DISK-SPHERE TRANSFORMATION IN MAMMALIAN RED CELLS

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(With Two Text-figures)

THE purpose of this paper is to try to explain the phenomenon of the transformation of the mammalian erythrocytes from disks to spheres when a small drop of a saline suspension is placed between glass slide and cover-glass. Ponder (1934) several years ago reviewed the work which had been done in connexion with this phenomenon. As was pointed out by Ponder, who was the first to show clearly the necessity of having the glass surfaces in close proximity for the formation of perfect spheres (1928–9), the two explanations which have been put forth to explain the phenomenon are both inadequate. As will be seen later, that of Waller (1930) is somewhat closer to the true explanation than that of Brinkman & van Dam (1920).

It will be shown that the phenomenon of sphering is a result of two changes which occur when the drop of suspension is placed between slide and cover-glass. One change is the increase in alkalinity of the suspension because of the alkaline nature of the glass surfaces. The other change is the adsorption on to the glass surfaces of a substance from the cell suspension. If this substance is present in the suspension, it will prevent the formation of spheres at the *p*H attained under the cover-glass, but if it is removed, as by adsorption, the cells will become spheres at that *p*H. Unless otherwise noted, all the cell suspensions used in the experiments about to be reported were suspensions of human erythrocytes in saline, made by adding 0·5 c.c. of whole blood, directly or within a few hours of drawing and oxalation, to 100 c.c. of 1 % NaCl. The observations on the cells of such a suspension were always made within 3 hr. of the formation of the suspension in order to avoid the changes in the red cells which occur on long standing.

Also, unless otherwise stated, the slides and cover-glasses used in the experiments were of new glassware, wiped clean with a soft towel prior to being used. Several brands and grades of slides and cover-glasses were tested, but all responded the same as far as the sphering was concerned.

ALKALINE EFFECT OF SLIDES AND COVER-GLASSES

The possibility of an increase in *p*H of the suspension between slide and cover-glass was put forward by Waller (1930), who, however, presented no positive evidence for such a change. This possibility was first suggested to me when I found that the

addition of powdered soft glass to a saline suspension of red cells in such a quantity as to make the ratio of glass surface to volume of suspension comparable to that obtained with a drop of suspension between slide and cover-glass, increased the *p*H of the suspension from approximately 7·0 to over 9·5; the red cells in a suspension so treated became spherical.

In another experiment a microscopic crystal of salicylic acid was placed beneath the cover-glass with a drop of cell suspension. On observing the cells beneath the cover-glass it was seen that all were spheres as usual except those in a ring around the crystal. In this ring, which gradually expanded (apparently as the acid diffused out from the crystal), the cells changed from spheres to crenated forms to disks before undergoing lysis by the acid. This experiment also suggested that an alkalinity increase (which was counteracted by the salicylic acid) between slide and cover-glass played an important part in the sphering phenomenon.

Indicators were used in the first attempts to measure the *p*H in suspensions between slide and cover-glass. Using cresol red, it was found that saline suspensions between slide and cover-glass had their *p*H shifted completely through the colour-changing range of the indicator (7·2–8·8). Moreover, the shift in *p*H appeared to occur almost immediately. This was well in accord with the observation that when a drop of a cell suspension is touched to the edge of a dry cover-glass resting on a slide and is drawn rapidly into the space under the cover-glass by capillary action, the cells become spheres almost as soon as the suspension enters the capillary space.

If the alkaline effect of slide and cover-glass plays such an important role in the sphering of red cells, one would, of course, not expect sphering between slide and cover-glass having no alkaline effect. Such slides were first obtained by cleaning the new slides in chromic acid cleaning solution. However, slides and cover-glasses so cleaned showed with indicators a slight acidifying effect on the saline suspension, even though they were rinsed well after cleaning. True enough, however, the cells in a drop of suspension between such acid-cleaned glass surfaces were not spheres, but were disks and cups.

Success in obtaining neutral reacting slides and cover-glasses, as far as the indicator method was able to detect, was finally achieved by boiling the new slides and cover-glasses for about 15 min. in distilled water and then rinsing them thoroughly in distilled water. Between a slide and cover-glass tested in this manner the cells in a drop of suspension, which had been disks in the bulk of the suspension, did not become spheres, but retained their disk shape. On standing awhile the cells under the cover-glass would often crenate a little or become cup-shaped, or in general, change somewhat from the good biconcave disk shape which they exhibited in the bulk of the saline suspension, but they never became spherical.

Exactly similar results were obtained with a quartz slide and quartz cover-glass as were obtained with the boiled slides and cover-glasses.

By rinsing new slides and cover-glasses for a few minutes with water before using, glass surfaces were obtained which had an intermediate alkaline effect. Between such slides and cover-glasses the cells were neither good disks nor good spheres, but were crenated discoids and spheroids. Such forms, as Ponder (1934)

has shown, are the intermediate forms between the biconcave disks and the spheres formed under cover-glass. Such forms were observed by Ponder in intermediate depths of a saline cell suspension placed under a long cover-glass resting on a slide at one end and raised above the slide by a small glass strip at the other end (thus forming a wedge-shaped chamber with the slide). At the narrow end of the chamber Ponder observed spheres, at the deep end he observed discoidal forms, and between the two he observed the gradual gradation of crenated forms. By using the indicator cresol red in such a wedge-shaped chamber, it was seen that the *p*H within the chamber gradually changed from about neutral at the deep end of the chamber to a *p*H of 8·8 or higher at the narrow end of the chamber.

