DISK-SPHERE TRANSFORMATION IN MAMMALIAN RED CELLS

II. THE NATURE OF THE ANTI-SPHERING FACTOR

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(With Four Text-figures)

THE transformation of red cells from disks to spheres in a drop of a saline suspension between slide and coverglass (Ponder, 1929, 1934) has recently been shown by one of us (Furchgott, 1939) to be the result of two changes. The first is a rise in the *p*H of the suspension above 9·2 because of the alkaline nature of the glass surfaces. The second is an adsorption out of the suspension by the glass surfaces of a substance which, if present, prevents the formation of spheres at the *p*H attained between slide and coverglass. The determination of the chemical nature of this substance, termed the "anti-sphering factor", and experimental attempts to gain an insight into the physical mechanism of the phenomena of shape changes are the subject of this paper.

I. THE METHOD AND THE SCALE OF ANTI-SPHERING ACTIVITY

As previously noted (Furchgott, 1939), quantities of cells, sufficient to work with, cannot be completely freed of the anti-sphering factor by thorough washing alone, but can be freed completely if they are treated with large quantities of clean glass beads, which adsorb the anti-sphering factor from the cells. In the removal of the factor from the cells for the experiments reported here, the cells were first washed free of their serum. They were then suspended in 1 % saline in a concentration of about 1 part in 800, and this suspension was evenly poured into a beaker full of glass beads. The beads were 3 mm. in diameter and afforded about 10 sq. m. of surface area for every 0·5 c.c. of cells. After standing about 5 min. with the beads, the cell suspension was decanted through cheese cloth. The cells were then centrifuged down and resuspended in a concentration of about 1 part in 400 in fresh saline.

In testing for the anti-sphering activity of serum, a given amount was added to

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5 c.c. of the treated, factor-free suspension, and then one or two drops of 0·15 *N* NaOH were added to the suspension so that the *p*H suddenly rose from around neutrality to between 10·2 and 10·7. After waiting about 5 min. to allow for the passing of the temporary tendency toward sphere formation (discussed in the previous paper), the shape of the cells at the high *p*H was observed in a drop in the concavity of a hanging-drop slide. If sufficient anti-sphering factor had been added to the suspension, the cells were smooth biconcave disks. If none or extremely little had been added, the cells were smooth spheres. If an intermediate amount had been added, the cells were intermediate crenated forms. All tests for anti-sphering activity with a given bead-treated suspension were performed within an hour of the

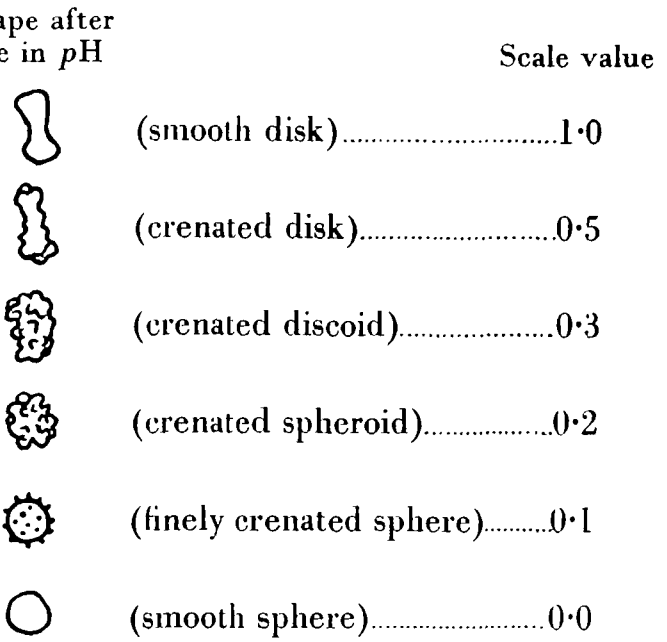

Fig. 1. Arbitrary scale of anti-sphering activity.

bead treatment, because the cells on somewhat longer standing showed less and less response to solutions containing anti-sphering factor.

