1936

Electrophoretic studies of isohemagglutination

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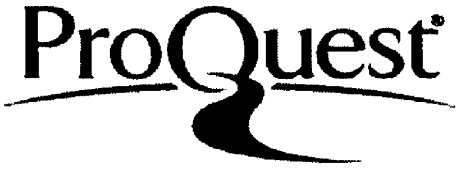

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ELECTROPHORETIC STUDIES OF ISOHEMAGGLUTINATION

By

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B. A., Montana State University, 1933

Presented in partial fulfillment of the requirement for the degree of Master of Arts.

Montana State University

1936

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Acknowledgment

Appreciation is expressed to Dr. C. Albin Watson for his many helpful suggestions and for the generous use of his time; to the library staff for liberal use of the periodicals; and to Frank Francis for direction in glass blowing.

ELECTROPHORETIC STUDIES OF ISOHEMAGGLUTINATION

INTRODUCTION

The mechanism of the agglutination reaction, and in particular that special case of it in which cellular antigens sensitized with specific immune serum are the floculating particles, has been extensively investigated. The details of the process vary from one type of particle surface to another, as well as from one type of suspending medium to another; but the underlying principles governing all the various manifestations of the reaction are applicable to each individual type of system.

Bordet in 1899[6] developed his two phase theory on the mechanism of antigen antibody reactions, the first phase being the combination of antigen and antibody and the second phase a reaction manifesting itself in various ways such as precipitation, agglutination, or lysis. Agglutination here is just a special case of several possibilities that can follow the primary antigen-antibody reaction. In his experimental work Bordet found that sensitized bacteria washed and suspended in distilled water formed stable suspensions. However, if a trace of salt is added to this suspension, agglutination immediately begins to occur. Thus he demonstrated the importance of electrolytes in producing agglutination.

Bechhold in 1904[5] discovered that the cation was the

effective agent causing precipitation and that its efficiency increased greatly with the valence. Tullock[23] explained agglutination by analogy, comparing it to heat and to alcoholic precipitation of protein. He believed that protein coating the surface of a particle became denatured in a similar manner as heat or alcohol can alter proteins. Buchanan[3] in 1918 stressed the negative charge carried by the suspended particle as the stabilizer and surface tension as the aggregator. Hertzfeld and Klinger[11], on the other hand, regarded the charge as of small importance but stressed the effect of hydration at the surface of the particle. These workers and many others have played a part in the development of the present theory of agglutination.

Northrop and de Kruif[18] in 1923 proposed a theory for the mechanism of agglutination based upon a great amount of experimental work which has formed the basis for the present generally accepted concept. Their theory has not been greatly altered nor seriously challenged since that time. In essence they conceived of two opposing forces acting between particles in suspension, one, the electrical charge on the particle promoting suspension stability and, the other, the "cohesive force" promoting aggregation and consequent precipitation. The important feature of their concept was that neither the force of repulsion nor that of attraction could be considered alone but each played a definite role in the

process of agglutination. In general they considered both the potential carried by the particles and the "cohesive force" as being affected by the hydrogen ion concentration, the salt concentration, and the presence of protein in the suspending medium, especially specific immune serum.

The charge was conceived as being a diffuse double layer at the particle medium interface and is most satisfactorily explained on the basis of a Donnan equilibrium. In general a cellular particle carries a negative charge. This charge is greatest in solutions of nonelectrolytes and diminishes in magnitude with increasing ionic strength. Reversal of the sign of charge can occur giving a measure of the amphoteric nature of the surface of the suspended particle.

The "cohesive force" between particles is difficult to explain. In the case of the agglutination of normal unsensitized cells the cohesiveness increases with diminishing electrolyte concentration.[19] This may be due to an increased surface tension or to a loose chemical combination; either possibility can be explained at least in part by considering the degree of surface hydration. Jacques Loeb[13] for example, has shown that the stability of inert collodion particles in suspension and nonspecifically coated with protein is not solely dependent upon the charge carried; but is also dependent upon the hydrophylic nature of the

coating substance. If the adsorbed molecules have a greater affinity for each other than for water, this tends toward instability. If the molecules have a greater affinity for water, then such suspensions may remain stable even in the absence of a demonstrable charge. In general increasing ionic strength from very dilute solutions up to about 0.15 normal increases the degree of surface hydration whereas further increase in salt concentration brings about dehydration. Finally salting out takes place in very concentrated solutions.[17]

With particles sensitized by immune serum the "cohesive force" could be a specific chemical combination as has been shown by Heidelberger and Kendall in the case of the pneumococcus polysaccharide S III,[10] or it could be due to a dehydrating effect incident to adsorption and "denaturation" of the coating substance.[13] In this connection Northrop and de Kruif[19] concluded that the "cohesive force" of sensitized cells remained unaffected by change in electrolyte concentration. This would indicate that the force is a specific chemical combination. As for degree of hydration, Rudd[17] has shown that normal red blood cells are relatively hydrophobic, and that when these are sensitized with immune serum they become relatively hydrophylic, but at the same time their power to agglutinate becomes greater.

From the above discussion it is evident that surface

dehydration does not satisfactorily account for the increased "cohesive force" of sensitized cells. If specific chemical affinity is also considered, the explanation is much more complete. To be sure one can also consider this increased chemical affinity of sensitized surfaces, according to Eagles[9], as due to dehydration or "denaturation" of the adsorbed protein film. Finally we can conclude that both specific chemical combination and the state of hydration can play very important parts in determining the power of cohesion.

Two definitions are in order at this point, one, the isoelectric point, and the other, the critical potential.

That point at which a particle fails to move in an electric field is known as the isoelectric point. This is the point where the cellular surface carries no charge or else carries an equal number of positive and negative charges. Here agglutination occurs most rapidly.[18]

The potential at which agglutination just begins to occur is known as the critical potential. In general it varies with the salt concentration, with the hydrogen ion concentration, with the presence of protein in solution, and with the presence of immune serum. All of these factors affect the magnitude of the "cohesive force" and the charge.[18]

According to Northrop and de Kruif[10], therefore, agglutination of both sensitized and unsensitized suspensions,

is dependent upon these two forces, potential and cohesiveness. If the potential is decreased while the cohesiveness remains the same, a critical potential will be reached below which the suspension becomes unstable. If both the "cohesive force" and the potential are decreased then the suspension may remain stable even in the absence of a demonstrable charge.

Northrop and de Kruif[10] did not theorize as to the nature of the cohesive force; but they were able to demonstrate its presence and to show that it was affected in the general manner described above. Their theory of bacterial agglutination has undergone little change since they first proposed it, and the principle line of its expansion has been a more adequate explanation of what the "cohesive force" really is.

It has been shown in some instances, that the "cohesive force" is dependent upon at least one or the other of the following factors: specific chemical combination, surface tension, and hydration. It is certain that change in the degree of hydration brings about change in surface tension; but it is a question as to whether or not hydration is the only factor that determines surface tension. Decreased hydration of the suspended particles causes increased instability.

Whereas the above studies have been limited to the mechanism by which bacteria are agglutinated by salts and

acids as well as by immune bodies obtained through animal inoculation, no adequate investigation has been made of the mechanism of isohemagglutination. In the latter case the antibodies occur naturally in human blood plasma, and the type of agglutinin present is determined by heredity. It therefore appeared to be desirable to make a study of the mechanism of this type of reaction.

The experiments herein reported are a study of isohemagglutination of human red blood cells, including the effect of salt concentration, acidity, serum protein, isohemagglutinins, heterohemagglutinins, and true antibodies. An effort was made to determine any differences between the four Landsteiner blood groups on the basis of cataphoretic mobility and to see if there were any differences between isohemagglutination, hetero-hemagglutination, and true antigen-antibody reaction.

Throughout this work the four blood groups will be referred to as O, A, B and AB on the basis of the antigens contained within the cells. The serum normally present with each type of cell contains $\alpha\beta$, β, α and o agglutinins respectively. O cells are not agglutinated by any of the four types of sera and the serum from AB cells does not agglutinate any cells, therefore the antigen in O cells and the antibody with AB cells are only hypothetical. Whenever the combinations $A\alpha$, $B\beta$, or $AB\alpha\beta$, etc. occur under the

proper conditions of temperature, time, and concentration agglutination takes place. The following diagram further illustrates the composition of the four groups:

Cells	O	A	B	AB
Serum	$\alpha\beta$	β	α	O

In their classical work of 1923 Northrop and de Kruif[10] demonstrated the effects of salt concentration and of acidity upon the potential of bacterial cells. For example, they found that Eberthella typhi in 0.1 molar NaCl carried a charge of about -2.5 millivolts and that as the solute in the suspending medium was made more dilute the negative charge became increasingly greater until at a concentration of 10^{-6} molar the potential was -30 millivolts. These authors also showed the effect of hydrogen ion giving iso-electric points for two types of bacteria studied.

According to Abramson,[2] however, the isoelectric points of mammalian red blood cells cannot be determined. He showed that the cells were always lysed or the surfaces altered by acid before any marked lowering of mobility occurred. He pointed out that reports in the literature giving isoelectric points to mammalian red blood cells were not those of the normal cells but those of decomposition products.

Schroder,[24] isolated from human red blood cells of

types A, B, and AB substances of a lipoid-lecithin nature which she claimed were active with isohemagglutinins and that were isoelectric at Ph = 2.7, a hydrogen ion concentration that would quickly destroy normal cells. Although Bruynoghe[7] has reported recently that the isohemagglutinogens are protein in nature it is still reasonable to assume that at least part of the cellular surface is composed of this lipoid type of substance. Mudd has shown that normal human red blood cells are more easily wet by oil than by water, a further indication of the lipoidal nature of their surfaces.[17]

A problem of theoretical interest here is whether or not the presumably lipoidal surfaces of blood cells can adsorb protein in a nonspecific way. It is held that when a particle is completely coated with a protein, the particle approaches the nature of the added substance having a mobility approximating that of the adsorbed protein.[1, 12, 16, et al] Abramson[1] showed that whereas gelatin would coat quartz particles and cholesterol it had no significant effect upon blood cells. Monaghan and White[16] showed that the mobility of red blood cells was unchanged by addition of protein to the suspending medium. The evidence indicates that red cells do not adsorb protein in a nonspecific way.

In 1926 Schroder[23] made a study of isohemagglutination from a physical-chemical standpoint. She determined the

charges on human red blood cells in both serum containing homologous isohemagglutinins and in serum in which the homologous isohemagglutinins were not present, using serum diluted 1:4 with isotonic sucrose slightly buffered with phosphate to Ph 7.0. The cells and serum under investigation were incubated for one-half hour at 37 C. before determinations of mobility were made. Under the conditions of her experiments she found that cells in the absence of any homologous isohemagglutinin carried a charge of 23. - 25. millivolts. In the presence of the homologous immune body there was always a diminution in the charge and the critical potential ranged from 12 to 13 millivolts. That is, agglutination always occurred somewhere within this range.

Pulcher[22] repeated this work of Schroder determining the titer of the isohemagglutinating serum along with the mobility. He did not state the exact dilution of serum used remarking that it was used in low dilution. He used isotonic sucrose slightly buffered with phosphate as a diluent. He concluded, as Schroder did, that isohemagglutination was always accompanied by a lowering of potential, and further that the decrease in charge was directly related to the titer of the serum. However, his calculations of charge ran consistently higher than those of Schroder; he gave as the critical potential 21.3 millivolts.

"Bacteria acquire an isoelectric point near that of the added protein"[21]* This being the case maximally sensitized cells will be isoelectric near the isoelectric point of the adsorbed substance. Bacterial antigens maximally sensitized with homologous immune rabbit serum have shown isoelectric points ranging from Ph 5.6 to 5.8. The globulin salted out of the same serum was isoelectric at Ph 5.1 to 5.2. Sheep cells strongly sensitized with immune serum have been isoelectric at about Ph 5.3.[15] These results indicate that in true antigen-antibody reactions, the specific immune substance is associated with the globulin fraction of the serum. In her work on isohemagglutination, Schroder[23] and [24] concluded that the isohemagglutinins are also associated with the globulin fraction of the serum, especially the euglobulin.