On using the transparent plastic methyl methocrylate as a slide and cellophane as a cover-glass, such a decided acidic reaction was given to the drop of cell suspension that much haemolysis occurred. Prior to haemolysis under these conditions, the cells became first cups and then large prolytic spheres, such as are found when one haemolyses cells with acid in the bulk of a suspension.

Rinsing the new slides and cover-glasses in organic solvents, such as ether, alcohol, petroleum ether, and benzene, had no effect on the ability of them to cause the formation of spheres.

To study the phenomenon of the disk-sphere transformation further it was desirable to determine the *p*H in drops of suspension placed between slide and cover-glass much more accurately than could be determined with indicators. A method for doing this with the use of a micro-glass electrode was therefore developed. A description of the apparatus and technique follows.[1]

The glass electrode was made by a modification of the method of MacInnes & Dole (1929). The tube to which the membrane was to be attached was drawn out in the form of a medicine dropper. The smooth tip, with an internal diameter of about 1·0 mm., was plunged while at a dull red heat into an irridescent bubble of Corning 015 electrode glass. If the fusing of the membrane to the tip was successful, 0·1 *N* HCl was poured into the sealed tube. Into this acid a silver-silver chloride electrode, prepared by the method of Brown (1934), was placed. The membrane was submerged in water for about a day before being used and was always kept submerged in water when not in use. This micro-electrode was used in conjunction with a regular macro-glass electrode potentiometer (model 3 C of the Coleman Electric Co.). To replace the cup as a receptacle for the suspension with *p*H to be measured, a flat-topped glass tube with a small capillary opening from the large bore of the tube through the centre of the flat top was used (see Fig. 1). The KCl bridge leading from the regular calomel half-cell of the apparatus passed up through the tube to the top of the capillary. On top of the tube and therefore in contact with the KCl solution in the capillary was placed a drop of the suspension of which the *p*H was to be measured. The tip of the micro-glass electrode was placed in the drop and the potentiometer balanced out and the *p*H obtained in the usual way. Both micro-electrode and the tube for supporting the drop were conveniently held in

[1] I wish to acknowledge here the suggestions of Dr L. S. Fosdick of the Department of Chemistry, School of Dentistry, North-western University, on the making of the apparatus.

clamps on an iron stand. Before each new *p*H determination the glass electrode was rinsed off with distilled water, the capillary was flushed out with KCl solution, and the top of the tube was rinsed off with water and then dried by lightly touching with a filter paper. This apparatus was capable of determining the *p*H of a drop of suspension of as small a volume as 0·005 c.c. with an error of less than 0·05 of a *p*H unit.

In determining the effect of slide and cover-glass on the *p*H of a drop of suspension with this apparatus, a drop of measured volume was first put on a slide from a micropipette, and a cover-glass of 24 by 60 mm. was placed upon the drop and allowed to remain in place for a half-minute or so. Then the cover-glass was removed by lifting from one end in such a manner that most of the suspension under it was collected in one drop on the slide. This drop was then transferred from the slide to the top of the tube containing the KCl bridge by inverting the slide and touching the drop to the top of the tube. The *p*H of the drop was then determined.

The results of the *p*H determinations with the micro-glass electrode are contained in Table I. It will be noted that observations were made not only of the effect of new slides and cover-glasses on the *p*H of saline suspensions, but also of the effect of boiled slides on saline suspensions, and of the effects of new and boiled slides on buffered suspensions. The *p*H values listed are all averages of several determinations on different drops of suspension.

The alkaline effect of the new slides and cover-glasses is surprisingly large. Even the values around 9·2 and 9·3 listed in the table as occurring in drops from between new slides and cover-glasses, which have caused complete sphering, are probably a few tenths of a *p*H unit below those actually attained between slide and cover-glass, for in the process of transferring the drop to the flat top of the tube there was always probably a decrease in *p*H due to the absorption of CO_2 from the air by the drop. This was indicated by the fact that the shorter was the interval of time between removing the new cover-glass from the saline suspension and the measuring of *p*H, the higher was the *p*H generally.

