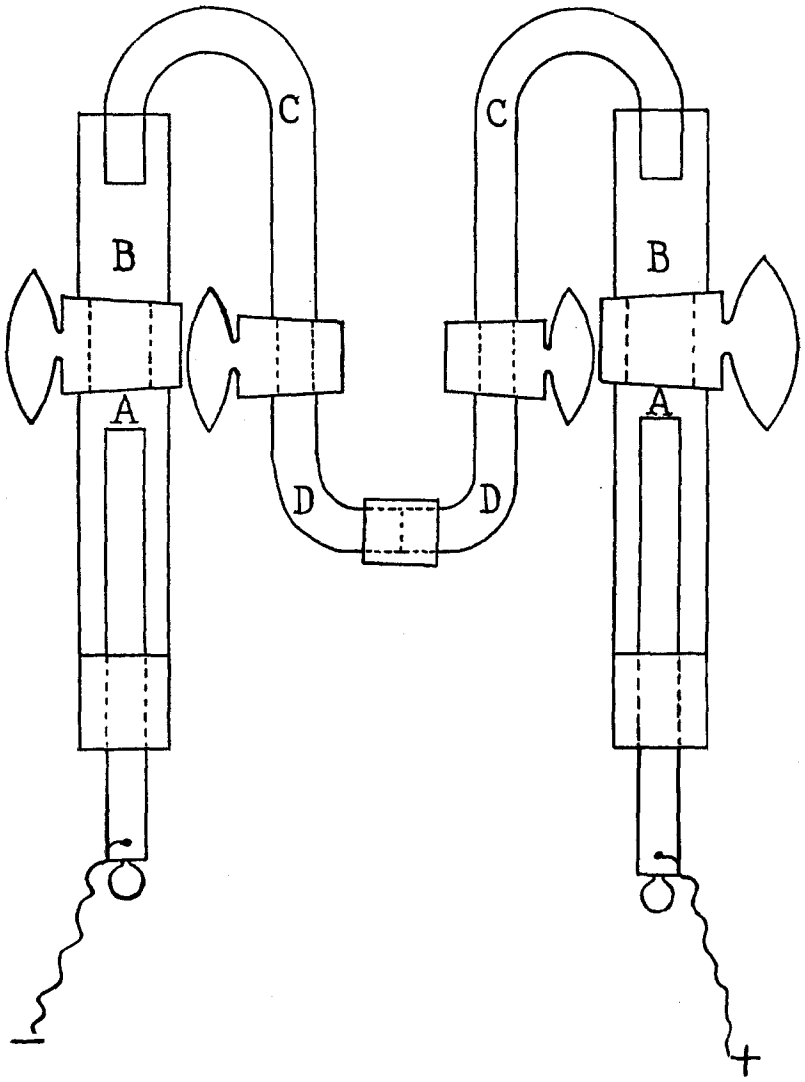

FIG. 1. Apparatus for cataphoresis of red blood cells. A, $ZnSO_4$; B, buffer; C, saccharose; D, cell suspension.

small stop-cocks with solution, the two halves of the middle portion can be filled separately with the test suspension and then pressed together, the short length of rubber tubing being left attached to one

of the halves. The ends of the side arms are then hooked into the electrode tubes containing buffer solution, and the cocks carefully opened. The fluid in the middle portion is brought to the same level on each side by adding a small amount, one or two drops, of buffer solution to the buffer chamber on one side or the other. With this type of apparatus the fall in electrical potential across the U-tube is relatively great, and the change in hydrogen ion concentration in the test fluid during the passage of current is reduced to a minimum. The determinations were made at 19 ± 1°C. without a thermostat in a room of even temperature. The current used was the Edison direct street current of 220 volts, giving a drop of almost exactly 7 volts per cm. in the U-tube.

Fresh defibrinated sheep blood was washed with two or more changes of saline solution and then freed from electrolyte by washing with four changes of ten volumes each of isotonic saccharose solution (M/4), and finally made up to 10 per cent concentration of cells in saccharose. It was found that the reaction of the saccharose solution should not be more alkaline than pH 6.5 as at more alkaline reactions the viscidity of the sedimented cell mass is so great that it is reemulsified only with difficulty and with the development of considerable hemolysis.