By determining isoelectric points of human red blood cells maximally sensitized with isohemagglutinins, heterohemagglutinins (cow serum), and with true antibodies obtained by rabbit inoculation it appeared feasible to demonstrate some differences between these three types of agglutination.

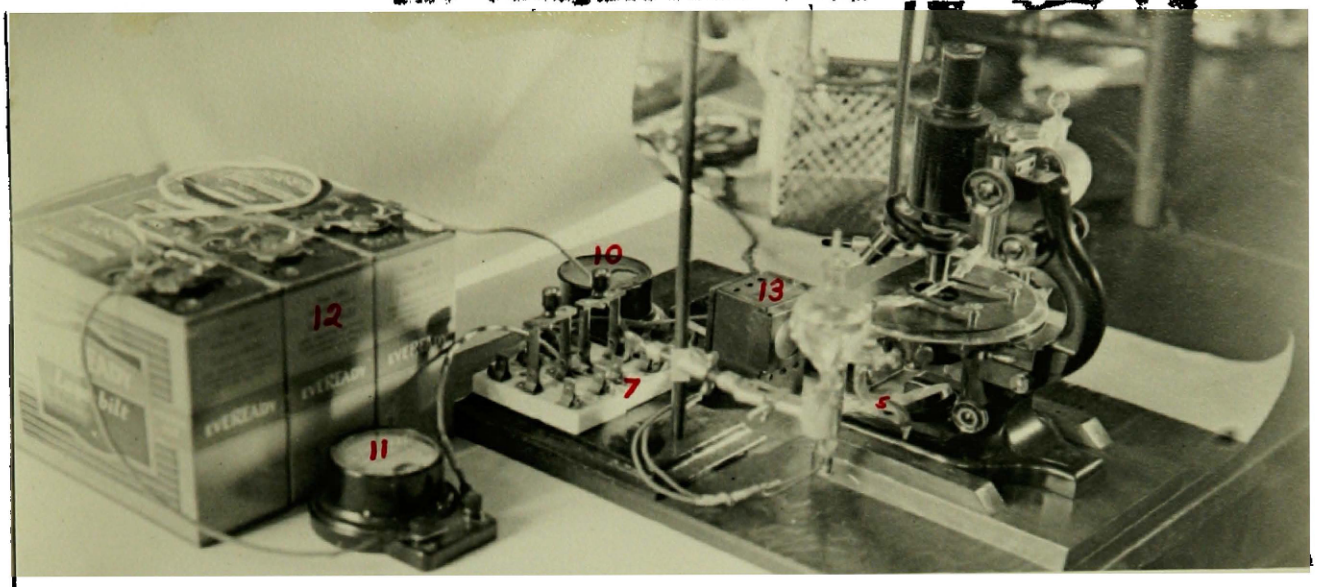
*On the question of nonspecific adsorption there is considerable question as to what happens at the cellular surface. In the case of immune reactions, one point is agreed, that substances in the sensitizing serum deposit upon the antigen surface.

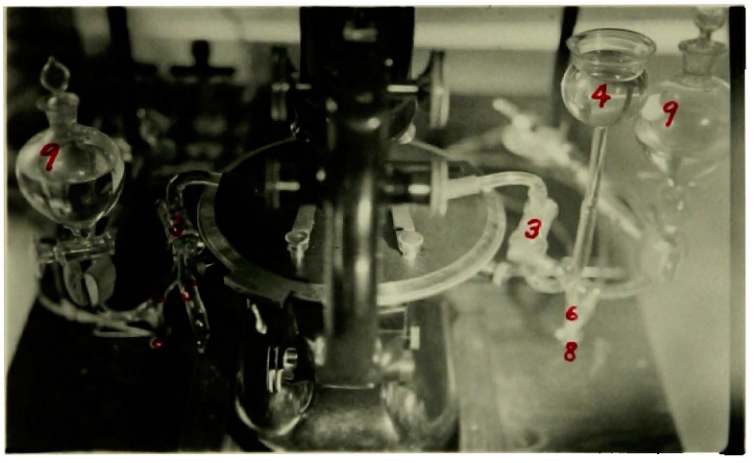
EXPERIMENTAL PART

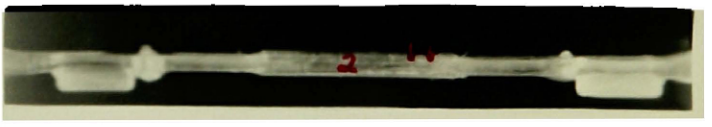
The Cataphoresis Cell

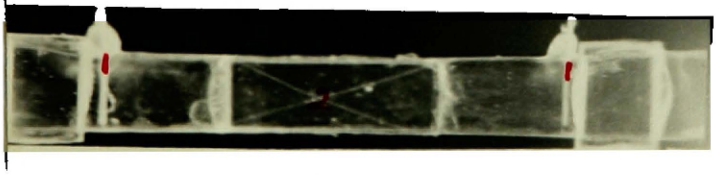
In these experiments the microscopic method for determining cataphoretic mobility was used. The cell was constructed after the Northrop - Kunitz apparatus,[19] the essential parts being a central flat capillary tube with Zn-ZnSO$_4$ electrodes attached to either end and Radio "B" batteries used to furnish an external source of e.m.f. By knowing the potential gradient within the flat capillary tube in which determinations of mobility were made as well as the time required for a particle to travel a certain distance the results can be converted to microns per second per volt per centimeter, in other words, can be converted to the number of microns traveled per second with a potential gredient of 1 volt per centimeter. Further, if the dielectric constant and the viscosity of the diffuse double layer between the particle and the surrounding medium are known, it is possible to convert u/sec/volt/cm. to potential difference between the particle and the medium. In practice the dielectric constant and viscosity of the suspending medium are used, it being assumed that they are not very different from those of the diffuse double layer.[4]

The Cataphoresis Cell









1. Platinum electrode
2. Flat capillary tube
3. Three-way stopcock
4. Filling tube
5. Draining tube
6. Electrode chamber
7. Double throw switch
8. Zinc electrode
9. Well containing saturated ZnCl
10. Volt meter
11. Ameter
12. Radio "B" batteries
13. Microscope lamp
14. Coverslip fastened on with Canada balsam

The above photographs were taken of the cell used in the experimental work. There are two small platinum electrodes sealed into the walls of the central flat capillary tube which were used in standardizing the cell. The two three-way stopcocks at either end of the central piece permitted ready connection to the filling and draining-tubes used to remove and replace suspensions and also connection to the electrode chambers for making determinations. The double throw switch to the side made it possible to take readings in both directions without appreciable loss of time. The system of lenses used in the microscope was a 16 mm. objective and a 10X ocular containing an eye piece micrometer. The zinc electrodes were in contact with saturated zinc sulfate during all determinations to prevent polarization. The wells on either side leading into the electrode chambers contained saturated zinc sulfate so that the electrode vessels could be washed out frequently to remove any protein precipitates that might collect at the stopcock openings.

Determination of Potential Gradient

It is necessary to know the drop in potential through the flat capillary tube. Rather than measure this potential for each determination, it was measured for several types of electrolytes to be used later as test media before

experimentation began. Given any applied voltage between the zinc electrodes, this voltage will bear a definite ratio to the potential gradient within the cell.

For a specific example, the distance between the two platinum electrodes was found to be 3.34 cm. The voltage from zinc to zinc with 0.01 molar phosphate made isotonic with sucrose and adjusted to Ph = 7.8 was 2.95 volts. At the same time the potential drop from platinum to platinum, measured with a Leeds-Northrop type K potentiometer, was 1.03 volts. Using this data then

$$X = \frac{P}{Vd} = \frac{1.03}{2.95 \times 3.34} = 0.105$$

where P is the voltage drop from platinum to platinum; V, the voltage from zinc to zinc; and d, the distance between the two platinum electrodes. X is the factor by which any applied voltage from zinc to zinc can be multiplied to give the drop in potential per centimeter within the flat cell itself, provided the same electrolyte was used in the test as was used in the standardization.[19]

Since there was no way to prevent polarization at the platinum electrodes it was necessary in standardizing to make a large number of determinations approaching the correct value from both sides until a reading was obtained which did not change after repeated trials with the same electrolyte.

The saturated zinc sulfate in the electrode chambers is an excellent conductor as compared to the dilute electrolytes used within the center part of the cell. Practically all of the resistance of the system takes place within the central portion.[18] With the various electrolytes up to 0.05 normal the factor for determining potential gradient did not vary noticeably with change of concentration. From 0.05 normal up to 0.15 normal there was significant variation due to the greater conductivity of the more concentrated electrolyte. For example the factor for 0.01 molar phosphate was 0.105; this held true for 0.05 molar as well. But for 0.15 molar phosphate the factor was 0.094.

Two troublesome sources of error in getting the correct potential gradient both in experiments as well as in standardization were: (1) the possible formation of gas at the electrodes and (2) the presence of air bubbles in the system, both of these causing increased resistance. These sources of error were avoided only by careful watching.

Determination of the Proper Level in the Cell At which To Make Mobility Readings

Due to the endosmotic flow of water along the walls of the cell the apparent mobility of particles is not the same at all levels. The velocity of the particles due to the electric influence on them at any depth is equal to the

observed velocity plus the velocity of the fluid. The velocity of the fluid may be either positive or negative depending upon the nature of the substance coating the walls of the cell and the nature of the suspending medium, that is, the direction of endosmosis depends upon these factors. In the case of flat cells it can be shown by mathematical reasoning and by experimentation that the observed mobility is the same as the real mobility of the particle with respect to the liquid at 0.21 and 0.79 the depth of the cell.[3] That is, there are two levels in the cell where the endosmotic flow of the water is only in a vertical direction. At these two levels the observed migration rate of the particles is not affected by any acceleration or retardation due to the motion of the liquid. In this work, the upper level of the cell was used for most readings.

Treatment of The Experimental Data Obtained from Cataphoresis Measurements

In making a reading the time required for a particle to travel a certain distance was measured with a stop watch and at the same time the voltage drop across the two zinc electrodes was observed. The data was then converted to u/sec/volt/cm. and reported as such or converted to potential difference if the dielectric constant and viscosity of the suspending medium could be obtained.

The dielectric constants were obtained from the

International Critical Tables. According to Abramson and Moyer[4] the dielectric constants of solutions up to .03 normal do not differ markedly from those of the solvents so that those of the solvents can be substituted for those of the solutions with reasonably accurate results. The dielectric constants of the exact solutions used could not be found so the dielectric constant of isotonic sucrose was used in most instances.

The viscosity was determined with an Ostwald viscometer.

The room temperature was recorded along with each experiment and then corresponding corrections in viscosity and dielectric constant were made.

The potential difference (zeta potential) was calculated by means of the Helmholtz-Perrin formula $J_E = \frac{4\pi\eta}{D} v$ where η = the viscosity of the medium; D = the dielectric constant and V = u/sec/volt/cm. (all units centimeter-gram-seconds and electrostatic units). In making a calculation, the numerator is multiplied by 10^{-4} to change microns per second to centimeters per second, and it is multiplied by 300^2 to change the practical voltage units to electrostatic units. The zeta potential is then expressed in electrostatic units. There is no unit of mass in the formula. The zeta potential is independent of the size of the particle.[14]

Hydrogen ion concentration was adjusted by the

potentiometric method. The apparatus was a quinhydrone outfit from the Welch Manufacturing Company capable of accuracy to within 0.03 of a Ph unit.

