THE INFLUENCE OF ELECTROLYTES ON THE STABILITY OF RED BLOOD CORPUSCLE SUSPENSIONS.*

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The experiments which are described in this paper are concerned with the electric charges of red blood corpuscles in fluid suspensions. They are based on the work of those investigators who have determined the physical properties of suspensions and colloidal solutions and are an attempt to apply these general principles to the special case in hand.

It was Jevons (1) who first suggested electrical repulsions as a source of suspension stability and Hardy (2) who pointed out the relation of the isoelectric point to flocculation, while the quantitative measurements of Powis (3) and Ellis (4) demonstrated that a critical potential exists for suspended oil droplets below which a suspension of them is no longer stable.

An application of these principles to biological phenomena has been recently made by Northrop and De Kruif (5), who have shown that a similar critical potential exists, under certain conditions at least, for suspensions of bacteria. In the case of suspensions of red blood cells, however, the application of these basic principles has been less successful. The possibility of such a physical explanation for the suspension stability of the blood has aroused the attention of clinicians recently and Fåhraeus (6), to whom the revival of interest in this subject is due, at first suggested that the stability of the different bloods depended on differences in the electric charge of the cells. In his later studies he was unable, however, definitely to establish such a relationship. The agglutination of red cells by chemicals has also been assumed to be due to differences in electric charge, but here again Rona and György (7) were unable to demonstrate such a correlation in the case of ricin agglutination.

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In our previous studies of the agglutination of red cells by arsphenamine (8), it was suggested that the mechanism of the action might be due to variations in the electric charges of the cells. From this specific problem we have proceeded to that of the mechanism of agglutination by chemical agents in general. In the present paper we will demonstrate the variations that different salts produce in the charges of cells in suspension and discuss the correlation of these differences with the stability of the suspension.

Method.

The Cell Suspension.—The primary requisite for a study of the properties of a suspension of red cells is that they be suspended in a fluid which is isotonic with them, and which at the same time does not contain substances affecting the electric properties of the cells. Electrolytes and proteins must therefore be excluded. The nearest approach to this ideal condition is obtained by a repeated washing of the cells in isotonic sugar solution and a suspension of them in it.

Such a suspension is unstable. In the course of a few minutes the clumps resulting from the aggregation of the individual cells may be seen with the naked eye and these clumps rapidly settle to the bottom of the container. A preliminary washing of the freshly drawn blood with isotonic NaCl solution makes the occurrence of this phenomenon more certain, provided the salt is thoroughly removed by subsequent washings with sugar solution, since the presence of a small amount of this electrolyte will stabilize the suspension. The instability of the final suspension, therefore, becomes a valuable criterion of its suitability in the experiments to be described. If this is lacking, it is probable that the stability is due to the presence of some electrolyte and this renders a proper estimation of the effect of the experimental procedure impossible.

In our experiments rabbit blood was used. It was defibrinated and washed three times with 0.9 per cent NaCl solution, five times with 10 per cent sucrose solution, and made up to a 5 per cent suspension in this fluid. If the suspension remained stable and did not agglutinate in a few minutes, it was rewashed in the sugar solution or discarded.

The Measurement of Instability.

The quantitative expression of the degree of agglutination, that is, of the instability of the suspension, was the most difficult part of our experiments. After trials of various methods it was decided that reliable comparative values would be obtained if the degree of agglutination was expressed by three arbitrary symbols, +, ±, and 0. The symbol + means that agglutination was complete, the suspension being entirely unstable and the cells settling completely so as to leave a clear supernatant fluid. By ± is meant a degree of instability such that agglutination had occurred with definite settling, but in which the supernatant fluid still contained cells in suspension. When no gross agglutination and no settling greater than that observed in the most stable controls occurred, the value 0 was used. Any attempt at further subdivision was found to lead to uncertain results.

In our experiments the settling of the suspension is taken as a measure of its stability. Settling of the cells was observed, however, in the most stable cell suspensions, so that there is no qualitative distinction between the "normal" and the more rapid settling due to agglutination. The measurement of the agglutination, therefore, depends on the measurement of the velocity of the reaction, and if this is to be estimated by a single determination at one point in the process (for the purposes of comparison with the same process under different conditions) a point must be selected which will properly represent the differences in the rates.[1] If the observations are made too early or too late in the course of the reactions, significant differences will be missed. After considerable experimentation we decided that the degree of instability should be determined at a period of from ½ to 1 hour after the mixtures had been prepared. After this time settling has occurred to some extent in all tubes and the instability due to the experimental procedure is masked by the "normal" process of settling. Unless otherwise stated the tubes were placed in a water bath at 37°C. In certain experiments the tubes were kept at room temperature and observed at earlier intervals.

[1] *Cf.* Freundlich (14), p. 570.

The Electric Charge.

The degree of the electric charge of the cells was determined by cataphoresis. In our experiments the microscopic method was used as described by Ellis (4), Powis (3), and Michaelis (9). A cell 2.0 × 1.0 × 75.0 mm. was placed on the stage of a microscope in the ocular of which a micrometer had been inserted. Each extremity of the trough was connected by an agar siphon to a $Zn\text{-}Zn_2SO_4$ electrode. The cell was filled with the red cell suspension, covered with a glass slide, and a direct current of 115 volts applied. Measurements of the speed of the particles at the proper depths were made and averaged in the usual manner and the results expressed as millivolts by means of the usual formula.

The Experiments.

The procedure in all the experiments was the same. A 5.0 per cent suspension of red cells in isotonic sucrose was mixed with the same amount of solution containing decreasing concentrations of the various electrolytes to be studied. The series of tubes was allowed to stand at 37°C. for 1 hour. The cells were now resuspended in the proper sugar-electrolyte mixture and the degree of charge determined by cataphoresis.

Some preliminary experiments were performed in the same manner with collodion particles. These were a repetition of Loeb's experiments (10). The results are reported in order that a comparison of the properties of these particles may be made with those of a red cell suspension under the same conditions. The presence of the sugar in the suspension fluid made no apparent difference in either the stability of the suspension or in the electric charge of its particles. The pH value of the original suspension of particles before the addition of electrolyte was 5.0.

The Effect of Electrolytes on the Charge of Collodion Particles and Its Relation to the Stability of the Suspension.