To determine the direction and rate of movement of red cells at varying hydrogen ion concentrations, a titration was first carried out to determine the H concentration resulting from the addition of graded amounts of HCl to the cell suspension at hand. 5 cc. of cells were introduced into twelve or more tubes, in two series. To the first series of tubes were then added 5 cc. of saccharose solution and increasing amounts of M/10 or M/100 HCl, the latter dilution made up in saccharose. Between manipulations the tubes and the stock flask were kept tightly stoppered to exclude atmospheric CO_2 as far as possible. After gentle agitation the tubes were allowed to stand at room temperature for 10 to 15 minutes, then centrifugated and the supernatant fluid was drawn off. Of this, one portion of 5 cc. with indicator added was compared with a standard series for the colorimetric determination of hydrogen ion concentration, the remaining portion serving as a color screen for the standard tube. The value thus obtained represents the H concentration with which the cells were in equilibrium.

The test emulsion for cataphoresis was prepared by adding the proper amount of acid to secure the desired H concentration to 5 cc. of saccharose solution; this was then poured gently into one of the second series of tubes of cells, and the mixture pipetted immediately into the U-tube. In this way it was possible even at reactions near the isoelectric point to observe the movement of cells before agglutination was complete and in many cases before macroscopic agglutination had appeared. For each determination 10 cc. of buffer solution and 10 cc. of saccharose solution, of the H concentration of the tube of cells to be examined, were prepared. If the apparatus was carefully manipulated the boundary of contact between the cells and the fluid in the side arms remained sharp, and the distance of displacement of the boundary during passage of current could be measured quite accurately with a pair of dividers. When agglutination appeared, a more dilute suspension remained, the movement of which could be determined likewise.

In addition to the determinations made in the absence, as far as possible, of electrolyte, measurements were made of the migration of cells in the presence of sodium chloride, sodium acetate, and sodium phosphate, using a dilution of 1 part of isotonic solution of these salts to 4 parts of saccharose solution. The acetate and phosphate were used in the form of buffer mixtures.

Sensitized cells were prepared by adding to a 10 per cent suspension of cells in saccharose approximately 50 hemolytic units of a high titer immune rabbit or hare serum. The reaction of the mixture was about pH 6.0. After incubation for 2 hours at 37°C. the cells were either used at once or were placed in the refrigerator over night. Before use the cells were sedimented twice and again made up to 10 per cent concentration. The amount of electrolyte in the immune serum was insufficient to cause agglutination. Kosakai[11] has shown that sensitizer can be dissociated from red cells by extraction in salt-free solutions of various sugars; we have been able to confirm this, but the dissociation is not complete and the cells used in our determinations were still combined with sensitizer, as shown by prompt hemolysis on the addition of complement and by agglutination when added to saline

[11] Kosakai, M., *J. Immunol.*, 1918, iii, 109.

solution of the usual strength. The titrations for H concentrations of the sensitized cells were carried out according to the method already described.

The determinations for normal and sensitized cells have been recorded in the form of curves (Figs. 2 and 3) in which the millimeters of movement during 10 minutes time are plotted as ordinates against the pH values as abscissæ. From these curves it appears that the