In the attempt to determine what substance normally contained by serum and cells is the anti-sphering factor, it was found useful to set up an arbitrary semi-quantitative scale of anti-sphering activity. With this scale the activity of a given amount of serum and a given amount of some fraction separated from serum could be compared, and the activity shown by a given amount of serum could be approximately measured by the shape of cells in the 5 c.c. of previously factor-free suspension to which the serum had been added and the *p*H of which had been raised to about 10·5. The arbitrary activity values corresponding to the different shapes are given in Fig. 1. If the shapes varied in the same suspension, the predominating shape was used.

Using this arbitrary scale, the anti-sphering activity of horse serum is plotted against the quantity of horse serum in Fig. 2. If any single substance fractionated from horse serum is the anti-sphering factor, it should show just as much activity per given amount as that volume of serum containing the same amount of the substance, i.e. its concentration-activity curve should be the same as the curve in Fig. 4.

As pointed out in the previous paper (Furchgott, 1939), cells and sera of different mammalian species are interchangeable so far as the anti-sphering factor is concerned. That is, human serum furnishes anti-sphering factor for dog cells, horse

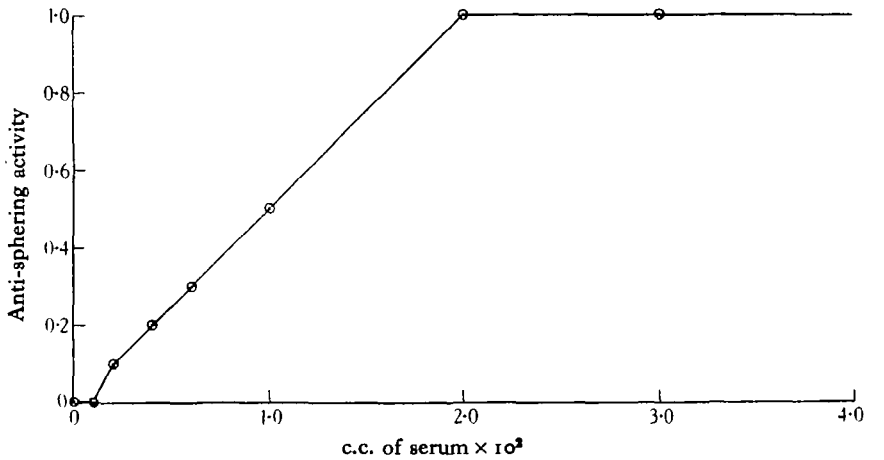

Fig. 2. Anti-sphering activity (ordinate) in a suspension of 5 c.c. of cells previously freed from the factor as a function of the volume of horse serum (abscissa) added to the suspension.

serum for human cells, etc. This was fortunate in the present work, for horse serum was the most suitable for fractionation, while human cells were the most readily available in a fresh condition. All the suspensions used were therefore prepared from freshly drawn human cells.

II. THE PROTEIN NATURE OF THE ANTI-SPHERING FACTOR

Several preliminary experiments indicated that the anti-sphering factor is a protein. The factor was adsorbed from cells and from serum on to glass surfaces as a protein might be. The anti-sphering activity of the serum was destroyed by heating the serum in a boiling water bath. When all the protein in serum was precipitated by saturation with Na_2SO_4, the supernatant fluid had no anti-sphering activity, while a solution of the precipitate had very much. If the precipitate was extracted with lipoid solvents, it did not lose its activity.

To obtain the various fractions of the serum proteins for the purpose of testing their separate activities, a fractionation of horse serum with Na_2SO_4 was carried out according to the method of Kekwick (1938). The total globulin fraction showed no activity, while the total albumin fraction showed a large amount of activity. On

separating the albumin fraction into a carbohydrate-rich and a carbohydrate-poor fraction, it was found that the carbohydrate-poor fraction had much more activity than the carbohydrate-rich fraction. A fractionation of the albumin fraction of horse serum was also carried out with $(NH_4)_2SO_4$ according to the method of Hewitt (1938*a*). Here again the carbohydrate-poor fraction, the crystalbumin of Hewitt, was extremely active, while the carbohydrate-rich fraction, the seroglycoid of Hewitt, had very little activity. This slight activity of the carbohydrate-rich fraction can probably be attributed to small amounts of the carbohydrate-poor fraction present as an impurity.[1]