The results of the experiments with collodion particles are shown in Fig. 1. It is seen that all substances depress the charge of the particles and that one of them, ferric chloride, reverses the charge. When

the P.D. is greater than a certain value, 32 mv., the suspension is stable; when lower than this critical value, aggregation of the particles

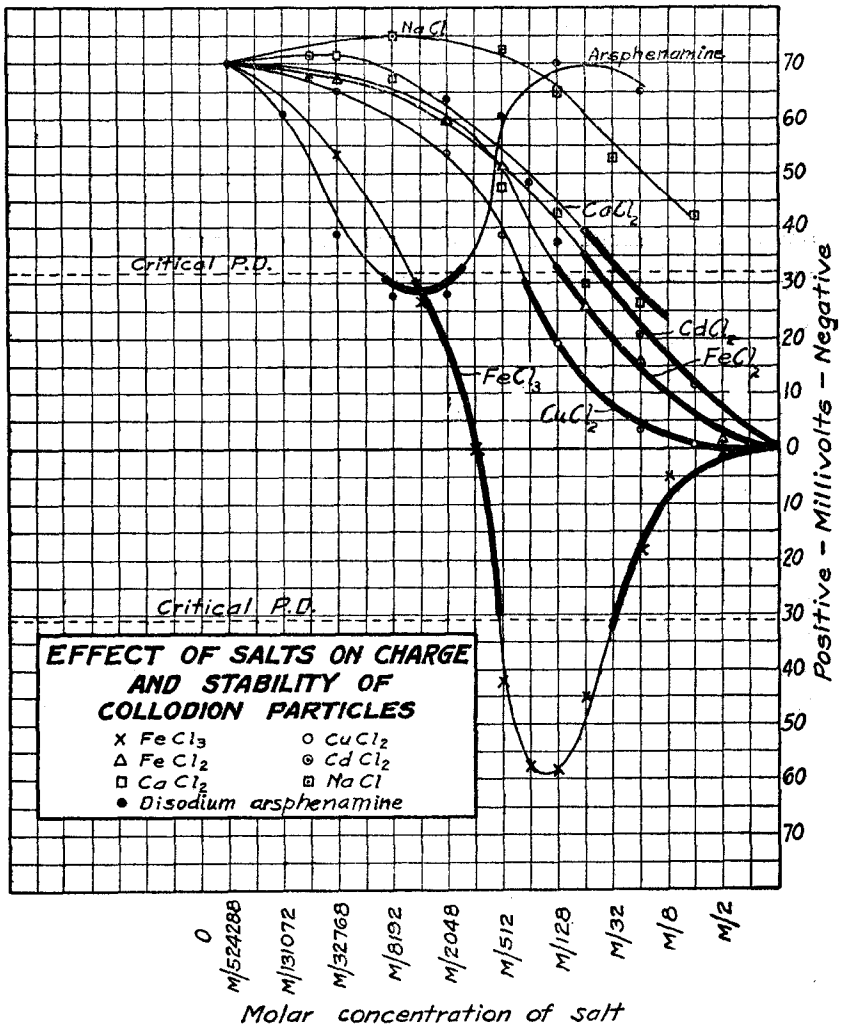

FIG. 1.

occurs and instability results. Conditions of both stability and instability alternate in the series of ferric chloride and arsphenamine. With ferric chloride this "irregular series" is produced by a secondary

rise in the reversed positive charge to a value above that of the critical value, and the following zone of instability is the result of a drop in the positive charge in the higher concentrations of the electrolyte below this value. In the case of alkaline arsphenamine the short zone of instability is the result of a depression of the original negative charge below the critical value, but the stability which follows in the higher concentrations is due to a sudden increase in this negative charge which follows this temporary depression.

These results are similar to those of Loeb, with the addition of the findings with arsphenamine, and will now be compared with the conditions that obtain with a suspension of red cells.

The Effect of Trivalent Ions on the Charge of Red Cells in Suspension, and the Relation of the Charge to the Stability of the Suspension.

For purposes of demonstration the effect of the trivalent ions will be considered first, as with them the variations in electric charge of the cells and in the stability of the suspension may be observed to their fullest extent. With the bivalent and monovalent ions the variations are similar though less completely developed.

As examples of trivalent cations Fe''' and Al''' were chosen, the anion being Cl, as is the case in all the experiments. The stability of the suspension and the charge of the cells were determined as in the previous experiments. The results are presented for Al_2Cl_6 in Fig. 2 and for $FeCl_3$ in Fig. 3. The graphic expression of the result of all the experiments is the same. At the bottom of the chart is shown the condition of stability of the suspension, expressed in terms of three values: + denoting complete instability, ± definite instability or agglutination, and 0 complete stability. These are represented as ordinates on the abscissæ of the concentration of the electrolyte. Above is shown the degree of electric charge of the cells, the negative charge lying above the heavy line and the positive below it.

Both Al_2Cl_6 and $FeCl_3$ cause considerable hemolysis of the cells in the high concentrations and this obscures the determinations of both stability and potential. A wider range of concentration is observed with Al_2Cl_6 but the type of variation is the same with both salts. There is a depression of the original negative charge which reaches 0 at

about M/32,000, followed by an increasing positive charge, which is in turn again depressed at a concentration of M/512. This secondary depression of the positive charge is less well shown by $FeCl_3$ because