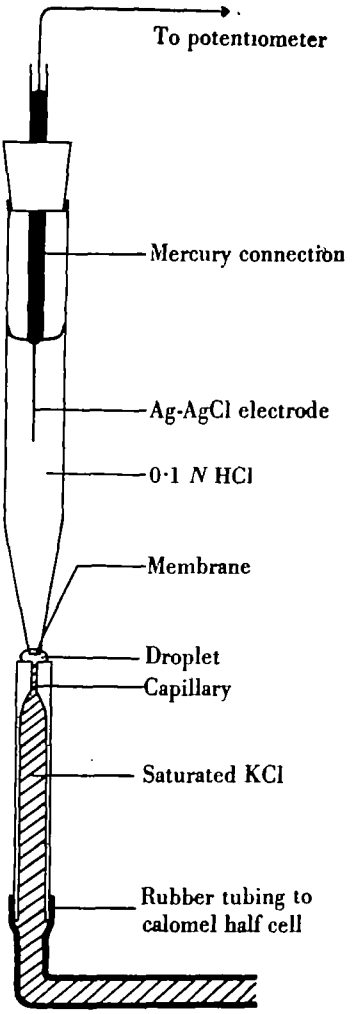

Fig. 1. Micro-glass electrode.

From the table it will be noted that the effect of the boiled slides and cover-glasses on a saline suspension does not appear to be a perfectly neutral one. The

lowering of *p*H in the case of boiled slides and cover-glasses, however, was so little that it might well have been due to absorption of CO_2 from the atmosphere by the drop in the various steps. Also it will be noted that even in the presence of considerable phosphate buffer the new slides and cover-glasses exerted a marked alkaline action. With a suspension containing 19 parts of saline to 1 part of *M*/15 phosphate buffer, the smooth spheres under the new cover-glass generally occurred in the thinnest depths of liquid, while the crenated spheres occurred in the thickest depths.

Table I

Nature of suspending solution	Nature of slides and cover-glasses	Volume under cover-glass in c.c.	*p*H of bulk of suspension	*p*H of drop from under cover-glass	Shape of cells under cover-glass
1% NaCl	New	0·03	7·21	9·20	Perfect spheres
1% NaCl	New	0·02	6·74	9·31	Perfect spheres
1% NaCl	Boiled	0·03	6·70	6·48	Disks, some crenated and cupped
19 : 1 saline to *M*/15 phosphate buffer	New	0·01	7·08	8·50	Spheres and finely crenated spheres
4 : 1 saline to *M*/15 phosphate buffer	New	0·01	7·08	7·87	Finely crenated spheres
9 : 1 saline to *M*/15 phosphate buffer	Boiled	0·02	7·08	6·94	Crenated disks

Two short experiments conclude this section on the alkaline effect of slides and cover-glasses. In the first it was found that by spreading a small drop of a saline suspension of red cells very thinly over a new glass slide the cells changed to spheres even though no cover-glass was placed over them. In the second the cells in a drop of suspension were all changed to spheres by placing the drop between cover-glass and slide. The cover-glass was then removed so as to leave the drop containing the spheres on the slide. After this a comparatively small drop of untreated suspension, containing cells in discoidal form, was added to the treated drop on the slide. On the addition the newly added cells also turned to spheres.

ADSORPTION OF ANTI-SPHERING FACTOR

Up to this point has been presented the experimental evidence for the decided alkaline effect of new slides and cover-glasses on drops of suspension between them and the important relation of this effect to the phenomenon of the sphering of erythrocytes in the drops. However, it would be incorrect to assume that the sphere formation is due to the rise in *p*H alone unless one could obtain spheres by merely increasing the *p*H of a saline suspension of red cells in bulk to the *p*H attained by a drop under a new cover-glass. And as it turns out this is not the case.

In the following experiments, which show that a second change accompanying the *p*H change is necessary for the sphering phenomenon to occur, a number of titrations had to be performed on cell suspensions. The standard procedure for a

titration was to put 5 c.c. of the cell suspension in the cup of the glass electrode apparatus (the macro rather than micro) and to add 0·15 *M* NaOH directly to this from a small burette. After each addition of alkali the *p*H of the suspension was determined with glass electrode, and about 0·10 c.c. of the suspension was transferred in a pipette from the suspension to the concavity of a hanging-drop microscope slide, where the shapes of the cells in the transferred suspension were observed with a microscope. A titration was always performed as rapidly as was conveniently possible.

When such a titration was performed on a saline suspension made in the usual way (1 : 200 of blood to saline), complete sphering did not occur until a *p*H of 11·3–11·4 was reached. This was definitely a much higher *p*H than could be obtained through the action of slide and cover-glass. Also the spheres which were finally formed at the high *p*H were unlike those formed under cover-glass in that they were larger in volume and were definitely prolytic spheres, always occurring just prior to haemolysis by the alkali.

The result of the titrations on saline suspensions of whole blood suggested that in such suspensions there was some substance which prevented the cells in the bulk of suspension from becoming spheres at the *p*H levels occurring between new slides and cover-glasses, that such a substance was removed from a drop of suspension between slide and cover-glass by adsorption on the glass surfaces, and that if the substance could be removed from red cells in the bulk of a suspension, the cells would become spheres at the *p*H levels occurring between new slides and cover-glasses. For the sake of convenience the hypothetical substance was called the "anti-sphering factor". If it actually were adsorbed on the glass surfaces of slide and cover-glass in the sphering phenomenon, such action would be compatible with the properties of serum proteins. The possibility of such a protein anti-sphering factor was further suggested by Ponder's surmise (1934) that it is an albumin fraction of the serum which causes the retransformation from spheres to disks when one adds a little serum to a drop of suspension in which the cells have previously been changed to spheres by placing the drop between slide and cover-glass.