All solutions used were made by diluting a calculated volume of an isotonic electrolyte(0.15 normal) to one liter, with isotonic sucrose. Tripple distilled water was used throughout.

Determination of "Cohesive Force".

Since the agglutination reaction is dependent not only upon the charge carried by the particle but also upon its cohesiveness, it seemed expedient to measure this "cohesive force" either directly or in some way which would give values proportional to it.

Attempts were made to measure this cohesiveness in a manner similar to that used by Northrop and deKruif[18]. The apparatus used was essentially two plain glass plates one piece of which could be attached by fine wire to the spring of a Jolly balance and the other piece of which was allowed to rest horizontally on the bottom or vertically on the side of a small beaker. Several plain ground glass coverslips were fashioned to be used as the top piece, some to hang horizontally and others to hang vertically. To run a measurement a surface of each piece of glass was smeared with blood cell and the film allowed to dry. The filmed surfaces

were then immersed into a test solution and allowed to rest against each other. After about a minute of contact, the force required to separate the plates was measured with the Jolly balance which was sensitive to within 2 milligrams.

Over 30 of these measurements were made with wholly inconsistent results, even with duplicate suspensions under the same conditions. Failure here can be attributed to at least two factors. (1) Hemolysis always occurred even if the films were fixed only by drying at room temperature, and (2) it was very difficult to keep the cells from peeling or washing off the glass. As this method of determining "cohesive force" was unsatisfactory as used here, there will be no further discussion of it.

A property which helps determine the "cohesive force" is the degree of hydration at the surface of the particle. A rough measure of this property can be made by employment of Mudd's oil-water interface technique.[20] This is done by placing a drop of oil and a drop of suspension on a slide and dropping a cover slip over the two so that there will be an oil-water junction somewhere under the slip. By carefully controlling the size of the drops used, the oil phase will advance slowly across the field pushing the suspended material along in front of it. Then by microscopic observation one can see what happens to the suspended particle

at the interface, whether it stays in the aqueous phase, whether it touches the oil phase, or whether it readily enters the oil. The degree of wetting by oil is a rough measure of the hydrophobic property of the particle surface. For further details about this technique see Nugent[80].

As for blood cell suspensions used in this work, no special care was taken in regard to concentration except that all suspensions used were under 0.5%. The cells were used within 20 hours after obtaining and usually within 10 hours.[1] At pH values near neutrality the age of the cells seemed to have little effect up to 20 hours old.

The experimental results given below were obtained with the apparatus and the methods which have been described. During experimentation the central flat capillary tube of the cataphoresis cell was broken and a new one had to be made which was of entirely different dimensions. It was satisfying and certainly of importance to find that results obtained with the new cell duplicated those of the old one within the limits of experimental error obtained with either cell.

RESULTS

From the literature one can conclude that increasing salt concentration diminishes the negative charge on bacteria in suspension. It seemed reasonable to assume that human red blood cells would be affected in the same general way as were bacteria. Therefore, the first experiment was designed to test this assumption; to see if erythrocytes did carry a negative charge, and to see if increasing salt concentration would decrease this potential. Incidentally on the basis of such an experiment it might be possible to determine some electrokinetic differences between the types of blood cells used.

Effect of NaCl Concentration upon Mobility.

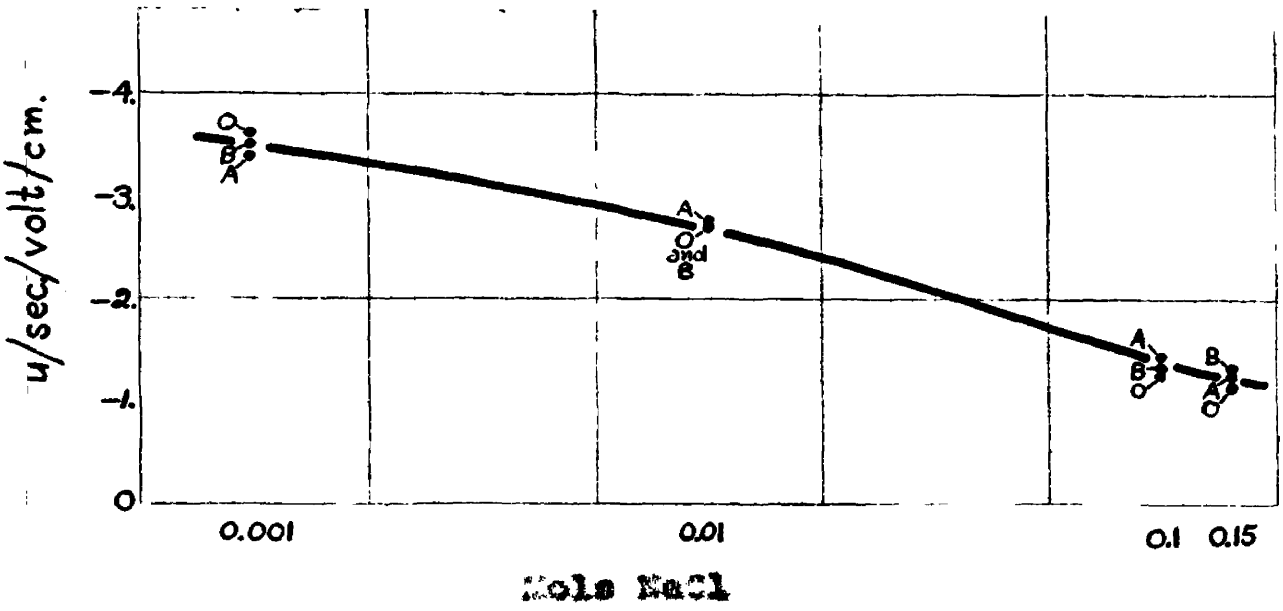
Solutions of varying concentrations of NaCl were made and were brought to isotonicity with sucrose as described in the experimental procedure. The pH of the solutions obtained ranged from 6.2 to 6.8. No effort was made to control hydrogen ion concentration. The blood cells used were washed three times in the solutions used for the cataphoresis tests. Graph number 1 gives the results of the effect of salt concentration upon the mobility of human red blood cells of types O, A and B. The results are expressed as u/sec/volt/cm. because it was impossible to estimate the dielectric constant, used in converting mobility to potential difference, of the concentrations used here above 0.01 normal. The blood groups of the cells used are indicated by the letters attached by line to the points on the chart. The curve on the graph represents a rough average of the points located. No agglutination was observed in any sample. The abcissa represents concentration of NaCl in mols and the ordinate indicates velocity of the blood cells converted to u/sec/volt/cm. The minus signs prefixed to the numbers on the ordinate indicate that the cells carried a negative charge.

The curve on graph number 1 gives a rough average of the mobilities observed with human red blood cellsof types O, A and B in graded concentrations of NaCl from .001 molar

up to .15 molar. From the dilute to the more concentrated the curve declines showing a steady loss of mobility with increasing concentration.

Graph 1

Effect of salt Concentration upon the Mobility of Human Red Blood Cells.



This diminishing mobility with increasing concentration, which is also very nearly proportional to the decrease in charge, shows that human red blood cells act in the same general way as do other organic particles capable of forming fairly stable suspensions. That is, they carry a negative charge, and increasing salt concentration depresses this potential.

The points on the graph representing determinations on

single types of cells as indicated by the letters, show that no difference between blood groups is evident on this basis. Reading the sets of letters O, A and B down the graph for each separate concentration and then comparing results at the different dilutions, shows no correlation for the sequence of the occurrence of these letters.

In .01 molar NaCl the points determined for the three types of cells, O, A and B, very nearly coincide showing almost exactly the same mobility for all three. At the same time the rate of migration at this concentration is quite high, 2.73 - 2.77 u/sec./volt cm. A high mobility is desirable for accuracy. Due to the high mobility and to the close checks obtained here with the different types of cells in 0.01 molar NaCl, this electrolyte concentration will be used in most of the subsequent experiments.

As mentioned above, just as increasing salt concentration depresses the negative charge on bacteria, so does increasing hydrogen ion concentration. The next experiment was designed to give an estimate as to the effect of hydrogen ion on normal human red blood cells and on cells agglutinated by isohemagglutinins.

It is possible to determine isoelectric points for many bacteria. This is the hydrogen ion concentration that just neutralizes the negative charge on the organism; further

increase in hydrogen ion confers a positive charge. According to Abramson[2], however, it is impossible to determine isoelectric points for red blood cells because acid hemolysis occurs long before the point is reached.

Effect of Hydrogen Ion Concentration on Normal and Agglutinated Red Blood Cells.

A series of potassium phosphate buffers of graded hydrogen ion concentration were made. A and B cells were then obtained and a portion of each (about 0.4 cc. of packed cells) was added to 20cc. of a 1:4 saline dilution of the homologous isohemagglutinating serum and incubated in the water bath at 37 C. At the end of one hour there was marked agglutination in both tubes but the supernatant in each tube was not completely clear. The sensitized cells as well as those not sensitized were then divided into several portions and washed three times in the respective buffers by means of centrifuging. The mobility and the degree of agglutination for each sample thus obtained were determined simultaneously after each specimen had been vigorously shaken to break up clumps of cells.

Graph number 2 gives the results. The abcissa represents hydrogen ion concentration expressed in pH units. The ordinate designates the magnitude of the charge carried by the cells. Again the minus signs before the numbers on

the ordinate indicate negative charges and the one plus sign at the bottom of the graph means a positive charge. The letters attached to points on the graph show the type of cell used in each individual test as they did on the first graph. In addition, sensitized cells are designated by suffixing the symbol for the homologous isohemagglutinin after the symbol for the cells. For example, Aα means cells sensitized with α agglutinin. Directly behind each symbol or pair of symbols, as the case may be, is recorded the degree of agglutination for that sample. The curve on the graph represents an approximate average of the charges determined.

The degree of agglutination here is estimated by the following figures: -, $\pm$, 1+ , 2+ , 3+ , and 4+. (-) means no agglutination; ($\pm$) means the slightest perceptible agglutination; and (1+) to (4+) means grades of definite agglutination, 4+ being the maximum. This method of estimating degree of agglutination by observation only is quite inaccurate; but nevertheless it will be used here and also throughout the rest of this work.

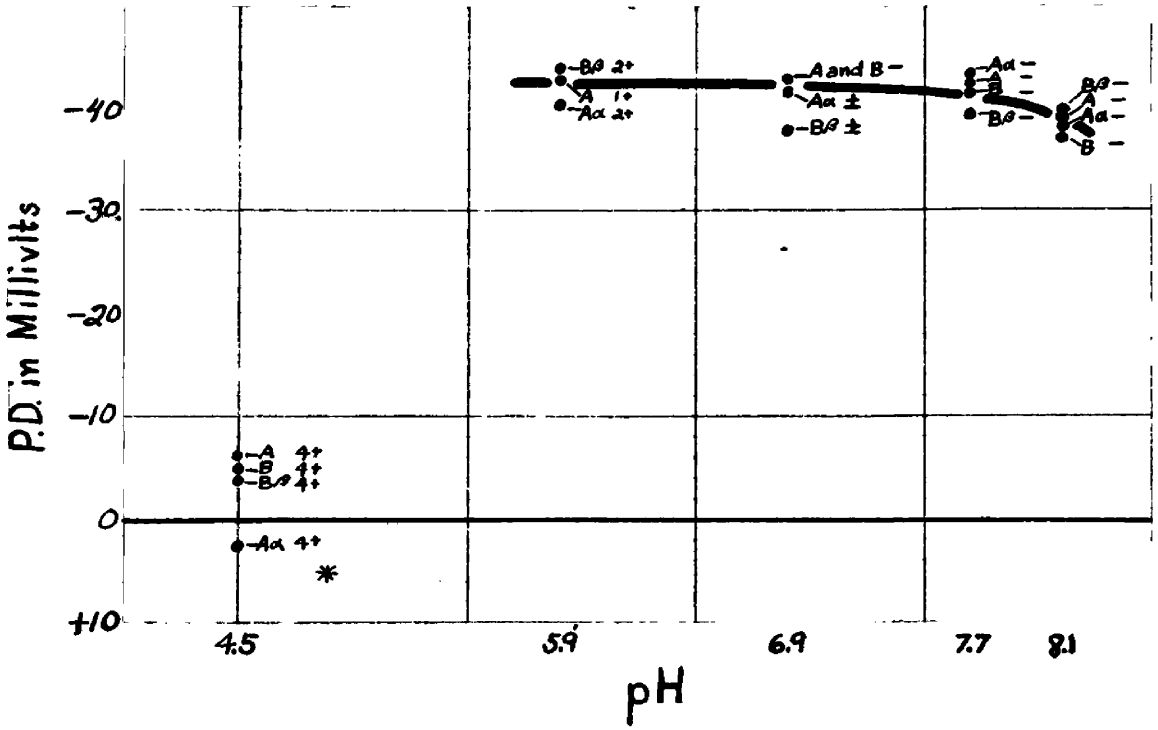
From the results shown on graph number 2 it can be seen that there is no definite relationship between sensitization and loss of charge in this experiment. In fact, between pH 5.9 and 6.1 the greatest charge is carried at

random by one or another of the four possibilities, A, B, Aα, or Bβ. Each one of these four combinations carried the greatest charge at one pH value or another.