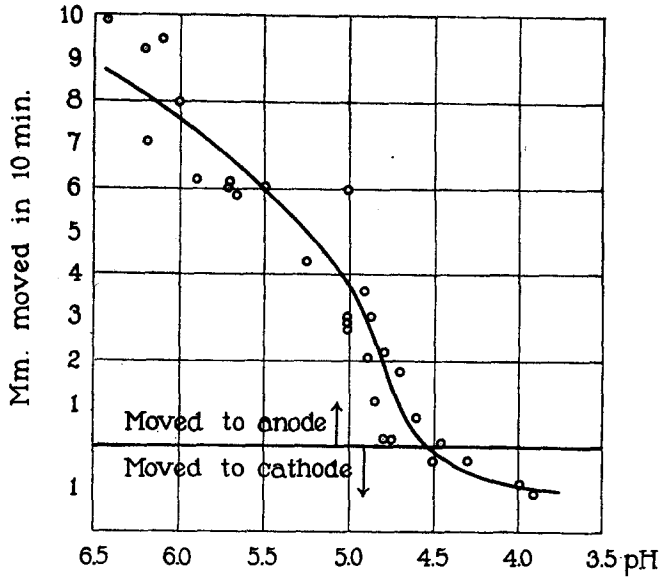

FIG. 2. Curve showing the movement in mm. during 10 minutes of normal red blood cells at varying hydrogen ion concentrations. The points above the curve between pH 4.0 and 5.0 represent determinations on different specimens of blood from those recorded in the points below the curve, so that the difference may represent actual variations in individual specimens.

direction and rate of movement in the electric field of both normal and sensitized red blood cells is a function of the hydrogen ion concentration. At concentrations less than pH 4.6 the charge carried is negative and increases in amount with the alkalinity; pH 4.6 represents the isoelectric point; at concentrations greater than pH 4.6 the charge carried is positive and increases in amount with the acidity.

A comparison of the two curves shows that on the alkaline side of the isoelectric point the charge of normal cells is greater and increases

more rapidly with increasing alkalinity than the charge of sensitized cells. The slower movement cannot be explained by the formation of agglutinated masses, since at the upper end of the curve at least no agglutination appeared even after 12 hours standing in the U-tube. On the acid side of the isoelectric point the measurements are not numerous and probably not accurate enough for a comparison.

The optimum H concentration for agglutination was determined by titrations exactly like those described above. Varying amounts of cells were used; the sharpest results were obtained by adding 1 or 2

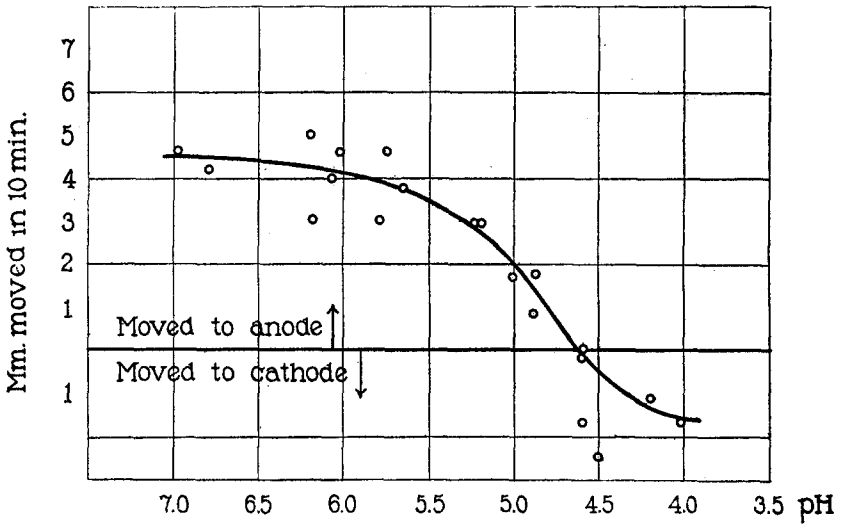

FIG. 3. Curve showing the movement in mm. during 10 minutes of sensitized red blood cells.

cc. of 10 per cent cells to 10 cc. of saccharose solution containing increasing amounts of HCl. At the optimum concentration agglutination is almost instantaneous; at greater and less acidities agglutination appears more slowly, but was practically completed within 30 minutes. The agglutination arranges itself asymmetrically on either side of the optimal point; with normal cells it never was observed at reactions more alkaline than pH 5.6, and seldom beyond pH 5.1. This variation is probably connected with variations in the amount of electrolyte present, and will be considered later. On the acid side

of the optimal point, however, agglutination appeared at all concentrations. As a result of over thirty series of determinations the optimum for agglutination of normal red cells may be given as pH 4.75. In the great majority of experiments the optimum corresponded quite sharply with this figure.