The activity of solutions of purified crystalbumin (for convenience we are using this term of Hewitt in place of carbohydrate-poor fraction prepared either by Na_2SO_4 or $(NH_4)_2SO_4$ fractionation) was measured quantitatively with the use of the arbitrary scale mentioned in the preceding section. The results were the same with both kinds of preparations. The activity of a given amount of crystalbumin was as much as that of a volume of serum containing approximately the same amount of crystalbumin. The amount of crystalbumin in horse serum was calculated from Hewitt's estimate that 33% of the protein in horse serum is crystalbumin (1938*b*). The amount of crystalbumin in a purified solution was determined from nitrogen analyses by the micro-Kjeldahl method of Koch & McMeekin (1924), but employing a Summerson photoelectric colorimeter (1939). As a conversion factor for nitrogen to protein 6·6 was used. On the basis of activity-concentration curves, therefore, it appears that crystalbumin is the anti-sphering factor. To make sure that it is crystalbumin and not some closely adhering impurity, further experiments were performed.

III. FURTHER EVIDENCE THAT THE FACTOR IS CRYSTALBUMIN

(1) *A denaturation experiment.* If the anti-sphering activity of crystalbumin were due to an impurity, it might well happen that the rate of heat denaturation of the crystalbumin would be different from the rate of heat inactivation of the active impurity. If this were the case, the curve for activity of partially denatured solutions of crystalbumin against concentration of undenatured protein remaining after heating would not be the same as the activity-concentration curve for unheated solutions of crystalbumin. However, if the curves were the same, the possibility of crystalbumin being the factor would be strengthened.

The solution of crystalbumin used contained about 0·5 mg. N per c.c. It was made isotonic by the addition of NaCl. Two c.c. portions were put into seven 15 c.c. conical centrifuge tubes. These tubes were then dipped for varying periods from a few seconds to a couple of minutes into boiling water. The amount of heat denaturation depended on the time of heating. After the tubes had been cooled in the ice-box for half an hour, the coagulated denatured protein in them was centrifuged down and the supernatant solutions, containing the undenatured protein,

[1] A sample of purified $(NH_4)_2SO_4$ fractionated seroglycoid furnished us by Dr Hans Neurath of Duke University Medical School had no anti-sphering activity at all.

were decanted off. One c.c. of each solution was analysed for nitrogen. Five-tenths of a c.c. of each was tested for anti-sphering activity.

In Fig. 3 are plotted the results of a few such experiments, using the arbitrary scale for activity. Also in the figure are points obtained by testing the activity of various amounts of a solution of unheated purified crystalbumin. In view of the arbitrary nature of the scale, the points obtained with undenatured solutions and partially denatured solutions lie sufficiently close to the same concentration-activity curve to be evidence against the anti-sphering activity of crystalbumin being the result of an impurity.

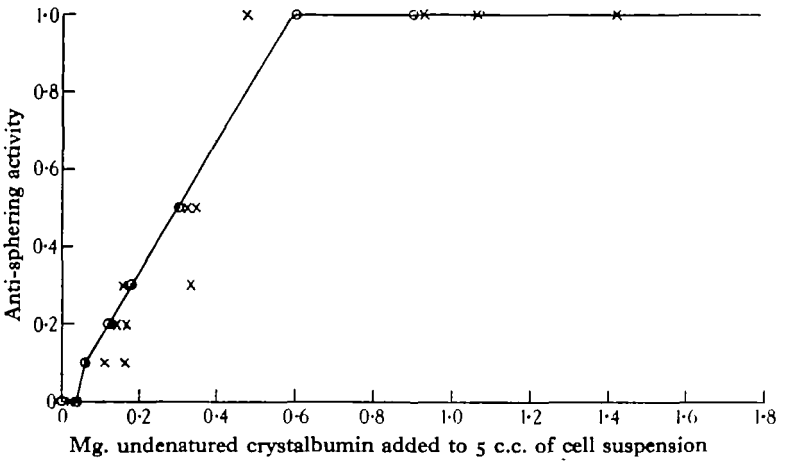

Fig. 3. Anti-sphering activity of undenatured (circles) and partly denatured (crosses) crystalbumin solutions as a function of the undenatured crystalbumin present.