Graph 8

Effect of Hydrogen Ion Concentration on the Charge of Normal and Partially Sensitized A and B Cells.

(.01 Molar phosphate buffers used as the suspending media).



*Acid hemolysis in every sample at pH 4.5.

It will be observed that no agglutination was evident at the time of mobility determinations on the alkaline side of neutrality and there were only ± reactions with the sensitized cells at pH 6.9 whereas in the preliminary sensitizing of the A and B cells with saline dilutions of the homologous isohemagglutinins, strong agglutinatio occurred.

This confirms the fact that increased ionic strength of the suspending medium lowers the potential thus permitting agglutination to occur more readily.

It is very evident from the data on Graph number 2 either that sensitization was incomplete or that the sensitizing substance carried a charge similar to that of the cells. The latter possibility is very improbable because in general antibodies are known to be modified globulin in nature or at least associated with the globulin fraction of the serum and cells do not show such high potentials as shown here when completely coated with globulin. According to Abramson[2], it is very unlikely that sensitized red cells are covered with a complete globulin film. So this as it may, it seems evident that only a small fraction of the red cell surface was sensitized and that although this partial film was sufficient to produce agglutination in serum diluted with saline, it was insufficient to produce any marked change in the mobility of the cells in .01 M potassium phosphate over a wide range of pH.

The charge (-37.4 to -43.8 millivolts) carried by cells in solutions ranging from pH 5.9 to 7.7 and the sudden drop occurring at pH 4.5 (-6.03 to +2.40 millivolts) and the occurrence of partial acid hemolysis here confirms the work of Abramson[2] and of Mudd[17], that the isoelectric points of

normal red blood cells cannot be measured due to acid decomposition of the cells before any marked lowering of mobility is observed.

The curve on the graph, representing a crude average of the measurements made between pH 5.9 and 8.1, takes a slight but definite dip toward the alkaline side for which no theoretical explanation could be given.

The data on Graph number 2 show that acidity does not affect the charge until acid decomposition of the red cells begins to take place. On the other hand, increasing salt concentration definitely lowers the charge. It now becomes of interest to determine the effect of serum proteins and of isohemagglutinins upon the potential and the degree of agglutination of human red blood cells. The following set of experiments will be carried out under conditions similar to those employed in setting up the usual laboratory agglutination tests except that .01 molar phosphate buffered to pH 7.3 ± .05 will be used instead of isotonic saline. The sodium phosphates will be used throughout the rest of the experimental work instead of the potassium salts.

<u>Effect of Isohemagglutinins and serum upon Potential and Agglutination.</u>

The mobility of cells was tested in serum diluted 1:4 with 0.01 molar phosphate buffer adjusted to pH 7.3 ± .05

and at the same time the degree of agglutination was recorded. After washing three times in isotonic sucrose, the cells were put into serum containing the homologous isohemagglutinin and for controls into serum containing no homologous isohemagglutinins. The suspensions were incubated for one-half hour in an incubator at 37°C. before determinations of mobility were made. Table number 1 gives the results of these experiments.

Table I

EFFECT OF ISOHEMAGGLUTININS UPON POTENTIAL AND AGGLUTINATION

Serum Diluted 1:4 with .01 Molar Phosphate Buffer; pH= 7.0±.05

Cells	Serum		Results Number of Samples Tested			
			1	2	3	4
O	αβ	P.D.	26.2	24.4	26.0	
		AGG.	---	---	---	
	β	P.D.	25.5	28.0	25.7	
		AGG.	---	---	---	
	α	P.D.	21.5			
		AGG.	---			
A	αβ	P.D.	13.4	10.2	13.8	
		AGG.	1+	3+	1+	
	β	P.D.	25.0	28.3	25.7	25.3
		AGG.	---	---	---	---
	α	P.D.	14.4			
		AGG.	---			
B	αβ	P.D.	15.5	11.9		
		AGG.	---	1+		
	β	P.D.	18.7	21.7	19.0	
		AGG.	---	---	---	
	α	P.D.	24.9	25.1		
		AGG.	---	---	---	
AB	β	P.D.	11.7			
		AGG.	1+			

P.D. = potential difference
AGG. = degree of agglutination

The results given in Table number 1 show that the potential carried by cells in serum containing no homologous isohemagglutinin was 24.4 - 26.3 millivolts. O cells in serum in which no agglutination is expected showed the extreme limits of charge just quoted. A cells in β serum and B cells in α serum, neither combination causing agglutination, gave similar charges which ranged within the limiting values mentioned for O cells in $\alpha\beta$ serum. When the isohemagglutinin was present, however, there was always a lowering of potential and whenever the potential dropped below about 15.2 millivolts agglutination occurred. In regard to the one exception where the potential dropped to 14.4 millivolts without causing agglutination it must be remembered that the suspensions were incubated for only one-half hour before determinations were made and that the rate of formation of agglutinated clumps depends in part upon the rate of collision of particles due to brownian movement.

In the case of sensitized cells, marked variation in mobility was characteristic, especially with those suspensions in which there was no agglutination. For example, B cells in β serum, tabulated in column number 3 and showing an average charge of 19.0 millivolts, gave values ranging from 14.3 to 23.0 millivolts. In these instances a large number of readings were made on each suspension and an average of the readings was reported. With cells in their own

serum, the velocity was remarkably constant, easily reproducible to within 1. millivolt. Presumably the marked variation in charge on sensitized cells was due to varying degrees of sensitization.

In general these values confirm the work of Schroder[23]. She concluded that human red blood cells suspended in serum not containing a homologous isohemagglutinin and diluted 1:4 with isotonic sucrose slightly buffered with phosphate at pH 7.3 carried a negative charge of 25.0 - 26.0 millivolts; that cells treated with the homologous isohemagglutinin always had a lower potential; and that whenever the charge was reduced to 12.0 - 18.0 millivolts, strong agglutination occurred.

Pulcher's value of 21.3 millivolts for the critical potential is not in agreement with the results here. He did not state the exact molar strength of his serum diluent and therefore, since we have seen the marked effect of salt concentration upon mobility, it is hard to judge his work critically.[22]

Due to the change in charge produced by change in ionic strength, it is evident that no work of this type is adequately described unless such details are brought out.

We have seen that washed partially sensitized cells as well as normal cells carry charges ranging from 37.4 to 43.8 millivolts in .01 molar phosphate over a fairly wide range

of pH and that increasing salt concentration lowers the mobility. On the other hand cells in their own serum diluted 1:4 with .01 molar phosphate at pH 7.8 carry a charge of about 25.5 millivolts. The question arises as to whether this lowered charge is due to non-specific adsorption of protein or to increased ionic strength acquired from the electrolytes in the added serum. The following experiment was undertaken with the hope that it might shed light upon these two questions.

The charge on glass particles and upon collodion particles was measured both in .01 molar phosphate at pH 7.8 and in human blood serum diluted 1:4 with the same buffer. Each suspension was incubated for one-half hour before mobility determinations were made. No agglutination was observed in any sample. Table number 2 gives the results of this experiment.

Table 2

Potential of Inert Particles in Pooled Human Blood Serum

Serum diluted 1:4 with .01 molar Phosphate; pH = 7.8

Collodion		Glass	
P.D. in buffer only	P.D. in Serum	P.D. in buffer only	P.D. in Serum
-43.1	-15.9	-38.1	-10.9
-43.7	-14.0	-37.0	-15.2

The results in Table number 2 show that serum greatly affects the charge upon collodion and glass particles. In these two cases we can consider the potential drop as being due to the adsorption of serum protein in a nonspecific way.[13]

It is evident that serum proteins do not affect these particles in the same way that they affect blood cells. Collodion particles in serum gave a charge of 13.5 - 14.0 millivolts and glass particles gave a charge of 13.0 - 13.2 millivolts whereas blood cells under the same conditions carried a charge of about 25.5 millivolts. These results indicate a definite physical difference between the surfaces in question. According to Monaghan and White[13] serum proteins do not adsorb to red cells in a nonspecific way. The greater charge on serum treated blood cells than on the other two types of particles further indicates that blood cells do not nonspecifically adsorb serum protein.

Since the charge on red blood cells in .01 molar sodium phosphate over a fairly wide range of pH ranges from 37.4 to 43.8 millivolts, and since the charge on cells in their own serum diluted 1:4 as described above is about 25.5 millivolts as well as the fact that these cells apparently do not adsorb serum protein, it is logical to assume that the lowered charge carried by cells in serum not containing any homologous isohemagglutinins and diluted with .01 molar phosphate is due to the increased electrolyte concentration

obtained from the added serum.

If only the electrolytes in the serum are responsible for the lowered potential upon cells in serum diluted 1:4 with .01 molar phosphate in which no homologous isohemagglutinins are present, it would be interesting to see what the effect of serum dilution would be upon the charge. Such an experiment might confirm or disprove the assumption that human red blood cells do not adsorb protein in a nonspecific way.

The Relationship Between Serum Dilution, Potential and Agglutination.

It has been shown that the salt concentration of the suspending medium has a marked effect upon the charge on red blood cells and that the presence of isohemagglutinins also affect their charge, but that in the case of washed partially sensitized cells the effect of isohemagglutinins is not definitely apparent. Therefore, it seemed expedient to run a series of experiments in which the cells were treated with a serum concentration which was a definite multiple of its titer. Eight times the concentration of the serum titer was chosen.

The details of the following experiment are similar to those of the two previous, .01 molar phosphate at pH 7.8 being used as the diluent, except that all suspensions were incubated for one hour instead of one-half hour before determinations were made. All serum dilutions of 1:4 and above

gave pH values of 7.8 ± .05 and those at a 1:2 dilution gave values of 7.65 ± .03. Table number 3 gives the results of this experiment.

Table 8

EFFECT OF ISOHEMAGGLUTININS UPON THE POTENTIAL AND AGGLUTINATION OF HUMAN RED BLOOD CELLS IN WHICH EACH SERUM WAS USED IN A DILUTION CONTAINING EIGHT TIMES THE CONCENTRATION OF ITS TITER. .01 Molar phosphate used as a serum diluent, pH=7.8±.05.