With sensitized cells, a greater variation was noted in individual experiments, due possibly to varying degrees of sensitization, but when the cells were still heavily sensitized after the final washing, the optimum for agglutination occurred regularly near pH 5.3. The optimum for precipitation of serum globulin, in which fraction of the serum the immune bodies are carried, is given as pH 5.2 by Rona and Michaelis,[12] and the isoelectric point of typhoid immune body[10] as pH 5.4 so that the flocculation of sensitized cells seems to be connected with that of the specific immune serum.

The Chemical Significance of the Isoelectric Point.

From the data given by the titrations above described, and carried out for the determination either of the isoelectric point or the optimum for agglutination, one may construct a curve giving the amounts of HCl added, as ordinates, on the pH values attained, as abscissæ. A similar curve may be drawn for the addition of acid to 10 cc. of saccharose solution alone. From these two curves a third may be drawn (Fig. 4) with pH values again as abscissæ, and as ordinates the differences between the ordinates of the first two curves, or the amounts of HCl required to bring a suspension of cells to a given H concentration in excess of those necessary in the case of the saccharose solution alone. There is evident a sharp inflection in this curve at pH 4.7. This point is so close to the value found for the isoelectric point that we may speak of it as the same. The protein of the cells exists then on the acid side of the isoelectric point as a different chemical substance from that occurring on the alkaline side. Since a true "puffer" action is manifest, it appears that the protein combines with hydrogen ion. An adsorption of hydrogen ion can hardly be thought of here as anything but a chemical combination.

[12] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

The observation by Joos[13] that the salt necessary for the agglutination of sensitized bacteria combined chemically with the bacteria, since it was not to be detected in the supernatant fluid after agglutination, although not confirmed by later investigators, led me to investigate the removal of salt after acid agglutination. With cells washed as free as possible from electrolyte, the amount of chloride present was very small and consisted chiefly in that added as HCl. A test for chlorine ion was made with $AgNO_3$ on the supernatant fluid from the cells, and it was found that at pH 4.8 no chloride was present, and

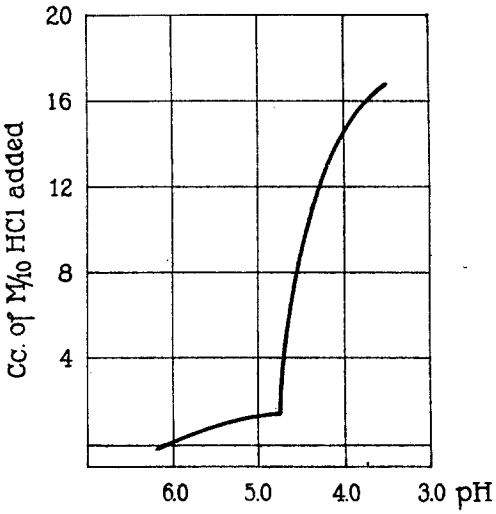

FIG. 4. Curve showing the combination of red blood cells with hydrogen ion. 5 cc. of 10 per cent normal cells. The same curve is given with sensitized cells.

only the slightest trace in the tubes to which considerable amounts of HCl had been added, while on the alkaline side of pH 4.8 a distinct turbidity appeared.

This experiment was repeated many times, always with the same result. The conditions were determined more accurately by volumetric analysis of the fluid from tubes of cells to which small amounts of M/10 NaCl were added along with M/10 HCl, so that each tube contained exactly the same amount of chlorine ion. The results are

[13] Joos, A., *Z. Hyg.*, 1901, xxxvi, 422.

given in the form of curves, Fig. 5 being that for normal cells and Fig. 6 that for sensitized cells. The curves give as ordinates the gram-ions $\times 10^{-5}$ of Cl lost by the supernatant fluid, or gained by the cells. It will be seen that the chlorine ion combines with both normal and sensitized cells in much larger amount on the acid side of pH 4.7 than