At first, on the assumption that the anti-sphering factor was contained in the serum alone, attempts were made to free the cells of the factor by washing them free of serum. The washings with saline were performed with the use of a centrifuge. After the washings, the cells were resuspended in saline in a concentration equal to that of cells in a suspension made by adding 1 part of whole blood to 200 parts saline. However, on titrating suspensions of washed cells with 0·15 NaOH, it was found that the cells still would not become perfect spheres in bulk until a *p*H of about 11·3 was reached. It was noticed that the anti-sphering activity (the determination of which will be discussed later) of the suspension of washed cells was not so great as that of a suspension of whole blood, but there was still a decided amount of anti-sphering activity even in a suspension of cells which had been washed up to ten times with a hundred times their volume of saline each time.

Evidently the cells, for practical purposes, could not be washed free of the anti-

sphering factor. Perhaps, however, the changes occurring between slide and cover-glass could be more exactly matched in the bulk of suspension; perhaps the cells in suspension could first have the factor adsorbed away from them by introducing into a suspension of washed cells a comparatively large amount of glass surface prior to the increase of alkalinity. It had already been noted that the cells of a suspension which were not sphered when the *p*H of the bulk of suspension was raised to about 10, became perfect spheres when a drop of the suspension at that *p*H was placed between boiled (and therefore practically neutral reacting) slide and cover-glass.

To verify the hypothesis that an anti-sphering factor, incompletely washable from the cells in bulk, might be completely adsorbed from the cells in bulk, the following technique was developed after a number of initial experiments. A dilute saline suspension containing the thoroughly washed cells from about 0·10 c.c. of blood was added to a 500 c.c. beaker full of glass beads. The volume of suspension was enough just to cover the beads. The beads were about 3 mm. in diameter, and prior to use had been cleaned by overnight standing in saturated Na_3PO_4 solution, thoroughly rinsed with water, and dried. As could be shown, they caused no appreciable change in the *p*H of the suspension of cells added to them. After adding the suspension, the beaker was kept in the ice-box 2–3 hr. The suspension was then decanted off through a cheese cloth. The cells in a suspension of this sort were generally crenated disks when the *p*H was around neutrality. However, on titrating such a suspension in the usual way, the cells all became perfect spheres at a *p*H between 9·15 and 9·25. Here, then, was a suspension in which the cells all became spheres at a *p*H level which actually was reached in drops of suspension between slide and cover-glass. The spheres, too, were in all respects similar to those obtained under cover-glass, and were unlike the larger prolytic spheres obtained around *p*H 11·3 in the bulk of untreated suspensions. On raising the *p*H of a bead-treated suspension above 9·2, the spheres persisted until a *p*H above 11 was reached, when they became larger prolytic spheres and haemolysed.

Now, if to a suspension of red cells which had been treated with glass beads a small amount of serum was added, the cells no longer became spheres at a *p*H of about 9·2, but behaved exactly like a suspension of untreated cells, not becoming spheres until a haemolysing *p*H of about 11·3 was attained. Also, if a diluted saline solution of serum, which when ordinarily added to a suspension of treated cells prevented them from being sphered below a *p*H of about 11·3, was first treated with a large quantity of powdered quartz so that all of the protein in it was adsorbed, it no longer prevented the cells from being sphered at a *p*H of 9·2 on being added to a bead-treated suspension.

Experiments of the types just described definitely indicate an anti-sphering factor which has to be removed from the red cells of a saline suspension before they will become spheres at a *p*H around 9·2. Treatment of a cell suspension with large quantities of glass beads evidently results in the anti-sphering factor being removed from the cells by adsorption on the very large surface of the beads.

All the titration experiments so far described were done on suspensions of human red cells in 1 % NaCl solution. For comparative purposes some experiments

were also performed on human cells in saline and phosphate buffer mixtures, and on the cells of a few other mammals in saline. It was found that unwashed human cells in mixtures of 9 : 1 and 4 : 1 saline to $M/15$ phosphate buffer (pH 7·2), became complete spheres at a pH of about 11·1–11·2. Human cells deprived of anti-sphering factor, however, in such saline-buffer mixtures became spheres at a pH level several tenths of a pH unit below the sphering level of about pH 9·2 found with pure saline suspensions.

Beef, dog, and horse cells all behave very similarly to human cells. In the presence of the anti-sphering factor in saline suspension they could not be changed to perfect spheres until just before haemolysis in the pH range of 11·2–11·4. Likewise, in the absence of the anti-sphering factor in saline suspension, these cells of different species all first became perfect spheres at a pH of about 9·2. Moreover, it was found that any one of the four mammalian sera investigated would serve as a source of active anti-sphering factor for a suspension of any of the other species of mammalian cells which had been previously deprived of their own anti-sphering factor.