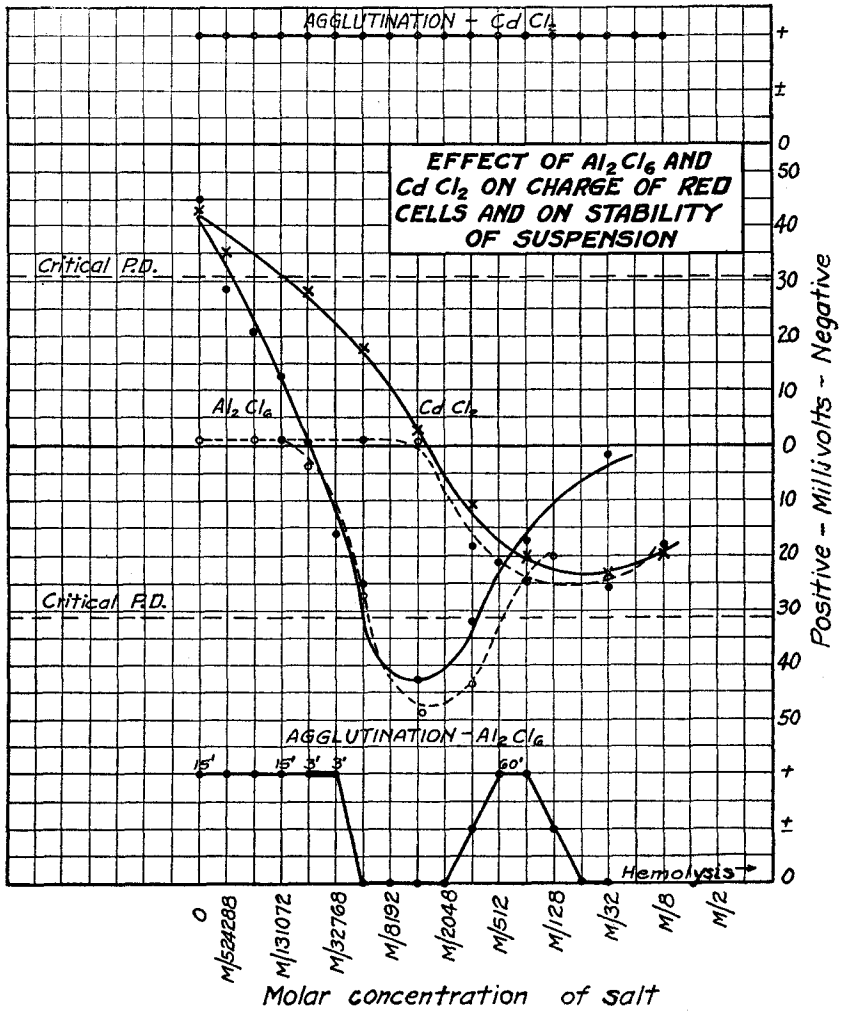

FIG. 2.

hemolysis obscures the readings in those tubes in which the changes would be most marked, although the beginning of the depression is readily seen.

The stability of the suspensions in the two salt solutions is also similar. In both, at a certain concentration of electrolyte the un-

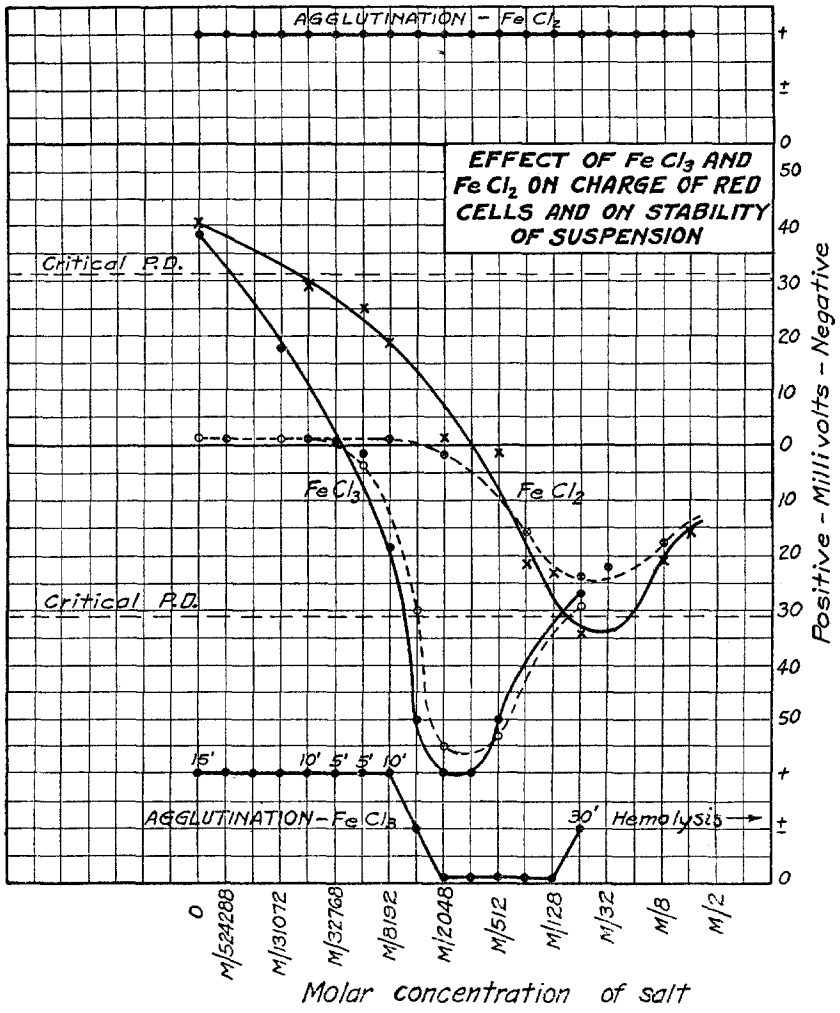

FIG. 3.

stable sucrose suspension becomes stable, and this is followed, in the higher concentrations, by a zone of instability. In the highest concentrations of the electrolytes, in which the cells are coagulated by

the action of the metallic salts and hemolysis is prevented, determinations are again possible, and stability occurs.

A comparison of the two phenomena, electric charge and stability of the suspension, gives a rather surprising result. In that half of the series in which the tubes contain the stronger concentrations of electrolyte, it is seen that the stability of the suspension might be explained by the electric charge of the cells. The first zone of stability that is noted as the concentration of the electrolyte increases coincides with the high positive charge, and the following zone of instability accompanies the depression of this positive charge. But this correlation apparently is not found in that half of the series whose tubes contain the weaker concentrations of electrolyte and in which the cells have a negative charge. Here all tubes, including the sucrose control which contains no electrolyte, agglutinate completely, although some of them, and notably the electrolyte-free control, apparently possess a strong negative charge. In some of the tubes this negative charge is equal in degree to the positive charge which may be assumed to be the cause of the stability of the suspension in the higher concentrations of the series.

This apparent paradox is explained when the negative charge in the weak concentrations of electrolyte is studied more carefully. The negative charge in the sucrose suspension, as well as in those tubes which contain only low concentrations of electrolyte, is not permanent. It gradually falls as the suspension stands, reaching practically the null point; and it is during this fall in potential that the aggregation of the cells and instability of the suspension occur. The changes are demonstrated in the following experiment.