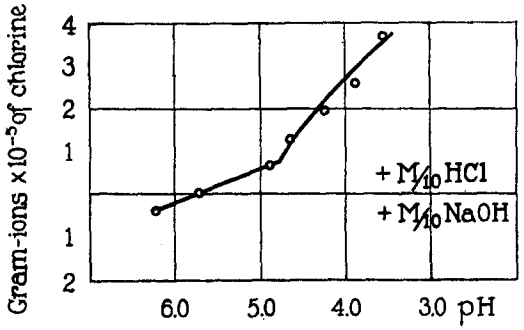

FIG. 5. Curve showing the combination of normal red blood cells with chlorine ion. 5 cc. of 10 per cent cells.

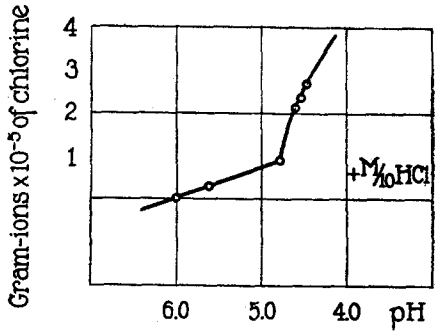

FIG. 6. Curve showing the combination of sensitized cells with chlorine ion. 5 cc. of 13 per cent cells.

on the alkaline side. On adding NaOH, Cl is actually given up by the cells between pH 5.7 and 6.2. This effect cannot be due merely to an increase in permeability of the cells, permitting the escape of chlorine ion, for in that case the amount of chlorine ion actually found should have been greatest at pH 3.5, where a trace of hemolysis appeared.

A similar determination was made on the combination with a cation at different pH values. Cells were suspended in isotonic $BaCl_2$, then washed, and again suspended in saccharose solution which had been brought to pH 6.0 with $Ba(OH)_2$ instead of NaOH. The amount of Ba present was therefore small, and could only be estimated from the turbidity of the fluid on adding Na_2SO_4. Many of the experiments showed no significant differences; in several, however, it was found that the amount of Ba in the supernatant fluid increased sharply at pH 4.7 and showed a further increase to pH 4.0. This must mean that the Ba was liberated from combination with the cells on the acid side of the isoelectric point.

Exactly the same chemical behavior has been found by Loeb[1] for gelatin, which combines with cation only on the alkaline side of its isoelectric point and with anion only on the acid side. This Loeb has shown by a series of volumetric analyses for Ag, Br, and CNS ions of the gelatin-ion compound. This work was unfamiliar to the present author at the time the observations detailed above were carried out. It is significant of the general importance of this relation that the same chemical behavior is manifested both by solutions of gelatin and by living cells. The old observation of Hamburger[14] that under the influence of HCl, H_2SO_4, or CO_2 red cells take up chlorine from the serum, while on the addition of alkali they give chlorine up to the serum, is thus to be interpreted, as suggested by Loeb, by the difference in chemical combining power on either side of the isoelectric point.

The conclusion appears justified by these facts that the character of the charge carried by a red cell depends upon the nature of its chemical combination; and that the amount of charge depends upon the amount of protein in ionic combination. This conclusion was facilitated by the observations of Loeb, already referred to.

The curve for velocities of normal cells (Fig. 2) on the alkaline side of the isoelectric point closely parallels the curve given by Loeb for swelling and conductivity of Na gelatin. Korányi and Bence[15] have shown that between saturation of defibrinated blood with CO_2 on the

[14] Hamburger, H. T., Osmotischer Druck und Ionenlehre, Wiesbaden, 1902, i.
[15] Korányi, A., and Bence, J., *Arch. ges. Physiol.*, 1905, cx, 513.

one hand and removal of the CO_2 by a stream of oxygen, on the other, the values for viscosity of the blood and refractive index of the serum pass through the minima. From the data already given, it appears that we must interpret these minima as corresponding with the isoelectric points of the serum and the cells, although in the lack of definite values for the reaction at which these minima occurred, this conclusion must be considered probable rather than proved. Indeed this conclusion was foreshadowed by Korányi and Bence in their interpretation of Höber's observations[4] on the migration of red cells.