(2) *An electrophoresis experiment.* Although it is impossible to free working quantities of cells of anti-sphering factor by washing, there must be, as pointed out in the last paper, a distribution of factor between the cells and the saline of the washing fluid with the equilibrium greatly in favour of the cells. Micro-Kjeldahl's were done on the saline washing fluids of cells previously washed free of serum, but the traces of nitrogen found were too small to be quantitatively determined. We decided to try to adsorb what little anti-sphering factor there might be in the saline washing fluids on to small amounts of quartz particles. The adsorption, if it occurred, could be detected by electrophoresis of the particles. Moreover, if the *p*H-mobility curve of the quartz particles treated with saline washing fluids was the same as that obtained with quartz particles coated with purified crystalbumin, it would be strong evidence that the anti-sphering factor is that protein.

The cells, generally from about 1 c.c. of fresh human blood, were first washed three times with 50 c.c. portions of saline. After each centrifuging practically all of the supernatant washing fluid was removed by a suction pipette, so that after the third washing the protein remaining from the original serum was negligible. The cells were then mixed with 50 c.c. of saline for the fourth washing, and after about 10 min. standing, the cells were centrifuged down. About .25 c.c. of the supernatant

saline were then decanted into another centrifuge cup, leaving all of the cells in the first cup. To the decanted supernatant was added about 25 mg. of finely powdered quartz. The mixture was allowed to stand for several hours, with stirring about every 10 min. The quartz particles were then centrifuged down and washed once with water before being tested in the electrophoresis cell.