The cells from the last washing in sucrose solution were suspended in this fluid, agitated, and their charge determined immediately. They were found to have a negative charge of 40 mv. The suspension was now allowed to stand for 5 minutes, the cells were removed by centrifugalization and resuspended in the supernatant fluid, and the charge determined. It was found to have fallen to 19 mv. After 10 minutes agglutination was observed in the original suspension and the determination was repeated and the charge found to be almost entirely absent, as more than 100 seconds were required for the cells to travel the required distance. These same cells were now sus-

pended in fresh isotonic sucrose solution and the charge was found to have risen to the original figure of 41 mv.

The same fall in charge occurred in the weaker concentrations of electrolytes, as shown by the following experiment. A series of tubes with increasing concentrations of Al_2Cl_6 and $FeCl_3$ was prepared as in the experiments just described and allowed to stand for 1 hour. The tubes were then centrifuged and the clear supernatant fluid removed. The cells were now resuspended in the supernatant fluid and also in fresh mixtures of similar electrolyte concentration and the charges determined immediately in both. The results are shown in Figs. 2 and 3. The charge in the old supernatant fluid is represented by the white circles and the dotted lines. The immediate charge of the same cells suspended in the same electrolyte-sucrose concentration is that previously recorded.

It will be noted in the weak concentrations of electrolyte up to the point at which reversal occurs, that the negative charge has fallen to 0, while there has been no change in the positive charges in the stronger concentrations. A comparison of these late curves of P.D. of the cells with the condition of stability of the suspension shows an essential agreement, except in the highest concentrations of electrolyte. Wherever there is a P.D. greater than 32 mv. stability of the suspension occurs, while with a lesser potential, aggregation and instability result. The critical potential observed by Powis and by Ellis for suspensions of oil droplets, by Loeb for collodion particles, and by Northrop and De Kruif for bacteria, holds for suspensions of red cells, except in high concentrations of electrolyte. There is this difference with a red cell suspension, however, that the initial charge in the weak concentrations of electrolyte is not permanent and that it is the final P.D. of the cells that is correlated with the stability or instability of the suspension.

The Effects of Hydrolyzing Salts with Bivalent Ions.

Two types of salts with bivalent ions were studied, those which in solution become acid in the stronger concentrations and those which remain neutral. Since the effect of the P.D. of the cells and the resulting changes in the stability of the suspensions are quite different with the two types of solutions they will be described separately.

The hydrolyzing salts with bivalent ions may be arranged in a series in which the degree of acidity decreases. In describing their

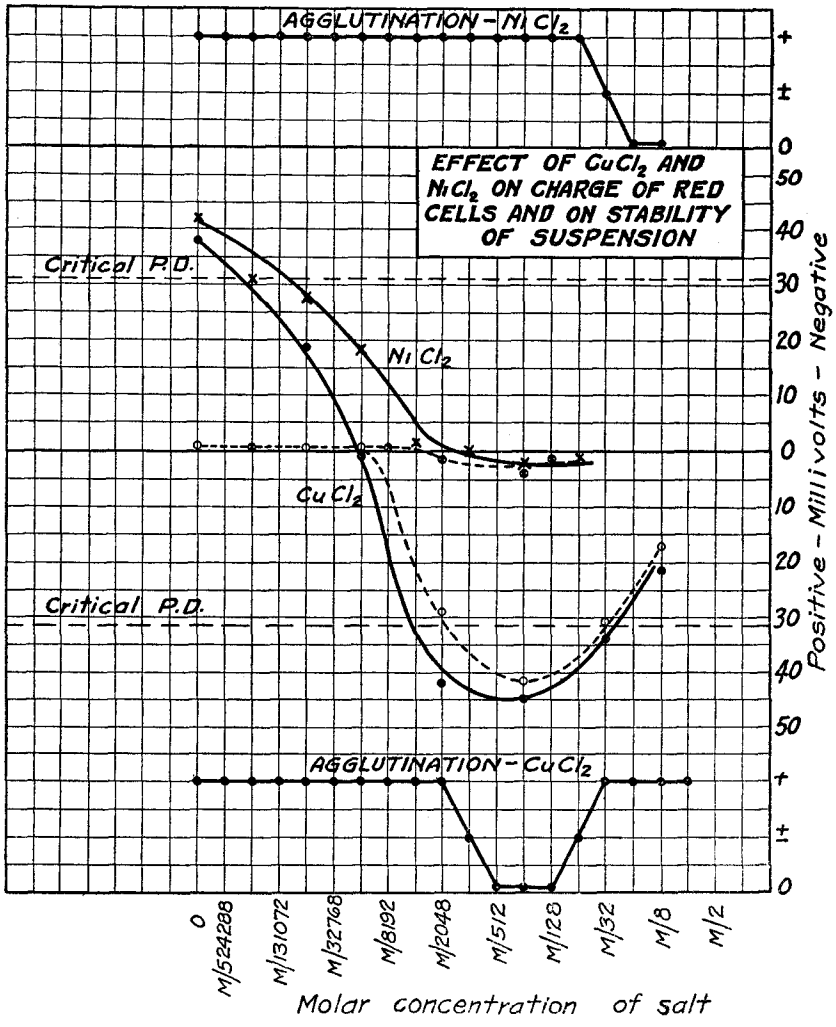

FIG. 4.

action we have considered them in this order, for in that way there is a gradual transition to the salts with bivalent ions which do not hydrolyze. The salts that were studied show the following order of

decreasing acidity in solution: copper chloride, ferrous chloride, cadmium chloride, nickel chloride. The experimental procedure was the same as previously described and in Figs. 2, 3, and 4, the results are presented in the same way as before.

With the strongly acid bivalent copper chloride the curve of P.D. resembles almost exactly that obtained with the trivalent ions, Al''' and Fe''' (Fig. 4). Reversal occurs at about M/16,000, and is followed by the development of a strong positive charge which falls toward a second isoelectric point in the highest concentrations. The initial negative charge in the weaker concentrations is not permanent, but in the course of a few minutes falls to 0. With these changes in the charge of the cells there occurs the same variation in stability that was noted with the trivalent ions. Where the charge is permanently above 32 mv. stability occurs, and this condition is found in the narrow zone of M/128 to M/512 in which the positive charge is at a maximum.