Cells	Serum		1	2	3	4	5	6	7	8
O	$\alpha\beta$	Dilution	1:8	1:8						
		P.D.	27.7	27.9						
		AGG.	-	-						
A	$\alpha\beta$	Dilution	1:8	1:8	1:16	1:8				
		P.D.	23.6	21.7	28.2	22.4				
		AGG.	-	-	-	-				
	β	Dilution	1:8	1:4	1:16					
		P.D.	26.1	24.6	29.2					
		AGG.	-	-	-					
	α	Dilution	1:2	1:2	1:8	1:8	1:8	1:16	1:16	1:2
		P.D.	10.5	14.2	25.2	27.3	26.2	29.2	28.8	13.8
		AGG.	3+	1+	-	-	-	-	-	1+
B	$\alpha\beta$	Dilution	1:8	1:8	1:8	1:8	1:8	1:8	1:8	
		P.D.	21.2	18.2	14.4	15.8	15.7	20.2	18.9	
		AGG.	-	±	1+	1+	1+	-	±	
	β	Dilution	Unc.	1:8	1:8	1:16	1:4	1:4	1:8	
		P. D.	6.7	19.6	22.0	27.6	21.7	19.0	15.6	
		AGG.	4-	-	-	-	-	-	1-	
	α	Dilution	1:8	1:8						
		P.D.	28.2	27.6						
		AGG.	-	-						
AB	$\alpha\beta$	Dilution	1:16	1:8						
		P.D.	19.6	13.1						
		AGG.	2+	3+						
	β	Dilution	1:4	1:8						
		P.D.	11.7	14.3						
		AGG.	2+	1+						

On close examination of table 3 a correlation between serum dilution and potential will be evident; the potentials determined are tabulated below to bring out this relationship

Effect of serum dilution upon the charge of cells in which the homologous isohemagglutinins were absent:

SERUM DILUTION	CHARGE
1:4	24:6
1:8	27.5 - 23.2
1:16	22:2

Effect of serum dilution upon the charge on cells in which the homologous isohemagglutinins were present:

SERUM DILUTION	CHARGE
1:2	10.5 - 14.3
1:4	11.7 - 21.7
1:8	13.1 - 27.3
1:16	19.8 - 29.2

The tabulated results show that as the serum is diluted with .01 molar phosphate the charge upon both normal and sensitized cells increases. In the case of sensitized cells the same marked variation in reduction of potential was observed as was shown in Table number 1; and the charge on sensitized cells was consistently lower than the charge on the unsensitized cells in serum of the same dilution.

Both normal and sensitized cells, as shown by Table number 3 and the tabulated results above, acquire a greater charge as the serum is diluted with .01 molar phosphate. This result is further evidence that the increase in potential is due to the increased ionic strength of the suspending

medium incidental to serum dilution with the dilute phosphate buffer used. An anomalous result occurred, however; as the isohemagglutinating serum was made more dilute the potential at which agglutination occurred became higher. This increased critical potential indicates a rise in the "cohesive force". Further discussion of the data in Table number 3 will be taken up after the results are presented in a more satisfactory form.

To demonstrate this rise in critical potential with serum dilution Graph number 3 below was constructed. It is a composit of all the experiments that have been run in which red blood cells were put into serum diluted with .01 molar phosphate buffered at pH 7.3. It includes the data from Tables number 1 and number 3 and data from other determinations not heretofore given.

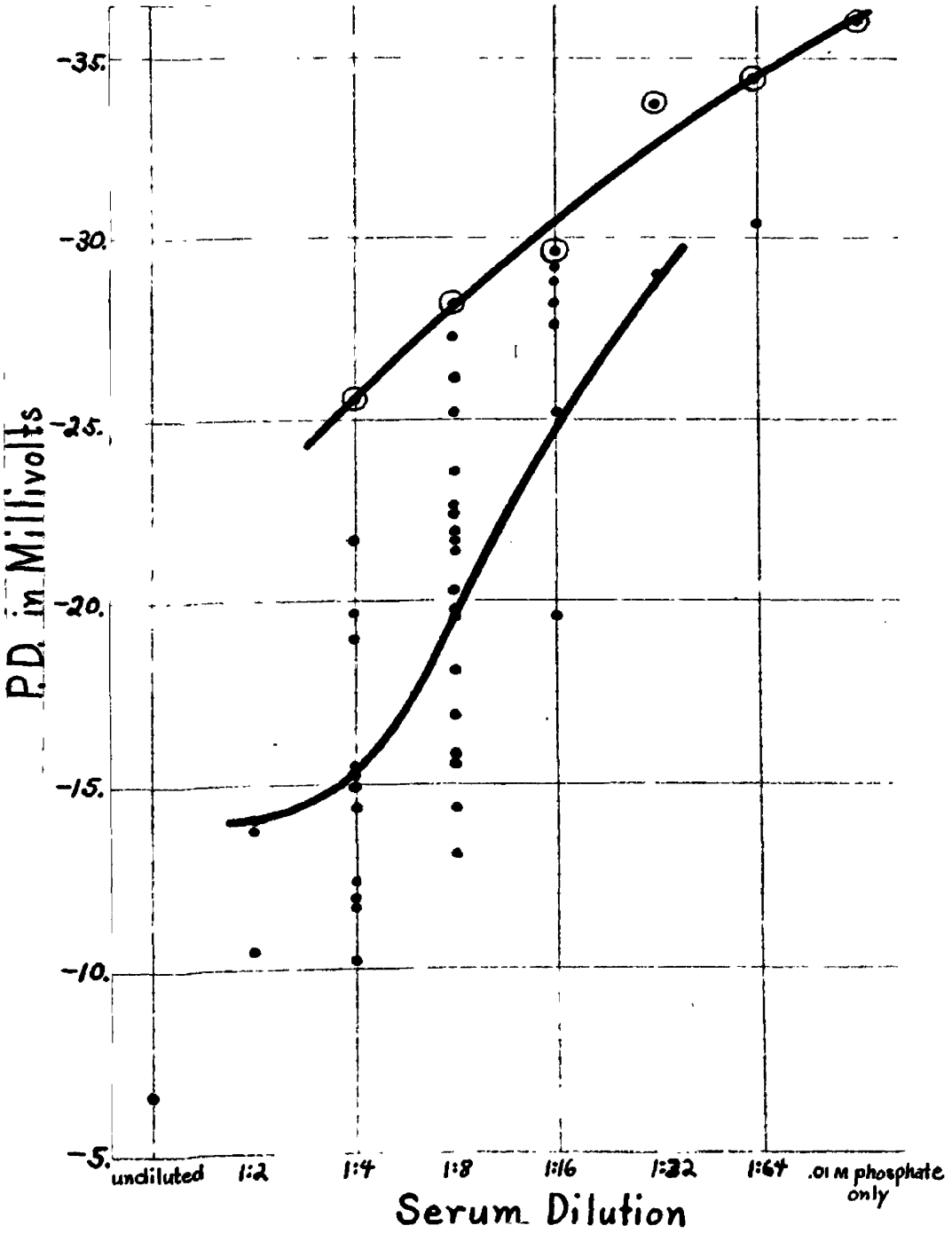
On Graph number 3 the abcissa represents serum dilution. The dilutions are indicated in the following manner: undiluted, 1:2, 1:4 etc. The ordinate designates the charge in millivolts carried by the cells. All cells carried a negative charge under these conditions. The red dots on the graph represent sensitized cells that showed agglutination and the black dots represent sensitized cells which showed no agglutination. The circled dots give the average potentials of cells in serum dilutions containing no homologous isohemagglutinin. The types of cells and serum used are

not marked. The black curve shows the rise in potential of unsensitized cells as the serum in their suspending medium is made more dilute. The red curve gives an estimation of the rise in the critical potential of sensitized cells incidental to dilution of the isohemagglutinating serum with the phosphate buffer.

Graph 3

EFFECT OF SERUM DILUTION UPON THE CHARGE ON NORMAL HUMAN RED BLOOD CELLS AND THE EFFECT OF SERUM DILUTION UPON THE CRITICAL POTENTIAL OF CELLS SENSITIZED WITH ISOHEMAGGLUTININS.

All serum diluted with .01 molar phosphate buffer at pH 7.3



The black curve on Graph number 3, representing the charge on cells in serum containing no homologous isohemagglutinin, shows again that as the serum is diluted with the phosphate buffer, the potential on the cells increases. The average charge on cells in serum diluted 1:4 is 23.8 millivolts whereas with cells in serum diluted 1:64 the average charge is about 34.4 millivolts. Cells in .01 molar phosphate at pH 7.8 carry a charge of 36.0 millivolts. It follows that as the serum is made more dilute with the buffer the charge on the cells approaches a limiting value which is the same as that for cells in the buffer alone. If protein were adsorbed nonspecifically to the red cells, there would be a greater difference between the charge in the 1:64 serum dilution and that in the buffer only. As it is, the value of 34.4 millivolts for cells in the 1:64 serum dilution and the value of 36.0 millivolts for cells in buffer only are the same within the limits of experimental error when compared to the potentials determined for cells in serum diluted 1:4 in which no homologous isohemagglutinins were present (34.4 - 23.8 millivolts). This is further indication that red blood cells do not adsorb protein nonspecifically and that the chief factor raising the potential with the serum dilution here is the decreased ionic strength of the medium.

The black dots and the red dots, both types of which signify sensitized cells, are all below the black curve. This shows again that whenever the isohemagglutin is present there is a lowering of the potential.

The great variation in charge on the sensitized cells as shown by the way the dots are strung out along the vertical lines can be regarded as due to varying degrees of sensitization or, in other words, as due to varying degrees of completeness of protein film. The way the red dots extend below the red curve, shows that a complete protein film is not in the least necessary to produce agglutination.

The critical potential in isohemagglutinating serum diluted 1:4 is 13.2 millivolts; in serum diluted 1:8 it is 19.5 millivolts and in serum diluted 1:16 it is about 25. millivolts. Since the critical potential rises with serum dilution under the conditions of this experiment, there must be an increase in the "cohesive force".

If there is an increase in "cohesive force", perhaps it could be measured in some way or another. As explained in the experimental procedure, all attempts to measure it directly by measuring the force required to separate two filmed glass plates were futile. However, the state of surface hydration is a factor which helps determine cohesiveness, and this property can readily be estimated by using Rudd's oil-water interface technique.[20]

The next experiment which makes use of Mudd's technique was carried out with the hope that it might help explain this rise in critical potential with serum dilution which suggests a greater power of cohesion.

Table number 4 shows the results obtained using Mudd's oil-water interface technique.[20] Four different types of suspensions were tested: (1) washed human red blood cells suspended in .01 molar phosphate only; (2) cells suspended in dilutions of their own serum diluted with the phosphate buffer; (3) cells suspended in isohemagglutinating serum made into dilutions with the same buffer; and (4) cells in anithuman red cell rabbit serum diluted with .01 molar phosphate. The details giving combinations of serum and cells, the dilutions of serum used as well as the method of incubation are all given on the table. The first vertical column of the table gives the numbers of the experiments according to the way they have been divided above.

The column marked Results shows roughly the comparative wetting of the cells and gives the degree of agglutination for each sample. The single line down the center of the column marked Preferential wetting of cells indicates diagramatically the oil-water interface observed under the microscope; to the left of it is shown the oil phase and to the right of it is the water phase. The circles near the oil-water interface represent the respective positions of red

blood cells in relation to the junction of the two phases. "Nujol" was used throughout for the oil. The details for making a preparation are described in the experimental procedure. In the discussion following Table number 4 the medium used for suspending the cells will sometimes be spoken of as "the water phase" for simplicity.