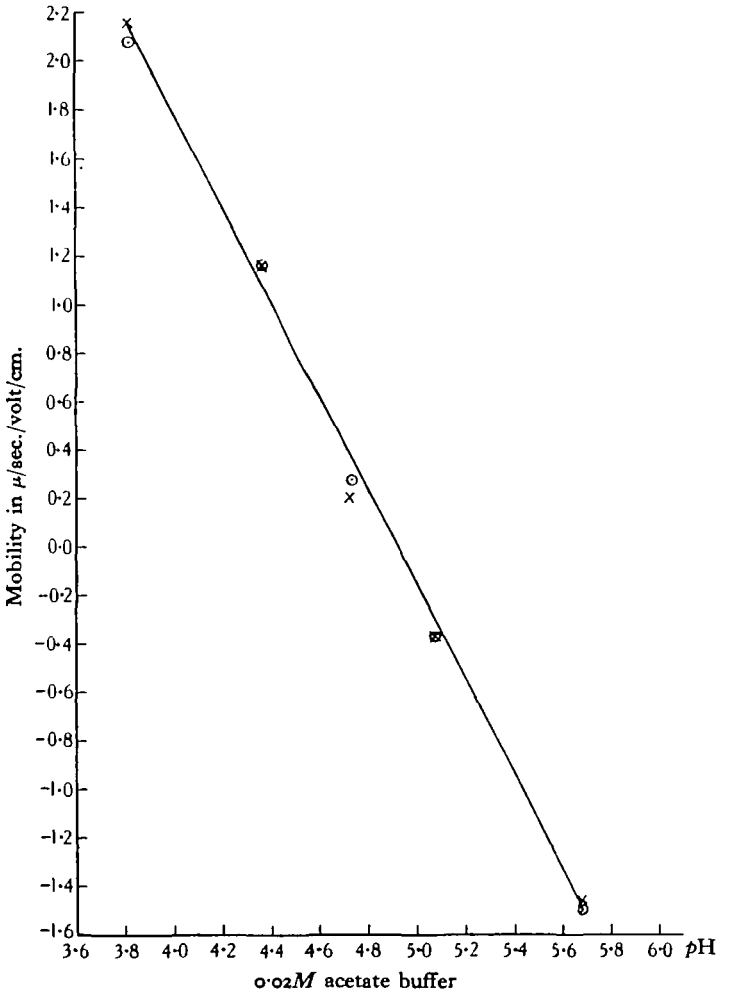

Fig. 4. Electrophoretic mobility in 0·02 *M* acetate buffer at 25° C. of quartz particles coated with crystalbumin (circles) and with protein from the supernatant washing fluid of serum-free red cells (crosses).

Since human cells were being used, the nature of this experiment demanded that human crystalbumin be used also. A preparation of it was made by the Na_2SO_4 fractionation of Kekwick (1938). Quartz particles were coated with it by mixing them with a solution of the preparation. These particles were also washed once with water before being tested in the electrophoresis cell.

The electrophoresis cell was of the Abramson type (1934). The technique used was that described by Abramson and more recently by Moyer (1936). The suspending solutions used in obtaining the *p*H-mobility curves of the particles were 0·02 *M* acetate buffer. The results of the electrophoretic measurements are shown in Fig. 4. Each point is the average of measurements on at least two different preparations.

The points for the particles coated by treatment with the saline wash agree remarkably well with those for the particles coated with purified crystalbumin. If anti-sphering factor is the substance adsorbed from the saline washing fluid, and evidence would indicate that it is, this electrophoresis experiment is further evidence that it is crystalbumin.

(3) *A quantitative experiment.* In view of the very large area of glass surface required to adsorb all of the anti-sphering factor from a small quantity of red cells, it looked as if the amount of the factor retained by washed red cells was of the order of hundreds of milligrams of factor per 100 c.c. of cells. Conversely, the amount of factor taken up by red cells, previously freed of anti-sphering factor, on addition of them to a solution containing the factor, might also be of that order. If crystalbumin were the anti-sphering factor, and if the concentrations of protein in a solution of it before and after the addition of factor-free cells were determined, a quantitative estimate of the amount of factor taken up by a given quantity of red cells might be obtained.

The red cells from 1 c.c. of blood were freed of anti-sphering factor. After the centrifuging following the bead treatment, the cells were suspended in about 6 c.c. of saline. Fifty c.c. of a solution of crystalbumin (about 0·07%) in 1% saline were placed in a centrifuge cup. Five c.c. of this solution were removed and analysed quantitatively for nitrogen. Then, to replace this 5 c.c. in the centrifuge cup, 5 c.c. of the cell suspension were added. The 50 c.c. of suspension now in the cup were stirred by rotation of the cup for 5 min. The cells were then centrifuged down, and two 5 c.c. samples were removed from the cell-free supernatant fluid and analysed for nitrogen. A count was made on the cell suspension of which 5 c.c. had been added to the crystalbumin solution. From this count, assuming that a count of five million correspond to 42% of cells by volume, we calculated the volume of cells and of solution added to the 45 c.c. of crystalbumin solution. From the total volume of supernatant fluid and the analyses made on aliquot portions of it, we calculated the total nitrogen content of the supernatant fluid. The theoretical total nitrogen content of the supernatant fluid, if the cells had taken up no protein, would have been 0·9 of the total content of the original 50 c.c. of crystalbumin solution. From the difference between the theoretical content and the content actually found by analysis, we estimated how much protein (using $6{\cdot}6 \times N$ to give the protein) was taken up by a given quantity of red cells.

The results of four such experiments are included in Table I. The rather large variations in the figures for grams of crystalbumin taken up per 100 c.c. of cells in the individual experiments may be attributed to the limitations of the micro-Kjeldahl analysis with such small amounts of nitrogen as were analysed for here

(of the order of 0·5 mg.). However, the results undoubtedly show that the cells take up a considerable quantity of crystalbumin—a quantity of the order of 800 mg. per 100 c.c. of cells.