Ferrous chloride, which is less strongly hydrolyzed than cupric chloride, shows the first departure from the type of correlated P.D. variation and stability that has been observed thus far (Fig. 3). Reversal of the original charge does not occur until a concentration of M/1,000 is reached. The positive charge does not reach the height previously seen and falls again in the highest concentrations. In other words, though the type of the curve of P.D. variation with concentration is the same as that observed with the trivalent ions and strongly hydrolyzed salts, the extent of the changes is less and stronger concentrations of electrolyte are needed to bring them about.

The result of these quantitative differences is a striking change in the variations of stability of the suspension. All of the suspensions of the series are instable, and this is seen to agree with the fact that the positive charge, which in former cases was great enough to cause stability, in the case of ferrous ion did not produce at any concentration a positive charge which surpasses the critical P.D. of 32 mv.

Cadmium chloride (Fig. 2), which hydrolyzes less than ferrous chloride, shows an even greater departure from the fully developed variation in charge. Reversal occurs at M/1,000 and the positive charge is not great. The negative charge in the weak concentrations

is not permanent, so that in no suspension is the final charge greater than 32 mv. and as a result none of the suspensions are stable and all agglutinate.

With nickel chloride (Fig. 4), in which the acidity increases only slightly, the variations are the least marked. The negative charge is reversed at M/1,000 but only a very slight positive charge is acquired in the higher concentrations. The charge, therefore, is insufficient to cause stability in any of the suspensions, yet in the two highest concentrations, M/8 and M/16, stability occurs. It will be seen in the next section that this is the type of stability which is found with non-hydrolyzing salts of bivalent and monovalent ions.

The Effects of Non-Hydrolyzed Salts with Bivalent Ions.

Two examples of this type of salts were studied, $BaCl_2$ and $CaCl_2$. The variations in stability and in the charge of the cells were identical, so the effects of the two salts may be described together.

With both these electrolytes (Fig. 5) there is a depression of the negative charge. The charge becomes 0 at a concentration of M/64. No reversal of charge occurs in the higher concentrations. The original negative charge falls as usual, so that in the end none of the suspensions have any significant charge.

The stability of the suspensions, which is seen in those mixtures whose electrolyte concentration is greater than M/1,000, must, therefore, be due to some other factor than the charge of the cells, as this is depressed and even abolished throughout the stable zone.

The Effect of Monovalent Ions.

NaCl was studied as an example of this class of electrolytes (Fig. 5). Its action on the P.D. of the cells and on the stability of their suspension is of the same type as described for the non-hydrolyzing bivalent salts, except that greater concentrations are needed to produce the effects noted. The original negative charge of the cells is gradually depressed, reaching the isoelectric point at about M/8. The negative charge in the weaker suspensions falls, so that ultimately the cells in all the tubes are without charge. The stability is practically identical with that observed with Ba and Ca, being present in all suspensions in

which the concentration is greater than M/512, and it therefore bears no relation to the charge of the cells.

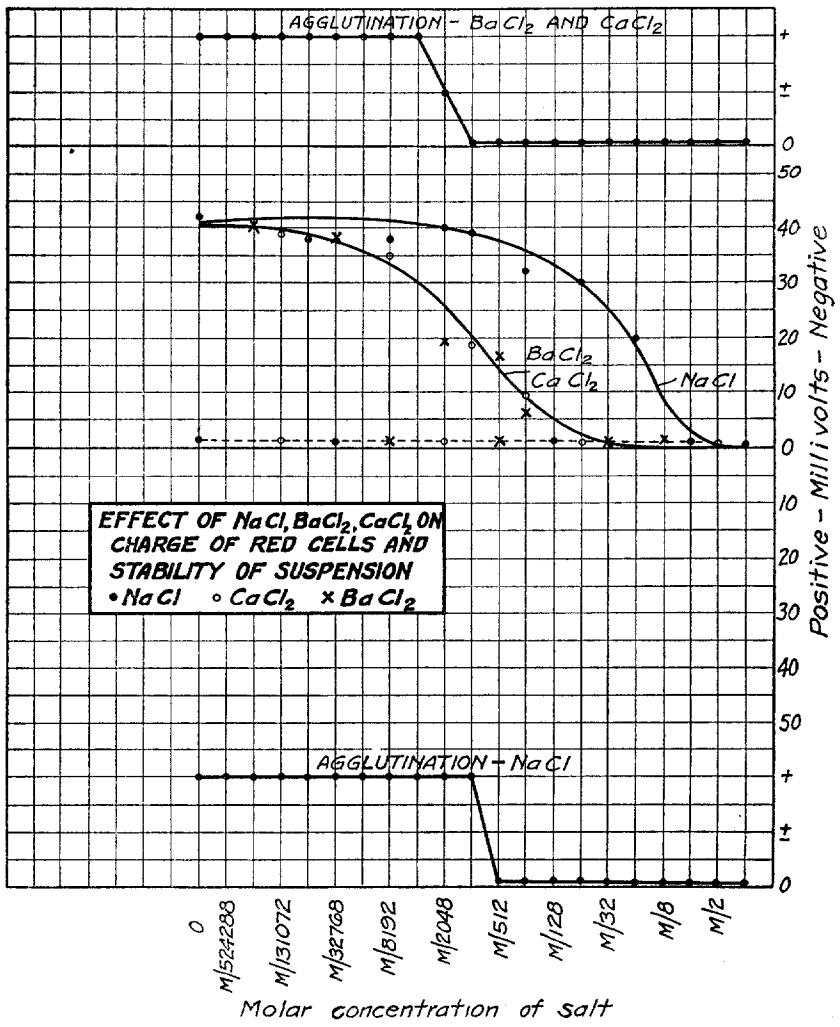

FIG. 5.

The Effect of Disodium Arsphenamine.

As stated in the introduction, our interest in the action of ions on the stability of red cell suspensions arose from a previous study (8)

of the agglutinating action of arsphenamine. We have therefore studied the action of this agent on the charge of the cells, though it differs considerably from the highly ionized salts whose effects have been described. This is particularly true of its chemical reaction, for its solutions are strongly alkaline.