INTERMEDIATE SHAPES OF RED CELLS

As has been shown by Ponder (1934), red cells undergoing the change from disk to sphere under a cover-glass pass through certain intermediate crenated shapes. The shapes which they successively pass through in the transformation may be arbitrarily listed as smooth disk, crenated disk, crenated discoid, crenated spheroid, finely crenated sphere, and smooth sphere. All of these intermediate forms could be observed when I gradually raised the pH, say by a few tenths of a pH unit per minute, of a cell suspension free of anti-sphering factor from a pH of about 6·0–7·0 to a pH of over 9·2. Indeed, with cell suspensions free of anti-sphering factor, crenated disks most often appeared even in neutral solutions. Crenated discoids appeared about pH 8·0, crenated spheroids about pH 8·5, finely crenated spheres about 9·0, and perfectly smooth spheres about 9·2. The smooth spheres swelled into prolytic spheres around pH 11·2. A diagram of these successive changes is given in row *A* of Fig. 2.

In row *B* of Fig. 2 is shown the shapes successively obtained when I gradually increased the pH of a suspension of whole blood in saline, which had not been deprived of any of its anti-sphering factor by washing or adsorption. It will be noted that smooth biconcave disks did not persist from neutral pH to the pH of prolytic sphering above 11, but that over a wide range of pH from slightly above neutral to almost 10, the cells were crenated disks and discoids. Then as one raised the pH into the region over 10, the cells surprisingly became smooth disks again. Around pH 11 the smooth disks generally tended to cup somewhat and in other ways became distorted. Before becoming swollen prolytic spheres in the vicinity of 11·2 they often passed through cup shapes.

If the cells were titrated with pure serum as a suspending medium sphering and lysis again set in about 11·4, but the cells remained smooth biconcave disks at all the pH levels from neutrality to over pH 11·0.

Rows *C*, *D* and *E* of Fig. 2 diagram what happened when cell suspensions containing varying amounts of anti-sphering factor were subjected to a sudden rise in *p*H from the region around neutrality to the region between 10·2 and 10·7 by a single addition of the proper amount of 0·15 *M* NaOH. The sudden increase enabled one to see a marked tendency of the cells to form spheres even in the presence of anti-sphering factor, which could not be clearly observed when one gradually raised the *p*H as already described. Row *C* represents the changes which occurred when a suspension of whole blood in saline was titrated. Immediately after the addition of NaOH and rise in *p*H, the cells became crenated spheres. But then, within a few minutes of the addition, the crenated spheres changed to the type of smooth disk which would have been obtained at the same *p*H had the *p*H increase been a gradual one.

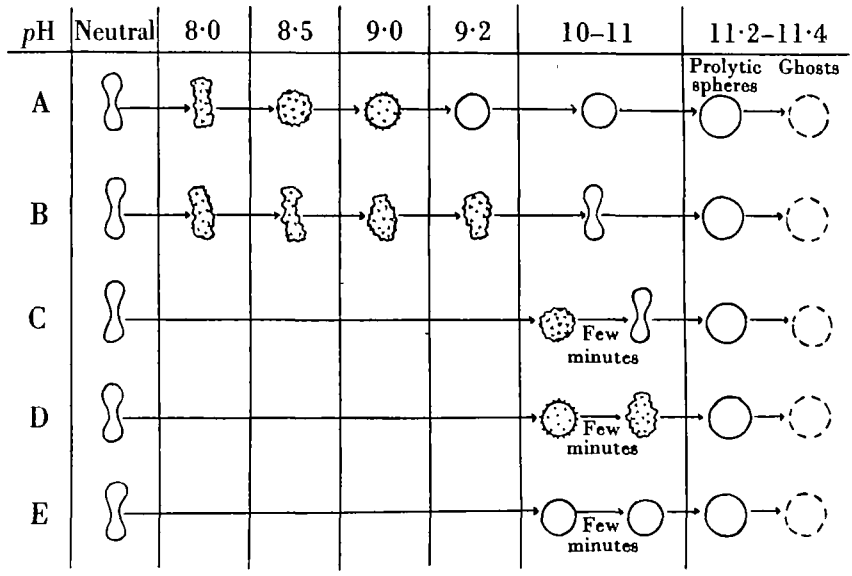

Fig. 2. Shape changes of red cells. *A*, gradual increase in *p*H in suspension free of anti-sphering factor. *B*, gradual increase of *p*H in suspension containing sufficient anti-sphering factor. *C*, sudden increase in *p*H from neutrality to about 10·5 in suspension containing sufficient anti-sphering factor. *D*, sudden increase in *p*H from neutrality to about 10·5 in suspension containing intermediate amount of anti-sphering factor. *E*, sudden increase in *p*H from neutrality to about 10·5 in suspension completely free of anti-sphering factor.

Row *E* represents changes which occurred when a suspension of washed cells which had been completely freed of anti-sphering factor by the glass bead treatment was subjected to a sudden increase in *p*H. Here, however, even after several minutes of waiting, there was no reconversion from the smooth spherical shape which had been formed immediately upon the addition of the NaOH. Row *D* represents the changes occurring when a washed suspension of cells which had been treated with considerably less beads than the suspension of row *E* was subjected to a similar sudden increase of *p*H. Apparently the bead treatment had still left some anti-sphering factor with the cells in this case. In the first sudden change the cells

became good spheres except for very fine crenations, but on standing a few minutes they changed to crenated discoids.