Table 4

PREFERENTIAL WETTING OF BLOOD CELLS

DETERMINED BY OIL-WATER INTERFACE TECHNIQUE

Exp. No.	Combination of Serum and Cells	Serum Dilution	Method of Incubation	Results: Preferential wetting of cells. Oil	Water	Degree of Agglutination
		-	1 hr. in the incubator (37°)	○		-
		1:4	1 hr. in the incubator - 37°	○		-
2*	"	1:8	"	○		-
	"	1:16	"	○		-
	"	1:32	"	○		-
	"	1:64	"	○		-
3	A cells in αβ serum	1:4	1 hr. in the incubator - 37°		○	1+
	"	1:8	"		○	1+
	"	1:16	"	○		±
	"	1:32	"	○		-
	"	1:64	"	○		-
4	O cells in anti-human rabbit serum	1:4	1 hr. 37° 3 hr. room temperature		○	3+
	"	1:8	"		○	3+
	"	1:100	"		○	3+
	A cells in anti-human rabbit serum	1:4	1 hr. 37° 3 hr. room temperature		○	4+
	"	1:8	"		○	4+
	"	1:100	"		○	4+

*0.01 molar phosphate at pH 7.8 used as the serum diluent in experiments 2, 3 and 4.

The first experiment recorded in Table number 4, showing the preferential wetting by oil of human red blood cells suspended in 0.01 molar phosphate only as well as the second experiment in which cells were put into graded serum dilutions containing no homologous isohemagglutinins, show that the cells have a hydrophobic surface and are more easily wet by oil than by water. In both of these experiments many cells were seen to enter the oil phase in each suspension tested.

The third experiment in Table number 4 gives the effect of serial dilutions of isohemagglutinating serum upon the nature of the red cell surface. In serum diluted 1:4 the representative position of the cells was on the oil-water interface. At this dilution the cells were apparently wet equally well by oil and by water. In the 1:8 dilution the cells were just inside the oil phase. With the subsequent dilutions of 1:16, 1:32 and 1:64 all cells were definitely wet by oil.

There was 1+ agglutination in the 1:4 and 1:8 dilutions and a ± reaction in the 1:16 dilution. No agglutination occurred in the other two. The agglutination is evidence of an antibody protein film. The tendency in the lower dilutions to be wet by water is also evidence of a protein film for according to Mudd[16] sensitized red blood cells become relatively hydrophylic compared to the unsensitized cells.

The fact that a ± agglutination occurred in the 1:16 dilution while at the same time the cells were wet by oil shows one or both of two things: (1) that only a small portion of the red cell surface need by sensitized to produce agglutination[2], and (2) that the decreased electrolyte strength brought about by serum dilution with the buffer had rendered the partially adsorbed protein film itself more hydrophobic.[12]

In the last experiment on table number 4 are shown the results obtained with O cells and A cells sensitized with antihuman red cell rabbit serum. In this experiment we are dealing with an immune antibody reaction. These cells in the 1:4 dilutions of antibody serum are decidedly hydrophylic. With serum dilution they become more hydrophobic as shown by the diagrams for the 1:8 and 1:100 dilutions; however, the degree of agglutination for both types of cells remained the same in as far as it could be estimated, 3+ for O cells and a 4+ for A cells.

It was conceivable that there might be a relationship between this preferential wetting by oil or water, in other words the degree of hydration, and the cataphoretic mobility of the cells sensitized with rabbit antiserum in the three dilutions studied. Accordingly the potential on the cells was determined, and it was found to be remarkably the same for both O and A cells in a given dilution of antiserum. The average charge on O and A cells in the respective

dilutions was as follows: cells in 1:4 antiserum dilutions carried a charge of 9.50 millivolts, in 1:8 dilutions a charge of 11.7 millivolts, and in 1:100 dilutions a charge of 13.0 millivolts.

Comparing these potentials with the charges carried by cells treated in a similar manner with isohemagglutinins as described on Graph number 3 it is evident that the much more potent rabbit antiserum (which had a titer of 1:1500) was more effective in lowering the potential on red blood cells than were the isohemagglutinins used, none of which had a titer higher than 1:128. The most striking comparison is shown with the potential of cells in the 1:100 dilutions carrying a charge of 13.0 millivolts as compared to the potential on the sample of sensitized cells in a 1:64 dilution of isohemagglutinating serum reported on Graph number 3 where the potential was 30.1 millivolts.

The discussion which is to follow is somewhat detailed and includes a treatment of the results shown on table number 4 as well as the data presented on Graph number 3.

It seems reasonable to assume that the greater lowering of potential on cells sensitized with rabbit antiserum as well as the very strong agglutination in all three of the dilutions studied was due to a more complete antibody protein film. In this connection it has been shown in previous experiments on isohemagglutinins that as the antiserum was

diluted with .01 molar buffer, the electrolyte concentration diminished; the same condition must hold true in the case of rabbit serum.

This diminishing electrolyte concentration renders the adsorbed protein on the cells sensitized with rabbit serum more and more hydrophobic and consequently more unstable as well as more easily wet by oil as shown in the table. The degree of agglutination for each dilution, therefore, could not have remained the same in the presence of an increased potential in the higher dilutions had there not been a simultaneous rise in the cohesiveness between cells.

In the experiment on homologous isohemagglutinins given on Table number 4 the fact that agglutination was less and that the cells were more hydrophobic than the cells sensitized with rabbit serum suggests again that these cells were not covered with a complete protein film. Nevertheless it seems that this partial film was rendered more hydrophobic as the serum was diluted and this made the "cohesive force" between cells greater.

It can be contested that with serum dilution, the degree of sensitization became less and less, and that the hydrophobic nature of the red cells in the more dilute serum solutions was due to the presence of too much normal hydrophobic cell surface. For example, however, the cells suspended in a 1:16 dilution of isohemaglutinating serum,

recorded in Table number 4, gave a * agglutination reaction, even though they were apparently as hydrophobic as any of the normal cells tested.

Regardless of how little sensitization had taken place, agglutination occurred at this dilution showing that there was a sufficient antibody film to cause the reaction. Therefore, since serum dilution with .01 molar phosphate buffer renders the cells sensitized by immune rabbit serum more hydrophobic, it is indicated that agglutination of cells sensitized with isohemagglutinins was caused in part by the dehydration of the adsorbed partial protein film.

If the sensitized cell becomes more hydrophobic with serum dilution under the experimental conditions employed here, and at the same time if the potential on the particles increases as shown in Graph number 3 under the same conditions, but still agglutination occurs at the higher potential, there must be an increase in the cohesiveness between particles. The facts presented lend strong evidence that the increased "cohesive force" incident to serum dilution as shown on Graph number 3 is due at least in part to the dehydration of the antibody protein film.

All of the experiments heretofore presented have dealt with such factors as salt concentration, hydrogen ion concentration and serum concentration in which the serum diluent was .01 molar buffer. The results obtained in these

experiments are not to be compared to the results that are obtained in routine laboratory procedures. In particular an increase in "cohesive force" is not to be expected with serum dilution in any routine work because the serum is ordinarily diluted with isotonic saline. No dehydrating effect here could be ascribed to any decreasing salt concentration for the reason that the ionic strength is not appreciably changed by serum dilution in ordinary laboratory work.

In the discussion of Table number 4 it was shown that rabbit antiserum had a more marked depressing effect upon the charge on human red blood cells than did isohemagglutinins. This fact suggests the possibility of showing some difference between isohemagglutination and so called true antigen antibody reaction. Cow serum contains normal agglutinins for human red blood cells. The next experiment was undertaken with the view of showing a possible difference between isohemagglutination, heterohemagglutination and the immune antigen-antibody reaction. This appeared to be possible by determining the isoelectric points of cells maximally sensitized with isohemagglutinins, heterohemagglutinins (cow serum), and true antibodies. In the following experiment these three types of agglutinins were studied.

Isoelectric Points of Human Red Blood Cells Sensitized with Isohemagglutinins, Heterohemagglutinins and Rabbit Antiserum.

0.03 molar phosphate buffers were made ranging from pH = 5.1 to pH = 6.1. The .03 molar buffers were used instead of .01 molar because it was reasoned that the pH of a somewhat stronger buffer would be less affected by any contaminating ions. Buffers were not used at a pH value below 5.1 because of the danger of acid hemolysis of the cells. O, A and B cells were obtained and an effort was made to maximally sensitize these with the rabbit antiserum and with cow serum. A and B cells were also sensitized with isohemagglutinins.

The method of sensitization was as follows: All specimens of sera used were first inactivated for one-half hour at 56 C. to remove complement, then 10 cc. portions of the various sera were diluted to 40 cc. with isotonic saline. Six drops of washed and packed O, A and B cells were then added to each 40 cc. sample of serum, and in the case of each tube thorough mixing was done immediately following the addition of the cells. After all suspensions had been made the whole set was placed in the ice box. For the next three hours all tubes of suspensions were shaken vigorously at frequent intervals. The tubes were then allowed to set in the ice box for 9 hours without further agitation. At the end of this time the tubes were removed from the ice box,

thoroughly shaken, and then put into a water bath at 37°C. for 1 hour. Again the tubes were shaken several times while in the water bath. At the end of one hour the whole set of tubes were put back into the refrigerator and left there to be used for the cataphoresis work which immediately followed.

At the time of mobility determinations the supernatant fluids from the sensitized cells were decanted, leaving in the bottom of each tube a concentrated mass of sensitized cells. With a given sample of cells about 6 drops of the concentrated suspension were added to a 30 cc. portion of one of the .02 molar buffer solutions and then shaken into an even suspension. Mobility readings were made within 4 minutes after mixing. The cataphoresis cell was previously washed out with the same buffer as was used to suspend the cells. Immediately following the mobility determination, the remainder of the 30cc. volume of sensitized cell suspension was used to determine the hydrogen ion concentration. The latter was done by the potentiometric method.

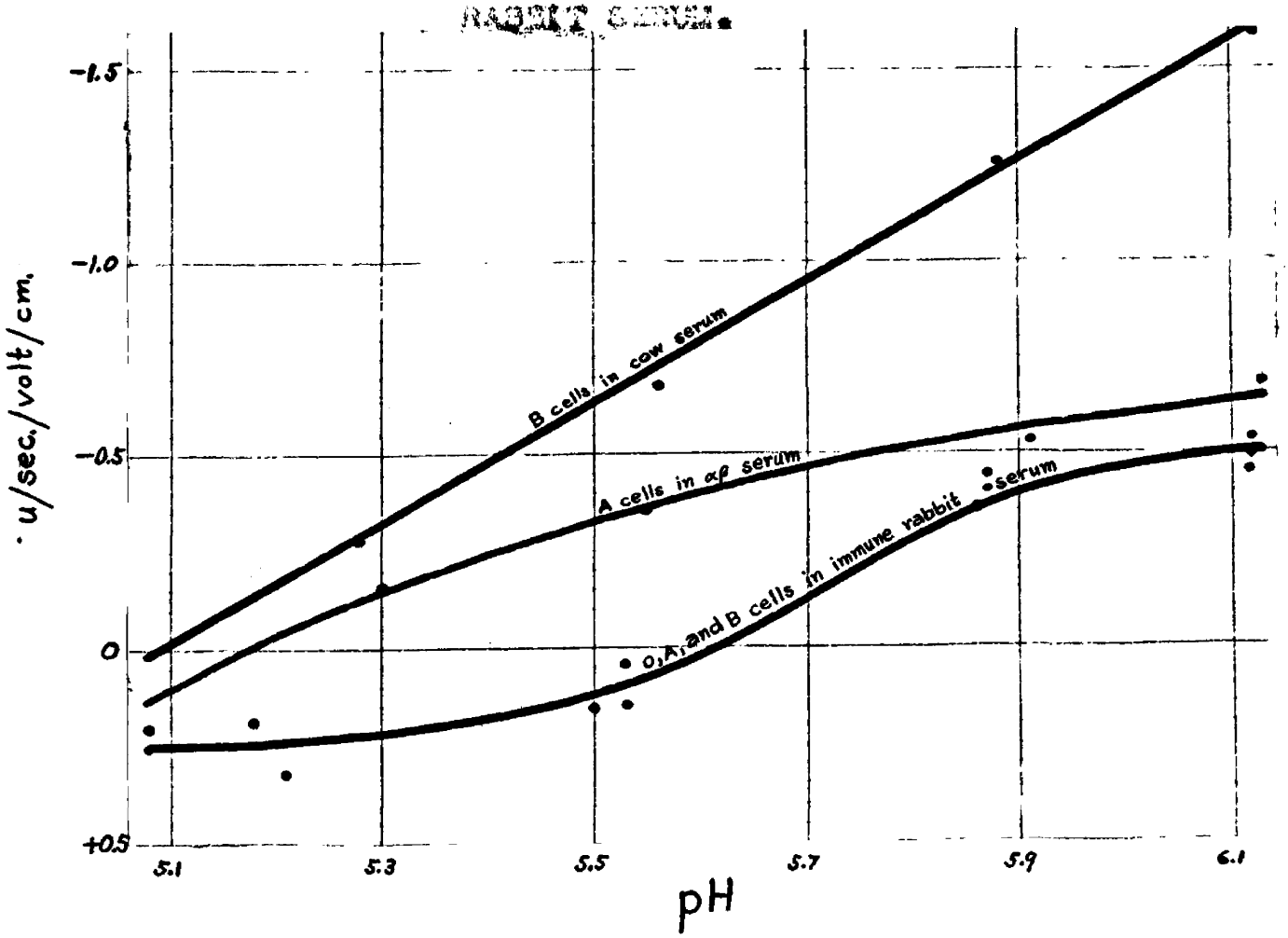
The object of the experiment was to estimate as closely as possible that hydrogen ion concentration at which the sensitized cells just ceased to move in the electric field, in other words, to determine their isoelectric points.