The experimental procedure was the same as with the salt solutions. Fig. 6 shows the results that were obtained.

The immediately determined P.D. shows a gradual depression of the original negative charge which reaches its lowest point at about M/16,000. In contrast to the effects of all the salts previously studied the fall in potential does not continue to 0, but suddenly turns at M/16,000 and rapidly climbs to the highest value thus far observed. The late determination of charges shows that the negative charges developed in the weaker concentrations of electrolyte have all fallen to 0 as in the former experiments, but that the strong negative charge in the higher concentrations has persisted.

In its distribution with respect to concentration of disodium arsphenamine the zone of stability resembles that seen with the non-hydrolyzing bivalent and the monovalent salts. The original instability of the suspension persists until a concentration of about M/1,000 is reached, and from then on the suspension is stable. However, in spite of this similarity in the distribution of the stability the mechanism of its production is entirely different. Stability in this instance resembles that seen with trivalent ions and hydrolyzed salts, in that it depends on the charge of the cells. The stable zone coincides with the high negative charge produced by the alkaline arsphenamine, instead of with a strong positive charge as was the case with these salts. The instability in the weaker concentrations is due to the fall of P.D. to 0, a phenomenon exhibited by all suspensions irrespective of the electrolyte present. The stabilizing effect of arsphenamine in this experiment is very evident, but the question may well be raised if it can be considered the direct cause of the instability noted in any of the tubes, or in other words if it "agglutinates" the red cells *per se*. A careful examination of the tubes in which the suspensions are instable shows that in certain of them the instability is the direct result of the arsphenamine and not the result of the general loss of negative charge that is observed in weak concentrations of all electrolytes.

This was definitely demonstrated when the tubes were allowed to stand at room temperature instead of at 37°C. in the water bath, so

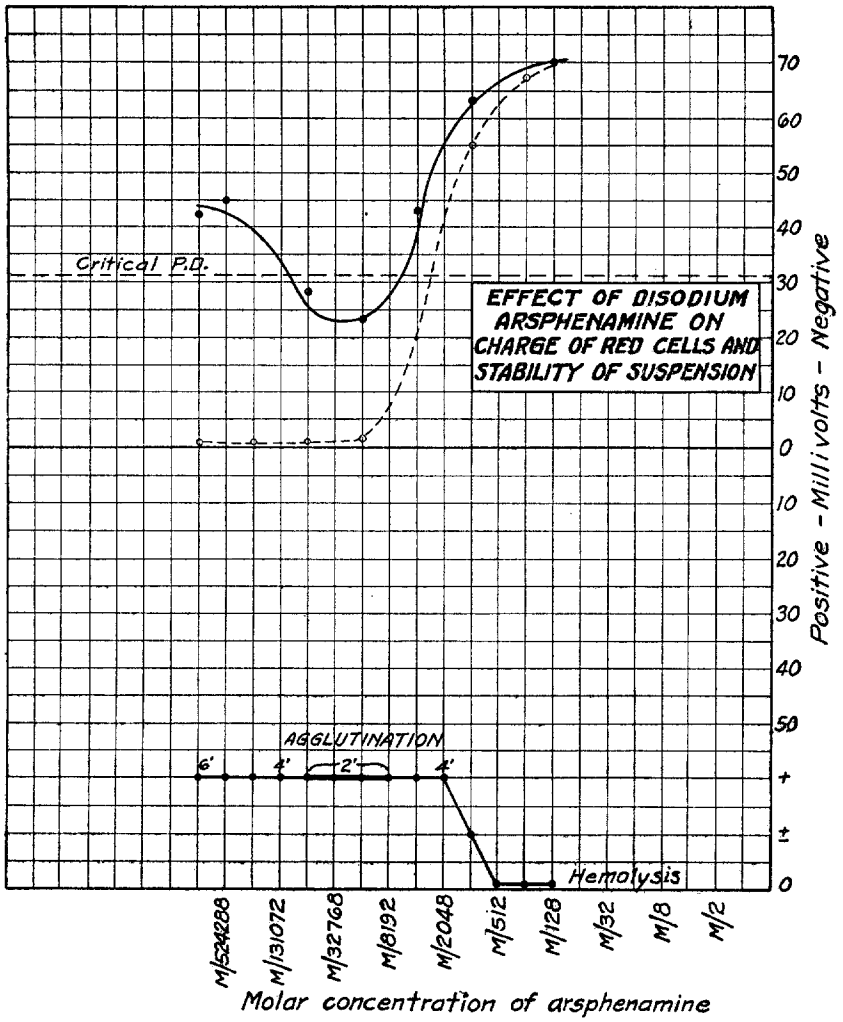

FIG. 6.

that agglutination of the cells and the resulting instability developed more slowly. Under these conditions it was found that in those tubes in which the original negative charge of the cells had been de-

pressed by the arsphenamine (M/32,000 to M/8,000) aggregation occurred considerably more rapidly (2 minutes) than in those in which the negative charge had not been so depressed (6 minutes). This increase in rate of agglutination is due to the immediate depression below the critical P.D. which occurs in these concentrations, whereas the agglutination which develops later is the result of the non-specific fall in negative charges that is found in weak concentrations of all electrolytes.

The occurrence of a rapid agglutination in certain of the suspensions in the instable zone was also found in the series of experiments with trivalent ions when the experiments were repeated at room temperature, the rate of the agglutination under these conditions not being so rapid as to obscure small differences. In Figs. 2 and 3 the time of appearance of aggregation of the cells is shown above the records of agglutinations. It is seen that in the neighborhood of the isoelectric point, agglutination occurred more rapidly than in those suspensions possessed of some charge. Agglutination of these was apparently delayed until the usual fall of P.D., which occurs in all suspensions weak in electrolyte, had brought them below the critical P.D. of 32 mv. With the hydrolyzing bivalent salts the differences were not well marked, probably due to the fact that the differences in charge are not so extreme as with the strongly active trivalent ions.

DISCUSSION.