I used this rapid titration method in roughly estimating the amount of anti-sphering activity of a cell suspension. The shapes of the cells was observed about 5 min. after the sudden *p*H rise. The less was the amount of anti-sphering factor, the closer to the spherical shape and the farther away from the discoidal shape were the cells. It must be pointed out, however, that any intermediate shape described here as occurring in a suspension under given conditions was not the shape of every cell in the suspension, but rather the shape of the majority of cells in the suspension. Some individual cells often appeared to retain a disk form more persistently than others, while other cells often tended to form spheres more readily than the rest. It was also of prime importance that the cell suspensions be as fresh as possible for one to obtain consistent results.

INFLUENCE OF FATTY ACIDS

At one period in the course of this research reported here I was much troubled by some apparently very inconsistent results. Some suspensions of cells, which had been washed several times, retained considerable anti-sphering activity, while other suspensions, washed just as many times, showed very little anti-sphering activity, and sometimes no anti-sphering activity at all. After some time the cause for the inconsistent results was determined. It happened that when mixing fresh saline with red cells in the washing process, I often used the ball of my palm as a stopper for the centrifuge tube while shaking. In the washing of other batches of cells, however, the mixing was accomplished either by stirring with a glass rod or by rotating the cup rapidly with the hands. Those cells which showed very little or no anti-sphering activity after washing were those which had been mixed with saline in tubes stoppered by the palm. Those cells which showed considerable anti-sphering activity after washing were those which had been mixed otherwise. It therefore seemed that in the mixing against the palm some substance (or substances) passed from palm to cell suspension and counteracted the action of the anti-sphering factor.

To test this idea three control experiments were performed. In one it was found that a 5 c.c. suspension of washed cells, showing considerable anti-sphering activity to begin with, could be completely deprived of anti-sphering activity by shaking it for a few minutes in a 50 c.c. centrifuge tube stoppered with the palm of the hand. In the second it was found that another similar 5 c.c. portion was not noticeably deprived of any anti-sphering activity by shaking it for several minutes in a 50 c.c. centrifuge tube stoppered with a cork. In the third it was found that another similar 5 c.c. portion of unshaken saline suspension, when mixed with a 5 c.c. portion of cell-free saline which had been previously shaken several minutes against the palm of the hand, lost all its anti-sphering activity. Mixing with saline shaken in a tube with a cork stopper, however, caused no noticeable reduction of anti-sphering activity.

A suspension of cells deprived of anti-sphering activity by the shaking against the palm behaved similarly to a suspension deprived of the anti-sphering factor by treatment with glass beads. The cells first became spheres at a *p*H of about 9·2. They haemolysed at a *p*H above 11·0. On the addition of a little extra serum to the suspension, the suspension would again show anti-sphering activity, evidently as a result of the anti-sphering factor in the serum being added in greater quantity than could be counteracted by the substance from the skin.

On the hypothesis that the "anti-anti-sphering factor" coming from the skin of the hand was one of the nature of fatty acids, the effects of addition of traces of oleic and palmitic acids to washed and unwashed cell suspensions, originally containing considerable anti-sphering activity, was investigated. The suspensions treated with either of these acids also were deprived of their anti-sphering activity, and behaved in all respects like suspensions deprived of anti-sphering activity by shaking against the palm.

BEHAVIOUR OF GHOSTS

Concentrated suspensions of washed red cells free of the anti-sphering factor were mixed with distilled water. After the cells had completely laked, the suspensions were made isotonic again by the addition of the proper amount of 7 % saline. The ghosts were washed a few times in saline with a high-speed centrifugation and then were suspended in saline. The amount of haemoglobin remaining in these watery ghosts depended on the concentration of red cells in the hyptonic haemolysing mixture, but whether very little or comparatively much haemoglobin remained, the ghosts all behaved in the same manner.

The ghosts in neutral saline suspension were generally disks or crenated disks. On placing a drop of a suspension between new slide and cover-glass, the ghosts all became spheres just like unlysed red cells. But within a minute of the initial sphering all the ghosts had changed back to discoidal forms. If unlysed red cells were placed under the cover-glass with the ghosts, they became spheres with the ghosts, but persisted as spheres after the ghosts had changed back to discoidal forms. Between boiled slides and cover-glasses the ghosts showed no temporary sphering.