Graph number 4 gives the results of these experiments on the determination of isoelectric points. The curves on

the graph are labeled to indicate the combinations of cells and serum used. The abcissa gives the hydrogen ion concentration expressed in pH units; the ordinate gives the mobility in u/sec./volt/cm.. The (O) on the ordinate indicates no mobility, and the number +0.5 below the zero means a velocity of 0.3 u/sec./volt/cm. toward the cathode. Therefore it represents a positive charge. All numbers above the zero represent negative charges and are prefixed by a minus sign.

Graph 4

ISOELECTRIC POINTS OF CELLS SENSITIZED WITH ISO-HEMAGGLUTININS, HETEROHEMAGGLUTININS, AND IMMUNE RABBIT SERUM.



The curve representing the data obtained with human red blood cells maximally sensitized with immune rabbit serum is an average of the determinations on sensitized O, A and B cells. In a preliminary graph drawn to a larger scale, curves were drawn through the four points determined for each type of cell used here. The isoelectric points of O, A and B cells as determined in the preliminary graph were pH 5.61, 5.58 and 5.62 respectively. These are close checks. The curve in the graph above, representing an average for all the points located, crosses the line showing zero potential at pH 5.6.

Bacterial antigens maximally sensitized with immune rabbit serum have been made isoelectric at pH 5.6 to 5.8 according to McCutcheon, Rudd, Strumia and Lucke[15], and the globulin salted out of the same antiserum was isoelectric at pH 5.1 to 5.2. Sheep cells strongly sensitized with amboceptor have been isoelectric at pH 5.3.[15] The quoted results above indicate that the coating substance on human red blood cells from immune rabbit serum is very similar to the adsorbing substance that sensitizes bacteria and sheep cells, namely globulin.

The results obtained on cells sensitized with isohemagglutinins and heterohemagglutinins were not as satisfactory as those in which immune rabbit serum was used. Several

suspensions of sensitized cells were experimented upon; but the cells in most of the suspensions were not reduced close enough to zero potential at any pH studied to warrant extrapolation of a curve to the line indicating no charge. The data on only two of these determinations is presented.

A cells in $\alpha\beta$ serum are isoelectric at about pH 5.2, and B cells in cow serum are isoelectric at about pH 5.1. These lower isoelectric points for cells sensitized with isohemagglutinins and heterohemagglutinins suggest either that these cells were not maximally sensitized or that there is a physical difference between these antibodies on the one hand and antibodies found in immune rabbit serum on the other. The data presented suggest further investigation.

There was a difference between the type of agglutination observed with cells in rabbit serum as compared to that observed in both cow and human serum. The immune rabbit serum produced a fine granular precipitate which was easily resuspended by shaking, whereas the other two types of sera produced a tenacious, gummy appearing mass of cells in the bottom of the tube which could be broken up only with great difficulty. It was nearly impossible to shake these precipitates back into an even suspension.

The results given on graph number 4 indicate that the immune bodies in rabbit serum specific for human red blood

cells is associated with the globulin fraction of the serum. Likewise, naturally occurring isohemagglutinins and heterohemagglutinins are associated with globulin; but cells sensitized with these latter types of sera are isoelectric at a slightly lower pH than are cells sensitized with the former. The types of agglutination observed differ as described above.

The results presented in this paper have been an investigation of the physical factors that characterize normal and sensitized human red blood cells and a study of the mechanism of isohemagglutination. The work by no means has been a complete and comprehensive investigation of the field. However, it gives an insight into some of the physical properties of the blood cells taking part in isohemagglutination and shows some of the characteristics of the antigen-antibody combination.

DISCUSSION AND SUMMARY

In the experimental work, the first question that arose was, whether or not human red blood cells are effected by change of salt concentration in the same way as are bacteria and other negatively charged particles. It was shown on Graph number 1 that as the salt concentration of the suspending medium is diminished the potential on the suspended cells increases. Using sodium chloride as the salt, the charge was always negative in sign with concentrations ranging from

0.15 normal down to 0.001 normal. This result agrees in a general way with those of Northrop and de Kruif[10] in their work on bacterial agglutination; that is, that diminishing salt concentration increases the potential on bacterial cells.

Similarly as salt concentration affects the charge on bacteria, hydrogen ion concentration has a marked effect, and isoelectric points can be determined for many species of bacteria. The experiment giving the effect of hydrogen ion on human red blood cells, shown on Graph number 2, demonstrates that acid hemolysis always occurs before there is any marked lowering of potential. From pH 5.9 to 8.1 the washed cells used carried a potential ranging from -37.4 to -43.3 millivolts. At pH 4.5 the charge dropped close to zero for all types of cells tested, and partial hemolysis was present in all the suspensions.

This result confirms the works of Abramson[2] and of Mudd[17], that the isoelectric points of normal red blood cells can not be determined due to acid decomposition of the cells before any marked lowering of potential is evident.

In the experiment on the effect of hydrogen ion concentration discussed above some of the cells used were partially sensitized with isohemagglutinins, sufficiently sensitized to cause agglutination of the cells when these

were suspended in isotonic saline. However these cells were indistinguishable from the normal cells on the basis of cataphoretic mobility in .01 molar phosphate buffer. This result indicates that only a small portion of the cell surface need be sensitized with the antibody to produce agglutination in saline, not enough surface to markedly change the mobility of the cells.

Change in salt concentration effects the potential on human red blood cells; but change in hydrogen ion concentration has little effect until acid decomposition begins to occur. The effect of blood serum and of isohemagglutinins used in a 1:4 dilution with 0.01 molar phosphate at pH 7.3 used as the diluent was tested. It was found that cells in serum containing no homologous isohemagglutinins carried a negative charge of 24.4 - 26.5 millivolts. When the isohemagglutinin was present, however, there was always a reduction in potential and agglutination always occurred when the potential dropped below about 15.2 millivolts.

These results agree with the work of Schroder[23], who reported that cells in serum diluted 1:4 with a weak phosphate buffer containing no homologous isohemagglutinins carried a charge of 25. - 26 millivolts, that there was always a reduction in potential when the isohemagglutinin was present and that agglutination occurred whenever the

potential dropped to between 12. and 13. millivolts. The results also compare in the same general way with the work of Mulcher[22]; but the experimental data does not check as closely as it does with Schroder's work.

It has been shown that cells in serum containing no homologous isohemagglutinins and diluted 1:4 with .01 molar phosphate buffer carry a charge of 24.4 - 26.5 millivolts. The question arose as to whether this lower potential upon cells in serum is due to the nonspecific adsorption of serum proteins or to increased salt concentration obtained from the added serum.

To answer this question collodion particles and glass particles were suspended in human blood serum diluted 1:4 under conditions that were the same as those employed in the experiment discussed above. The charges on these particles in serum was 13.5 - 14.0 millivolts for collodion and 13.0 - 13.2 millivolts for glass. The same particles had potentials of 44.1 - 45.7 and 37.0 - 38.1 millivolts for collodion and glass respectively when suspended in the dilute phosphate buffer only. The presence of serum evidently had a marked effect upon the potential of these particles.

Loeb[13] has shown that collodion particles adsorb protein in a nonspecific way; Abramson[1] concluded that whereas gelatin would coat quartz particles and cholesterol, it had

no significant effect upon blood cells; Monaghan and White[16] report that the mobility of red blood cells is unchanged by the addition of purified protein to the suspending medium.

The facts that red blood cells in serum diluted 1:4 with the .01 molar buffer carry a maximum charge of about 25.5 millivolts and that the charge on the inert particles studied is only a little over one-half this potential suggests that red blood cells do not adsorb protein in a non-specific way. Furthermore the lower charge on blood cells in the serum dilution as compared to the charge carried by cells in the weak diluent only suggests that the lower potential of cells in serum is due to the electrolytes added from the serum itself.

If serum dilution with .01 molar phosphate buffer increases the potential on suspended red blood cells and if serum proteins do not affect the charge on blood cells, then the potential on cells in very dilute serum containing no homologous isohemagglutinins will approach that of the cells in the serum diluent alone.

Such a result was shown with the data presented on graph number 3. Cells in serum containing no homologous isohemagglutinins and diluted 1:64 with the weak buffer had a potential of 34.4 millivolts as compared to a charge of 33. millivolts for cells in the buffer only. The above

result is confirmatory evidence that the increasing potential with serum dilution using the dilute buffer is due to the decrease in salt concentration of the suspending medium and also that red blood cells do not nonspecifically adsorb proteins.

When the isohemagglutinin was present, the charge on the suspended cells was always lower than it was for cells in serum at the same dilution containing no homologous immune bodies. With a given dilution the lowering of potential from one sensitized suspension to another varied markedly; this is interpreted as indicating that the completeness of the adsorbed protein film or, in other words, the degree of sensitization was by no means the same for all sensitized cell suspension. The greater the sensitization, the lower the potential.

As the isohemagglutinating serum was diluted the potentials at which agglutination could occur became higher. The critical potentials for some of the dilutions of isohemagglutinating serum used were as follows: 1:4 dilutions = 15.2 millivolts; 1:8 dilutions = 19.5 millivolts; and 1:16 dilutions = about 23 millivolts. The much lower potential of some agglutinated suspension below the critical potential shows again that a very complete protein film is not in the least necessary to cause agglutination.

The rise in critical potential along with serum dilution indicates an increase in "cohesive force".

No direct measurement of the "cohesive force" nor of any force proportional to it was found possible. However, it is known that the degree of hydration at the surface of particles has a direct bearing upon their suspension stability. According to Loeb[13] the less the hydration the more unstable are the particles. In addition to the state of hydration, specific chemical affinity has been shown definitely to play a part in some immune reaction. Heidelberger and Kendall[10] showed this to be true in the case of the pneumococcus polysaccharide S .

In regard to hydration of red blood cells it was shown in Table number 4 that the normal cells either in serum or in dilute electrolyte solution were decidedly hydrophobic. Nevertheless red blood cells form suspensions that are fairly stable. If we assume that there is no specific chemical affinity between normal red cell surfaces, this helps to account for their stability.