Table I

1	2	3	4	5	6
Nitrogen content of original 50 c.c. of crystalbumin solution	Volume of cells in 5 c.c. of suspension added to 45 c.c. of crystalbumin solution	Theoretical nitrogen content of supernatant solution after addition of cells	Actual nitrogen content of supernatant solution from nitrogen analyses	Amount of crystalbumin taken up by cells (6·6 × difference between columns 3 and 4)	Amount of crystalbumin taken up by 100 c.c. of cells
mg.	c.c.	mg.	mg.	mg.	g./100 c.c.
5·32	0·24	4·79	4·57	1·45	0·61
5·89	0·25	5·30	4·93	2·44	0·98
5·42	0·18	4·88	4·70	1·19	0·66
6·48	0·17	5·83	5·59	1·58	0·93
				Average	0·80

Removal of crystalbumin from a solution by red cells previously freed of anti-sphering factor.

IV. ELECTROPHORESIS EXPERIMENTS

With the hope of finding out something about the physical mechanism of the phenomena of shape changes in red cells we made electrophoretic measurements on cells treated in the various ways described in this paper and in the previous one (Furchgott, 1939). If changes in the exterior surface of the cell were related to the shape changes, such changes might be detected by changes in electrophoretic mobility. The method of determining the electrophoretic mobilities of the cells was the same as used for determining those of the coated quartz particles, discussed earlier in this paper.

The first set of measurements was made on cells in $M/15$ phosphate buffer of pH 7·4 and of specific resistance of 126 Ω at 25° C. Measurements were made on washed cells containing anti-sphering factor, on cells freed of anti-sphering factor with glass beads, and on washed cells of which the activity of the anti-sphering factor had been counteracted by first shaking a suspension of the cells against the palm of the hand. This counteracting of anti-sphering activity, as pointed out in the previous paper (Furchgott, 1939) is probably due to fatty acids coming from the palm. Measurements were also made on ghosts which had been put through the temporary sphering process discussed in the previous paper. These ghosts, made by hypotonic lysis, were sphered by suddenly raising the pH of a suspension of them in isotonic saline to pH 9·5. After a few minutes, during which the spheres changed back to disks and discoids, the pH was readjusted to neutrality, and the ghosts were centrifuged down for the electrophoretic measurements.

The results of the measurements in phosphate buffer are included in Table II. The mobilities (all corrected to 25° C.) of the differently treated cells are easily within experimental error of one another. They agree very well with Abramson's

(1934) figure of 1·31 μ/sec./volt/cm. for the mobility of human cells in the same phosphate buffer solution.

The cells in the phosphate buffer were all discoidal. After finding no differences in their mobilities in that buffer, we decided to make measurements at a *p*H high enough to actually cause the cells to be either disks or spheres, depending on the treatment given them. For this purpose a glycine buffer was made up containing 5·5 parts of a glycine and NaCl solution (7·505 g. glycine and 5·85 g. NaCl per litre) to 4·5 parts of 0·1 *N* NaOH. To 10 c.c. of a mixture of 1 part of this buffer with 5 parts of 1 % saline was added a small amount of cells for electrophoresis measurements plus 0·05 c.c. of either a dilute solution of crystalbumin or distilled water, depending on the treatment desired. The *p*H of the resulting suspensions was 10·1 and the specific resistance at 25° C. was 66·7 Ω. Measurements were made on spherical cells deprived of anti-sphering factor by glass beads, on smooth discoidal cells, resulting from the presence of the crystalbumin added to the buffer, and on washed cells which were spheres in the buffer because a suspension of them had previously been shaken against the palm of the hand.

Table II

Conditions of cells	Mobility in μ/sec./volt/cm.
Cells containing the factor	1·30
Cells freed of the factor	1·29
Cells with activity of factor counteracted by fatty acid	1·30
Ghosts after temporary sphering	1·31

Electrophoretic mobility at 25° C. of differently treated human red cells in *M*/15 phosphate buffer at *p*H 7·4.