The changes of shape of watery ghosts brought about in a drop of suspension between new slide and cover-glass could also be brought about in the bulk of suspension. By adding enough alkali to raise the *p*H of the suspension suddenly from about neutrality to about 9·5, one could obtain the exact temporary formation of spheres seen under the cover-glass. This temporary formation of spheres somewhat resembled the temporary formation of crenated spheres caused by rapidly increasing the *p*H in a suspension containing unlysed cells in the presence of anti-sphering factor (see Fig. 2). However, in the case of the unlysed cells the reconversion to disks was brought about by the presence of the anti-sphering factor, whereas the factor was absent from the suspensions of ghosts. After the ghosts had changed back to disks, they could not be changed to spheres again by lowering the *p*H back toward neutrality nor by raising the *p*H up to about 10·5. Over *p*H 10·5 many of the ghosts appeared to disintegrate and disappear from view. The few fragments

which could still be detected at higher *p*H levels appeared to form spheres around *p*H 11·3. If the *p*H of a suspension of ghosts was gradually raised from neutrality to above 10·0, there was no temporary sphering of all cells at once, but in the region around *p*H 9·0, there was some tendency for the crenated forms to become somewhat spheroidal for a short while.

REVERSIBILITY OF DISK-SPHERE TRANSFORMATION

Upon alternately raising and lowering the *p*H of a red cell suspension free of anti-sphering activity from a neutral *p*H to over *p*H 9·2, and from over *p*H 9·2 back to neutrality, the disk-sphere transformation was found to be reversible. When the *p*H went over 9·2 the cells became spheres; when it was reduced to neutrality they again became disks. In changing again to disks they passed through, in reverse order, the same intermediate crenated forms they had passed through on the way to spheres. By shifting the *p*H back and forth with the use of 0·15 *N* NaOH as the base and either 0·15 *M* HCl or CO_2 gas as the acid, the cells could be made to go back and forth through the transformation an indefinite number of times. With long continuation of this increasing and decreasing of *p*H, the *p*H level of complete sphering generally fell several tenths of a *p*H unit below 9·2.

The reversibility of the disk-sphere transformation could also be demonstrated on a new glass slide, using the alkaline glass surface as the source of base. A drop of suspension was put between new slide and cover-glass in the usual way, so that spheres were formed. The cover-glass was then removed so as to leave the drop of saline containing the spherical cells on the slide. Then if a small jet of CO_2 was directed over the drop, all the cells suddenly changed to disks. On withdrawal of the source of CO_2, the cells all changed back to spheres. These spheres could again be changed to disks by blowing on more CO_2. The number of reversals of shape one could produce this way in a single drop was only limited by the evaporation of a drop. In some preparations the transformation from sphere to disk could be brought about by merely blowing the breath strongly on the drop. Agitation of a drop with a metal wire or with a blast of pure air would not bring on such a transformation. Evidently, the addition of CO_2 at first lowered the *p*H of the drop enough to cause the transformation to disks; but on short standing after CO_2 treatment, a *p*H above the sphering level *p*H was again attained because of the reduced CO_2 tension and the counteracting of the acid effect of the added CO_2 by more alkali from the glass and possibly to some extent by the haemoglobin in the cells.

By using the technique just described, an observation was made which has some bearing on the question of whether the red cell has any fixed structure. If I carefully observed the cells in a series of disk-sphere reversals, I could see that *any one cell always had its biconcavities reappear in the same parts of its surface in changing from perfect sphere to disk* (easily noted in the case of cells stuck on edge to the glass slide), and that even the crenations in the transitional forms always reappeared at the same points on the cell surface. Individual cells, however, appeared to have individual differences as far as the exact shape of the crenated transitional forms went.

OTHER PHENOMENA

As already mentioned, the observations of the shapes of cells in a suspension being titrated in bulk were made on about 0·1 c.c. portions of the suspension transferred by pipette to the concavity of a hanging-drop microscope slide. The shapes reported in the previous sections were the shapes occurring toward the middle of the drop under observation in the concavity. In the case of red cells free of anti-sphering factor and in the case of ghosts, the shapes toward the middle of a drop were about the same as the shapes of cells near the surface. But in the case of suspensions of washed and unwashed cells containing anti-sphering factor the shape of the cells within a few micra of the surface of the drop were different from the shape of those in the bulk of the drop. As the *p*H was raised gradually, those cells near the surface successively took on the shapes which those cells in a suspension free or almost free of anti-sphering factor would take on under a similar *p*H change. That is, they tended to sphere at *p*H levels above 9·2, while the cells below them in the bulk of the drop were in discoidal shapes. I suggest that this tendency toward sphere formation at the surface of a suspension containing anti-sphering factor is a result of the adsorption of the anti-sphering factor away from the cells near the surface on to the suspension-air interface.

Occasionally some of the cells in a suspension, after treatment with glass beads, would become crenated discoids, which, despite the removal of the anti-sphering factor, would remain crenated discoids even when the *p*H was raised above the sphering level. In fact, these cells would not even show a temporary tendency to sphere with a sudden rise in *p*H. Evidently, they had somehow been altered in the treatment for removing the anti-sphering factor.

In contrast with these cells which never showed a tendency to sphere were the spherical cells formed in blood which had stood a long time after drawing. Such cells never showed a tendency to acquire any other shape than the spherical, no matter how much anti-sphering factor was present or at what level in the range between acid and alkali haemolysis was the *p*H.