With sensitized red blood cells, it was shown in Table number 4 that the cell surfaces became relatively hydrophylic. This result agrees with the work of Mudd[17]. At the same time agglutination occurred, and this reaction was due in part to the greatly lowered charge on these particles. In

addition we can speculate that it could have been due to specific chemical affinity between partially sensitized cells where antigen receptors on a portion of normal cell surface could combine with chemically specific antibody radicals of molecules which were already attached by similar radicals to another cell.

In this connection Loeb[13] gave evidence that protein adsorbed to inert particles became more hydrophobic than the normal protein. Schroder[24] showed that the surface tension of a lipoidal extract of red blood cells containing isohemagglutinogens sensitized with the homologous agglutinin gave a surface tension higher than that for the euglobulin fraction of the serum containing the isohemagglutinins. With increased surface tension or decreased hydration, which may be the same thing, the suspension is rendered less stable. The surface molecules have a greater attraction for each other than for water.

On Table number 4 it was shown that with serum dilution, sensitized cells became more hydrophobic. In the results reported on four of the different samples that showed agglutination the sensitized cells were more easily wet by oil than by water.

Two of these samples were sensitized with immune rabbit serum in a dilution of 1:100 and evidence was presented which indicated that they carried an extensive protein film.

Human red blood cells 1 rabbit serum diluted 1:4 were hydrophylic, however; but still showed strong agglutination.

It was shown that with serum dilution using .01 molar phosphate as the diluent the electrolyte concentration of the solution diminished. Decreased electrolyte concentration under the conditions employed here brought about dehydration of the particle surface.[17]

Summing up the evidence given in Table number 4 and in some of the previous experiments it is indicated that isohemagglutinins adsorbed to red blood cells become more hydrophobic than the naturally occurring agglutinins; that with sensitization there may be a specific chemical affinity between red cell surfaces which was absent before sensitization; and that with decreased electrolyte concentration of the suspending medium, the adsorbed protein film becomes more hydrophobic. It is suggested that this decreased hydration of the protein film, which is accompanied by an increase in the charge on the particle, is one of the major factors which renders the suspension sufficiently more unstable to raise the critical potential.

The more marked effect of immune rabbit serum in lowering the potential on blood cells as compared to isohemagglutinins suggested the possibility of demonstrating some difference between the two types of agglutination. Heterohemagglutination was included also using cow serum for the

agglutinating agent.

Isoelectric points were determined for cells maximally sensitized with isohemagglutinins, heterohemagglutinins, and immune rabbit serum.

The isoelectric point of cells maximally sensitized with immune rabbit serum was pH 5.6. This value agrees well with the isoelectric points of bacteria maximally sensitized with rabbit serum.[13]

The results on cells sensitized with cow serum indicated that these were isoelectric at about pH 5.1. A cells sensitized with $\alpha\beta$ serum were isoelectric at about pH 5.2. The charge on these cells was reduced very close to zero so that exterpolation of a curve to zero potential through the points located for these sensitized cells seemed possible without coming to erroneous conclusions. These isoelectric points of about pH 5.1 and 5.2 for cells sensitized with heterohemagglutinins and isohemagglutinins respectively are suggestive of a difference between isohemagglutination and true antigen antibody reaction.

Cells sensitized wi h immune rabbit serum produced a fine granular easily suspendable precipitate, whereas cells sensitized with heterohemagglutinins and isohemagglutinins gave a coarse, gummy precipitate that was very difficult to resuspend.

In all of the experiments presented no definite physical

differences were demonstrated between the blood groups.

From the experimental work and from the literature certain facts and certain probabilities can be summarized which characterize isohemagglutination.

It has been shown that normal human red blood cells carry a negative charge, have hydrophobic surfaces, and do not adsorb protein in a nonspecific way. Increasing salt concentration lowers their charge, and acidity can cause lysis. In addition there probably isn't any specific chemical affinity between unsensitized cells.

In order to bring about isohemagglutination, three conditions must be fulfilled: (1) Collisions between cells must take place. This requirement is automatically made possible because all particles having a size on the order of blood cells and bacteria are in a constant state of brownian motion when in suspension. (2) The potential on the cells must be made sufficiently low so that the momentum of cells toward each other will not be overcome by the like charges that they carry. (3) The cells must have a sufficiently high cohesiveness so that they will remain together once they come in contact.

The lower potential is obtained in two ways: (1) The more salt in the suspending medium up to isotonicity as shown in the experiments above, the less the potential on the

cells. (2) The sensitization of the cell surface with isohemagglutinating serum has been shown to lower the potential further. When cells are coated with a protein film their surfaces approach more and more closely the nature of the adsorbed substance.

As for the "cohesive force" there is evidence that protein adsorbed to particle surfaces becomes less hydrophylic.[13,24] That is, the protein molecules acquire less affinity for water and more for each other. This result promotes clumping.

If the isohemagglutinating serum diluent is a very dilute electrolyte solution then a rise in this cohesiveness with serum dilution can be expected. This is due to a diminishing electrolyte concentration which in turn has a dehydrating effect upon the adsorbed protein film. At the same time cells under these conditions carry a greater potential.

It is suggested that a definite chemical combination between sensitized cells can take place also and thus be another factor determining the strength of the cohesiveness.

CONCLUSIONS

1. Decreasing salt concentration from 0.15 normal down to .001 normal increases the negative charge upon human red blood cells.

2. Increasing hydrogen ion concentration does not greatly affect the potential on normal red blood cells until acid decomposition starts to take place. Decomposition begins to occur somewhere between pH 5.1 and 4.5.

3. Red blood cells in serum diluted 1:4 with .01 molar phosphate at pH 7.0 carry a charge of about 25.5 millivolts when the homologous isohemagglutinin is not present. When the homologous isohemagglutinin is present the potential is always lower and agglutination always occurs when the potential drops to about 15.2 millivolts.

4. With serum dilution using .01 molar phosphate buffer as the diluent the charge on the suspended cells approaches the same value as that on cells in the buffer alone. It was shown that the potential on collodion and glass particles was much more markedly reduced by the presence of serum than was the charge on red blood cells. This evidence indicates that red blood cells do not nonspecifically adsorb protein.

5. Normal red blood cells are hydrophobic, more easily wet by oil than by water. Sensitization, however, renders them more hydrophylic.

6. Under the conditions of this experimental work there is a rise in the critical potential of sensitized cells with

serum dilution. It is indicated that this rise in critical potential is due at least in part to dehydration of the adsorbed protein film. The dehydration is caused by a decrease in electrolyte concentration incident to serum dilution with .01 molar phosphate buffer.

7. The isoelectric point of human red blood cells maximally sensitized with immune rabbit serum is pH 5.6. It was indicated that A cells sensitized with $\alpha\beta$ serum are isoelectric at about pH 5.2 and that B cells sensitized with heterohemagglutinins are isoelectric at about pH 5.1. However, this apparent difference between the types of agglutination might have been due to incomplete protein films in the cases of iso and heterohemagglutination.

8. Human red blood cells maximally sensitized with immune rabbit serum form a fine, granular precipitate which is easily disrupted. Cells sensitized with heterohemagglutinins and isohemagglutinins form coarse gummy precipitates which are very difficult to disrupt. These results show a difference between isohemagglutination and heterohemagglutination on the one hand and true antigen-antibody reaction in which immune rabbit serum is used, on the other.

9. No physical differences were shown between the different blood groups studied.

BIBLIOGRAPHY

1.Abramson, Harold A. "The Cataphoretic Velocity of Mammalian Red Blood Cells", Journal of Gen. Physiol., (1929), 12, p. 711.

2.Abramson, Harold A. "The Isoelectric Point of Normal and Sensitized Mammalian Erythrocytes", Journal of Gen. Physiol. (1930), 14, p. 163.

3.Abramson, Harold A. "The Influence of Size, Shape and Conductivity on Cataphoretic Mobility, and Its Biological Significance", Journal of Physical Chemistry, (1931), 35, part 1, p. 838.

4.Abramson, Harold A. and Moyer, Laurence S. "The Electrical Charges on Mammalian Red Blood Cells", Journal of Gen. Physiol. (1936), 19, p. 601.

5.Beehhold, H. "Die Ausflockung von Suspensionen bzw. Kolloiden und die Bakterienagglutination", Z. Physik Chem. (1904), 48, p. 385.

6.Bordet, Jules Ann. Inst. Pasteur, 1899, 13, p. 225. Cited by Rudd, et. al.; J. Phys. Chem., 1932, 36, part 1, p.229.

7.Bruynoghe, C. "La Nature Des Agglutinogenes", Comples Rendus, Soc. de Biol., (1930), 122, 16, p. 94.

8.Buchanan, R. E. "Agglutination", J. of Bact., 1919, 4, p.73.

9.Eagles, Harry "Specific Agglutination And Precipitation", Jour. Immunol., (1930), 18, p. 393.

10.Heidelberger, M. and Kendall, F. E. "Some Physicochemical Properties of Specific Polysaccharides", J. Biol. Chem. (1932), 95, p. 127.

11.Hertzfeld and Klinger "Biochem Z., (1917), 83, p. 223. Cited by Rudd, et. al.; J. Phys. Chem.(1933), 36, part 1, p. 229.

12.Joffe, Eleanore M. "A Physical-Chemical Difference in Antibodies Against the S and R Variants of a Single Bacterial Strain", Jour. of Gen. Physiol. (1935), 18, 5.

13. Loeb, Jaques "Stability of Suspensions of Solid Particles of Protein and Protective Action of Colloids", Jour. of Gen. Physiol., (1923), 5, p. 479.

14. Mattson, Sante "Cataphoresis and the Electrical Naturalization of Colloidal Material", J. Phys. Chem., (1928), 32, p. 1532.

15. McCutcheon, M.; Mudd, S.; Strumia, M.; and Lucke, B. "On the Mechanism of Opsonin and Bacteriotropin Action", IV The Isoelectric Points of Certain Sensitized Antigens. Jour. of Gen. Physiol., (1930), 13, p. 669.

16. Monaghan, B. R. and White, H. L. "Effect of Proteins on Electrophoretic Mobility and Sedimentation Velocity of Red Cells", Jour. of Gen. Physiol. (1936), 19, p. 715.

17. Mudd, Stuart; Nugent, R. L.; and Bullock, L. T., "The Physical Chemistry of Bacterial Agglutination and Its Relation to Colloid Theory", J. Phys. Chem. (1932), 36, part 1, p. 229.

18. Northrop, John H. and de Kruif, Paul "The Stability of Bacterial Suspensions", Jour. of Gen. Physiol., (1922), 4, p. 639.

19. Northrop, John H. and Kunitz, M. "An Improved Type of Microscopic Electrocataphoresis Cell" Jour. of Gen. Physiol., (1923), 7, p. 729.

20. Nugent, Robert Logan "The Application of the Mudd/Interfacial Technique In The Study of Protective Protein Films in Oil-in-Water Emulsions", J. Phys. Chem., (1932), 36, part 1, p. 449.

21. Olitzki, Leo "Electrical Charges of Bacteria Sensitized With Purified Agglutinins", Jour. Immunol., (1934), 27, p. 105.

22. Pulcher, Caludia "Untersuchunger uber Isoagglutination und Elektrokinetisches Potential der Erythrocyten Mittels Einer Neuren Kataphoresekammer", Pfluger's Archiv Fur Physiologie, (1933), 232, p. 248.

23. Schroder, Vera "On Some Physical-Chemical Advances on Isohemagglutination", Pfluger's Archiv Fur Physiologie, 1926, 215, p. 32.

24. Schroder, Vera "The Physical-Chemical Properties of Isohemagglutinogens", Zeitschrift Fur Immunitats-forschung Und Experimentelle Therapie, (1932), 75, p. 77.

25. Tullock, Biochem. Journal, (1914), 8, p. 223. Cited by Mudd, et. al.; J. Phys. Chem. (1933), part 1, p. 229.