The negative charge of red blood cells suspended in an isotonic solution of non-electrolyte, such as sucrose, is temporary, falling in the course of a few minutes to a point so low that it can no longer be accurately determined by the methods we have used. With this fall in the P.D. of the cells occurs aggregation, or agglutination, and the suspension becomes unstable. This impermanency of the negative charge is in striking contrast to the permanency of the negative charge of collodion particles when they are suspended in water, for there is no change in the P.D. of these particles and the electrolyte-free suspension is correspondingly stable for long periods. This difference presumably depends on differences in the mechanism of the production of the charge in the two cases and will be discussed at another time. For the present the fact alone will be considered. In this

discussion the charge which exists when the cells are first placed in the suspension fluid will be called the "immediate charge," and that which ultimately results when equilibrium is established will be termed the "final charge."

Electrolytes affect the charge of suspended red blood cells in the same manner as they affect that of all suspended particles. At certain concentrations they depress the charge to an isoelectric point. The negatively charged cells are apparently affected by the positive cation, for the valency of the latter determines in part the concentration of the salt which is required to bring about this result. Considerable variation was noted in each valency group as to the exact concentration of salt required. With trivalent salts M/32,000 to M/64,000 concentrations sufficed, while with the monovalent M/4 was required. Among the bivalent group wide variations occurred, from M/16,000 in the case of $CuCl_2$ to M/256 for $CaCl_2$, $FeCl_2$, and $CdCl_2$; $NiCl_2$ required M/1,000. Obviously some other factor than valency must enter into the cause of these wide variations, and this factor must operate only when the suspended particles are red blood cells, for no such discrepancies were noted in our experiments with collodion particles. Collodion particles were also much less susceptible than red cells to the action of the salts. For example, a concentration of M/1,000 $FeCl_3$ was necessary to bring them to the isoelectric point, whereas only M/32,000 was required in the case of red blood cells.

A possible explanation of these differences may be sought in the chemical composition of the two types of particles. The collodion particles are relatively inert, while the red cells, comprising both proteins and lipoids, act as amphoteric electrolytes and are therefore peculiarly subject to the effects of H ion concentration. Many of the salts whose actions were studied in our experiments hydrolyze, and in the higher concentrations their solutions are strongly acid. From the work of Loeb (11) and of Coulter (12) it is known that the combination of ions with gelatin and with red cells varies with the pH of the medium, not only quantitatively but also qualitatively. The addition of a salt which by hydrolysis increases the H ion concentration of the medium in which the cells are suspended will therefore modify the action of the ions present. It is our purpose to report later on the relative effects of H ions and of other cations and on the P.D. of the cells.

With certain electrolytes, notably those yielding trivalent ions, reversal of the charge occurs; a strongly positive potential is established, which falls again towards a second isoelectric point in the higher concentrations. The positive charge thus produced is a permanent one, and so differs from the negative charge in the weaker concentrations of electrolyte. Two curves of the P.D. of a red cell suspension in a series of electrolyte concentrations can therefore be recognized. A transient "immediate curve" in which the full effect of electrolyte is observed, which corresponds in type to the curves established by other investigators for several different types of particles, and a "final curve" in which that part which lies before the isoelectric point in the weaker concentrations of the series, and which shows the negative charge of the cells, has fallen to zero. In the stronger concentrations, showing the positive charge after reversal, the "final curve" coincides with the "immediate curve." The recognition of the different conditions represented by these two curves is essential for a proper understanding of the effects of electrolytes on the charge of the red blood cells. The "immediate curve" must be considered if the effects of electrolytes on the charge of the cells are to be fully demonstrated and compared with similar effects on other particles, such as collodion particles. The "final curve" is the one that must be correlated with other phenomena which develop in the standing suspension; notable among these are variations in stability with different electrolyte concentrations.

A correlation of the two curves, "final curve" of P.D. and curve of stability of the suspension, shows that, excepting in the highest concentrations of electrolyte, whenever the potential of the cells is above a certain critical value stability occurs. In the pure sucrose suspension the fall in P.D. of the cells which occurs as the suspension stands is accompanied by agglutination and instability. With trivalent and strongly hydrolyzing bivalent salts the strong positive charge which occurs after reversal is accompanied by a zone of stability; and when this positive charge is again depressed in the higher concentrations of electrolyte, instability again ensues. With bivalent salts which hydrolyze less, and so do not produce a positive charge which surpasses the critical value, this intermediate zone of stability is not found. A red cell suspension therefore resembles the oil droplet

suspension (Powis), the bacterial suspensions (Northrop and De Kruif), and the suspensions of collodion particles (Loeb), in that it shows a critical flocculation potential. There is considerable variation in the absolute value of the critical potential found by different investigators, and this variation does not seem to depend on the chemical nature of the suspended particles as each investigator has found the same value for particles of different composition. Powis and Ellis found a critical P.D. of 30 mv. for oil droplets and colloidal arsenous sulfide, while Northrop and De Kruif obtained a value of 16 mv. for bacterial suspensions and Loeb a similar value for collodion particles. Our value for collodion particles and red cells corresponds with that determined by Powis and Ellis.

The curve of "immediate charge" is not, however, without importance for the stability of the suspension, for if the velocity of the development of instability in the series of electrolyte concentration is closely observed under proper conditions, it will be seen that with those electrolytes which produce extremes of negative and positive charges (Fe and Al), aggregation of the cells occurs first in those mixtures in which the immediate charge has been depressed to the neighborhood of the isoelectric point, and that agglutination develops later in the other tubes as the equilibrium of the "final curve" is reached. In this zone of electrolyte concentration the electrolyte is the direct cause of the rapid agglutination of the suspension; elsewhere agglutination is due to the failure of the electrolyte to stabilize the suspension. One may speak of "rapid agglutination" in the region of the isoelectric point and of "slow agglutination" in those regions where the charging action of the electrolyte was insufficient to stabilize. This "slow agglutination" is well shown in the case of the bivalent salts $FeCl_2$ and $CdCl_2$. There is more extensive agglutination with them than occurs with trivalent ions, as all the tubes are agglutinated, but the agglutination is due to a lack of charging activity of the ions rather than to any "marked" action on their part as might be supposed by the extensive range of the agglutination.