DISCUSSION

The investigation of the causes of the transformation of red cells from disks to spheres in a drop of saline between new slide and cover-glass has led to the realization that there are two important factors influencing the shape of red cells whether between slide and cover-glass or in the bulk of a suspension; namely, the hydrogen-ion concentration of the suspension and the amount present of a substance which militates against sphering. Taking these two factors into consideration, one is able to explain certain hitherto inexplicable findings of former investigators relative to the sphering phenomenon.

The reason why one does not obtain complete sphering if the depth of suspension between slide and cover-glass is too great (Ponder, 1928–9) is that the depth depends on the ratio of the volume of suspension to area of glass surface in contact with the suspension; this ratio must be small in order for the glass surface to adsorb all the

anti-sphering factor from the suspension and to furnish enough alkali to raise the *p*H of the suspension above the sphering level.

Cells will not become spherical between slide and cover-glass coated with paraffin (Ponder, 1928–9) because such slides and cover-glasses can neither adsorb anti-sphering factor nor give off alkali. If enough serum is in the suspension under the cover-glass the cells will not become spheres because there is more anti-sphering substance present than can be adsorbed and probably also because the serum exerts a buffering action against the alkaline effect. The ability of ammonium oxalate (Gough, 1924) and phosphate buffers of *p*H less than 7·0 (Waller, 1930) to act somewhat like serum in changing cells sphered beneath a cover-glass back to discoidal shapes is due to their ability to buffer the alkali from the glass. The lack of sphering between slide and cover-glass coated with calcium stearate monolayers (Ponder & Neurath, 1938) may be due to a loss of alkalinity with the formation of the new surface and the inability of such a surface to adsorb the anti-sphering factor.

I regard as completely untenable the hypothesis of Brinkman & van Dam (1920), which attributes the sphering phenomenon to the electrostatic charge on the glass surface and the lack of cholesterol in the cell suspension. Like Ponder, I have been unable to confirm the reports that flaming the slides (Kesten & Zucker, 1928–9; McGlone, 1926) or washing them with ether and drying them in air (Waller, 1930) affected their ability to cause sphere formation. Perhaps as Ponder & Neurath suggest (1938), the ether used by Waller was impure and left a hydrophobic layer on the glassware. Such a layer might prevent adsorption of the anti-sphering factor. There is also the possibility that the ether used by Waller contained certain acidic impurities which neutralized the alkali of the glass surface. This possibility is suggested in view of the fact that one sample of ethyl alcohol which I used for rinsing some of my slides and cover-glasses definitely did away with the alkaline effect of the glass surfaces.

From the results of work now in progress it appears that the anti-sphering factor is very likely the carbohydrate free fraction of the serum albumin.[1] It seems that this protein is in equilibrium between red cells and serum, and when the serum is washed away, it is in equilibrium between cells and saline. However, the red cells hold so tenaciously to what anti-sphering protein is left them after the removal of the serum, that in the equilibrium between cells and saline very little of the protein is in solution in the saline. This accounts for the inadequacy of repeated washings to remove all of the anti-sphering protein from cells. When, however, one brings a saline suspension of cells in contact with a comparatively large area of clean glass surface, as when placing a drop between slide and cover-slip, the anti-sphering protein molecules in solution coming in contact with the surface are irreversibly adsorbed. With the protein being removed from solution by adsorption more protein leaves the red cells to enter the solution in the attempt to establish an equilibrium. This also is adsorbed in turn, and very soon all the protein is thus adsorbed away from the cells on to the glass surface.

As to the actual physical and chemical changes in the red cell which result in

[1] The work being done on the determination of the chemical nature of the anti-sphering factor will be reported in a future paper.

changes in shape when one alters the hydrogen ion or anti-sphering factor content of a suspension, nothing can be said at present, but attention should be called to the observation that in repeated reversing of the disk-sphere transformation the biconcavities and particular crenations of any one cell always reappear on the same parts of the surface of that cell. This is strong evidence for some sort of fixed cellular structure.

SUMMARY

1. Experimental evidence has been presented to show that the sphering of mammalian red cells in saline suspension between new slide and cover-glass is a result of these two changes: (1) the *p*H of the suspension is raised over 9·2 by the alkaline effect of the glass; (2) the glass adsorbs out from the suspension a protein, which, if present, prevents sphering until a *p*H of about 11·3 is reached, but if absent allows sphering around a *p*H of 9·2.

2. Anti-sphering factor is contained by both serum and cells. It cannot be adequately removed from cells by washing them with saline, but it can be removed from them by adsorption on clean glass surfaces.

3. Changes of cell shape occurring between slide and cover-glass may be duplicated in the bulk of a suspension.

4. The *p*H and amount of anti-sphering factor present also influence the intermediate crenated shapes between the disk and sphere shapes.

5. Substances from the skin which counteract the activity of the anti-sphering factor may be introduced into a suspension by shaking it against the palm of the hand. Oleic or palmitic acids in a suspension also counteracts the activity of the anti-sphering factor present.

6. Watery ghosts show a peculiar temporary sphering between new slide and cover-glass.

7. The disk-sphere transformation in the absence of anti-sphering factor is reversible. In repeated reversing of the transformation the biconcavities and particular crenations of any one cell always reappear on the same parts of the surface of that cell.

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