DISCUSSION.

The effect of the addition of sodium chloride, sodium phosphate, or sodium acetate upon the velocity of cells, on the alkaline side of the isoelectric point at least, is a minor one. As compared with the values in the absence of electrolyte the velocities are somewhat greater in the presence of NaCl, and somewhat less in the presence of the other salts. These variations are in all probability due to alterations in the viscosity of the medium, which was not corrected for in the measurements given in the curves. This close correspondence of values is what we should expect if the cells exist at these reactions only as sodium proteinate, in these experiments.

When normal cells are washed in four or more changes of saccharose solution, a very slight absorption of CO_2 from the atmosphere is sufficient to raise the H concentration to pH 4.75, at which the cells agglutinate almost instantaneously. In this condition they exist most nearly in the pure state, uncombined with acid or base.

The addition of NaCl on the other hand lessens their susceptibility to change in reaction. When this salt or $CaCl_2$ is added the pH reached is less acid for a given amount of HCl than if the cells are suspended in saccharose solution alone. It is this decrease in H concentration in the presence of salt, without doubt, which carried the reaction in Höber's experiments from the acid to the alkaline side of the isoelectric point and thereby prevented the saturation of the solution with CO_2 from bringing about a positive charge on the cells. The pH value for the isoelectric point is not altered, but it is attained only on the addition of larger amounts of HCl. The curve for pH

values shows the same inflection at pH 4.7 as Fig. 4, and is therefore omitted.

This effect is very difficult to analyze. It appears to be a chemical one, and may involve the formation of a small amount of protein chloride on the alkaline side of the isoelectric point, due to the excess of chlorine ion, but since the effect is noted on both sides of the isoelectric point, any explanation of it in the light of the facts presented here would be largely speculative.

The agglutinability of sensitized cells in the presence of an electrolyte as compared with the stability of normal cells is not an absolute difference, even when the electrolyte is NaCl. Specific agglutination is known to occur over a wide range of pH values, as shown by Krumwiede and Pratt[16] among others; that it is not independent of the hydrogen ion concentration, however, is indicated as shown above by the existence of an optimum, at which it is practically independent of the presence of neutral salt. On either side of this range as common experience shows it is accelerated by the presence of neutral salt. Similarly normal cells which may not agglutinate in salt-free media at pH 4.9 have been found in our titrations to agglutinate readily at pH 5.5 in the presence of NaCl. At less acidities, while flocculation may not occur, we have found the speed of settling to be affected in a similar way by the hydrogen ion concentration.

It is apparent then when we compare the influence of the hydrogen ion concentration upon the electrical charge and chemical behavior of red cells with the influence upon agglutination that we have to deal not with one phenomenon, but with two, which are, however, closely related.

CONCLUSIONS.

1. The movement of normal and sensitized red blood cells in the electric field is a function of the hydrogen ion concentration. The isoelectric point, at which no movement occurs, corresponds with pH 4.6.

2. On the alkaline side of the isoelectric point the charge carried is negative and increases with the alkalinity. On the acid side the charge is positive and increases with the acidity.

[16] Krumwiede, C., Jr., and Pratt, J., *Z. Immunitätsforsch., Orig.*, 1912, xvi, 517.

3. On the alkaline side at least the charge carried by sensitized cells is smaller and increases less rapidly with the alkalinity than the charge of normal cells.

4. Both normal and sensitized cells combine chemically with inorganic ions, and the isoelectric point is a turning point for this chemical behavior. On the acid side the cells combine with the hydrogen and chlorine ions, and in much larger amount than on the alkaline side; on the alkaline side the cells combine with a cation (Ba), and in larger amount than on the acid side. This behavior corresponds with that found by Loeb for gelatin.

5. The optimum for agglutination of normal cells is at pH 4.75, so that at this point the cells exist most nearly pure, or least combined with anion and cation.

6. The optimum for agglutination of sensitized cells is at pH 5.3. This point is probably connected with the optimum for flocculation of the immune serum body.

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