Table III

Condition of cells	Shape of cells	Mobility in μ/sec./volt/cm.
Cells containing the factor	Smooth disks	1·04
Cells freed of the factor	Smooth spheres	1·07
Cells with activity of factor counteracted by fatty acid	Smooth spheres	1·04

Electrophoretic mobility at 25° C. of differently treated human red cells in a saline-glycine buffer solution of *p*H 10·1.

The results of these measurements are given in Table III. Again the mobilities of the differently treated cells are within experimental error of one another.

To complete the electrophoresis experiments we investigated the mobility of cells made spherical by the addition of small traces of lecithin (Ponder, 1936). The suspending medium was a solution of 5 parts of 1 % saline to 1 part of *M*/15 phosphate buffer of *p*H 7·4 and it had a specific resistance at 25° C. of 64·4 Ω. Here again the mobilities of spherical and non-spherical forms were within experimental error of one another: 1·00 μ/sec./volt/cm. for the disks, and 0·98 μ/sec./volt/cm. for the cells sphered by lecithin.

V. DISCUSSION

This investigation appears to identify the anti-sphering factor definitely as crystalbumin, yet it does nothing, in a positive sense, toward clarifying the physical mechanism behind the phenomena of shape changes in red cells. If anything, it leaves the situation more puzzling than ever. The red cells take up crystalbumin in an amount of the order of 800 mg. per 100 c.c. of cells; such an amount is enough to form more than one monomolecular layer on the surface of the cells, yet from the electrophoresis data one can see that the exterior surface of the cells, which controls their electrophoretic mobility, definitely remains the same whether the cells have or have not taken up crystalbumin. Whether the albumin molecules go below the exterior surface of the cell membrane cannot be said.[1]

The electrophoresis experiments probably rule out surface tension changes as a possible explanation of the shape-change phenomena. The constancy of the mobility of cells put through the various treatments discussed here is in keeping with the constancy of mobility found for ghosts made by various means of haemolysis (Abramson, Furchgott & Ponder, 1939).

From the electrophoretic data it appears that spherical cells have the same charge per unit area of surface as the discoidal cells. This points to the conclusion that the transformation to the spherical form does not involve the compression of an exterior molecular film into a more condensed film. Rather, it may indicate a very fine submicroscopic crenation of the periphery of the cell in the spherical form, this being the culmination of the successively smaller microscopic crenations found in the intermediate forms between disk and sphere.

We cannot say how fatty acids counteract the action of the crystalbumin. Perhaps the molecules of the fatty acid compete with the protein molecules for certain positions in the membrane, in which the protein molecules may have to be if they are to exert their anti-sphering action.

VI. SUMMARY

1. Experimental evidence has been presented showing that crystalbumin (the carbohydrate-poor fraction of serum albumin) is the factor which prevents mammalian red blood cells from becoming spherical at *p*H values over 9·2.

2. The amount of crystalbumin taken up from a solution of it by red cells previously freed of it is of the order of 800 mg. per 100 c.c. of red cells. If this amount is all taken up at the red cell surface, it would form a layer only a few molecules thick.

[1] If all the crystalbumin taken up by the cells is supposed to be at the cell surface, it would form a layer of 45–60 A thick (assuming a density of unity and excluding possible contribution of water). Calculated on the same basis, the thickness of the red cell envelope is only about 120 A (Fricke, Parker & Ponder, *J. Cell. and Comp. Physiol.* **13**, 69, 1939), so the layer of crystalbumin would be from one-third to one-half the thickness of the cell's surface structure, but only a few molecules thick.

3. The electrophoretic mobility in phosphate buffer of *p*H 7·4 is the same for cells containing crystalbumin, cells free of crystalbumin, cells with anti-sphering activity counteracted by fatty acid, and ghosts which have been temporarily sphered by a rise in *p*H. The mobilities in a saline-glycine buffer solution of *p*H 10·1 for the first three classes of cells just mentioned are also the same. The mobility of cells sphered with lecithin in a saline-phosphate buffer solution is the same as that for untreated discoidal cells.

We wish to thank Dr Henry B. Bull of Northwestern University Medical School for his interest and for helpful suggestions relative to this work.

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