An analogous condition in the flocculation of colloid solutions by electrolytes has been studied by Zsigmondy (13), who distinguishes two types of flocculation, a rapid and a slow. In a review of this work Freundlich (14)[2] discusses the pos-

[2] Freundlich (14), p. 592.

sible causes for the occurrence of the rapid and slow flocculation, and suggests that the transition from the slow to the rapid type may depend on the electrical properties of the particles. He states

"Man muss demnach vor allem das Konzentrationsgebiet der *raschen* konstanten Koagulationgeschwindigkeit von dem der *langsamen* veraenderlichen Koagulationgeschwindigkeit sondern. Es ist sehr wahrscheinlich dass die Grenze zwischen den beiden Gebieten, oder richtiger, der verhaeltnismaessig schnelle Uebergang von dem einen zu anderen, wiederum mit elektrischen Eigenschaften der Micellen zusamenhaengt. . . . Betrachtet man in diesem Zusamenhang die Kurve (curve of P.D.) so wird man jetzt zwei kritische Potentiale bzw. zwei kritische Ladungen zu unterschieden haben; das frueher schon eroerterte erste kritische Potential, das ereicht werden muss, damit die Flockung deutlich stattzuhaben beginnt; dann eben das zweite kritische Potential unmittelbar beim Nullpunkt bei dem die langsame Koagulationsgeschwindigkeit in das rasche uebergeht."

The occurrence of "rapid agglutination" in the neighborhood of the isoelectric point and of the development of "slow agglutination" in other parts of the P.D. curve is demonstrable with suspensions of red cells and trivalent ions, and Freundlich's theory is therefore confirmed for these conditions.

Another type of stability which is unrelated to the electrical properties of the suspended cells is also seen in our experiments. This is the occurrence of a "stable zone," best shown in the highest concentrations of non-hydrolyzing salts with bivalent and monovalent ions. It also occurs with other ions, but is in most cases obscured by the hemolysis which occurs in the high concentration of these salts. It is the same type of stability described by Northrop and De Kruif with these same salts and bacterial suspensions, and which they showed to be due to a depression of attractive forces (cohesion) between the bacteria. We have not studied this force in our experiments as the procedure used by these investigators to determine the degree of cohesion would completely denature the red cells. It is obvious that this zone of stability cannot depend on the electric charge of the red cells, for this is almost completely removed at the concentration in which the phenomenon is observed. Since the conditions of the red cell suspension resemble those of Northrop and DeKruif's bacterial suspension, it may be assumed for the present that the cause of the stability is the same in both.

Under the general head of stability of suspensions must also be considered the questions of "prozones" and "irregular series," first

described by Bechhold (15) and by Neisser and Friedemann (16). These phenomena have been intensively studied by Buxton, Teague and Schaffer (17). The latter authors distinguish between "irregular series" and "prozones," and the later work of Northrop and De Kruif shows that under certain conditions such a distinction is valid, for the mechanism of the production of the two phenomena may be different. The same relations hold true in red blood cell suspensions. In the case of Al_2Cl_6 both an "irregular series" and a "prozone" are present, and the differences in the mechanism of production are well illustrated. The zone of stability in the middle of the series ("irregular series") is the result of the strong positive charge of the cells; the zone of stability in the highest concentrations of the electrolyte ("prozone") may be assumed to be due to the effect of the electrolyte on cohesion, for it cannot be the result of the P.D. of the cells, which is low at this point.

The "prozones," which are observed in strong concentrations of $BaCl_2$, $CaCl_2$, and NaCl, likewise cannot be due to the P.D. of the cells and may be explained by variations in cohesion. All "prozones" are not, however, to be explained by this hypothesis, since the "prozone" which occurs in high concentrations of disodium arsphenamine is more likely the effect of the high negative charge which this alkaline solution produces on the cells. Nor can it be stated that "irregular series" are produced by all trivalent salts and by them alone, for we have shown that they are produced by strongly hydrolyzed bivalent salts like $CuCl_2$; and we will show in a later paper that they may be absent with trivalent ones. It is not the valency of the salt but the degree of positive charge following reversal that determines the "irregular series," and this may be the result of H ions in the solution.

The action of disodium arsphenamine on the red cell suspension merits special discussion. In a previous study (8) we suggested that the agglutination might be due to changes in the electrical properties of the cells, and the experiments reported in this paper show this to be the case. The curve of alterations in the charges of the cells and that of the stability of the suspension go hand in hand, and the relation resembles exactly that found in the action of arsphenamine on the suspension of collodion particles. Considering the series of mixtures from the weakest to the strongest concentrations, two effects of this

agent may be observed: an agglutinative and a stabilizing action. In the weakest concentrations no effect is noted and the cells agglutinate spontaneously as their negative charge slowly falls. From M/32,000 to M/8,000 "rapid agglutination" occurs, a direct result of the action of the arsphenamine which has depressed the P.D. of the cells below the critical value. From M/4,000 onward stability is present, and also the direct effect of the alkaline arsphenamine, which produces a high negative charge on the cells.

As has been shown in our former study (8) the presence of another electrolyte (NaCl) in sufficient concentration markedly modifies the occurrence of agglutination. Agglutination in 0.9 per cent NaCl suspension is quite different from that observed in an electrolyte-free sucrose suspension. From what we have seen of the action of NaCl on the properties of red cells it is evident that the selection of "physiological salt solution" as a suspension fluid for red cells in experiments on agglutination is a particularly unfortunate one, as with such a concentration of this electrolyte both the P.D. of the cells and the attractive forces (cohesion) between them are profoundly modified.

Since this paper was completed Northrop and Freund (18) have published the results of their studies on the agglutination of red blood cells. As is the case with suspensions of collodion particles, we find a critical potential for the blood cell suspension which is much higher than the value (8 to 10 mv.) they found, although the difference in the actual value of the potentials of the particles of both suspensions with the varying electrolyte concentrations is not so marked. This difference may perhaps be explained by a phenomenon which Northrop and Freund describe in the same article, namely that the value of the critical potential may be considerably altered by the presence of various substances in the suspension. Our suspensions, both of cells and collodion particles, may well have differed from theirs in the actual constitution as a result of different methods of preparation. The essential fact of the matter, concerning which there is no disagreement, would, therefore, seem to be that a critical potential exists for the agglutination of red blood cell suspensions and that the value of this potential varies with conditions in the suspension.

CONCLUSION.

Two types of stability are observed in suspensions of red blood cells. In weak concentrations of electrolytes the stability depends on the electric charge of the cells and suspension is unstable below a certain critical P.D.

In strong concentrations of electrolyte, the stability bears no relation to the charge.

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