THE SPHERICAL FORM OF THE MAMMALIAN ERYTHROCYTE

IV. DISK-SPHERE TRANSFORMATIONS BETWEEN SURFACES COVERED WITH MONOLAYERS

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(With One Text-figure)

The most suggestive observation which we have regarding the remarkable disk-sphere transformation which occurs when mammalian red cells in saline are enclosed between slide and coverslip is that the velocity and extent of the shape change depends on the distance between the surfaces, and upon whether or not both surfaces are wetted by the suspension medium (Ponder, 1929). Thus the transformation occurs rapidly and completely between two glass surfaces 50 μ apart, but slowly and incompletely between two glass surfaces 1 mm. apart, and paraffining of both surfaces, or the substitution of surfaces unwetted by saline, prevents the shape change completely. That the transformation is due to electrostatic charges on the surfaces, pressure,2 or diffusion of alkali from the glass, has been disproved experimentally (Ponder, 1929, 1936), and the only suggestion which still remains is that the surfaces have an orienting effect, of an obscure nature, on a "liquid-crystal" structure in the red-cell envelope.

Since the extent to which the opposing surfaces are wetted by the suspension medium is of such importance, we have carried out experiments in which the glass surfaces were covered with hydrophilic and with hydrophobic surface layers. It is with these, and with certain unexpected results which have been obtained, that this paper is concerned.

EXPERIMENTAL

The results are best described under the heads of the experimental procedure.

Exp. 1. Slides and coverslips were coated with gelatin by dipping the glass into 0.1% gelatin (American Carbon Company, and subsequently electrodialysed) for 10 min., washing several times with water, and drying at room temperature. Washed human red cells in 1% NaCl, when placed between such gelatin-coated surfaces, become perfect spheres, just as if placed between two glass surfaces. The cells remain unhaemolysed for hours (Fig. 1A).

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2 Dr Douglas Marsland tells us that he has subjected red cells to as much as 7000 lb. pressure in a bomb without the spherical form being assumed.
Exp. 2. Slides and coverslips were coated with monolayers of Ca stearate according to Blodgett's (1935) technique. The monolayers were made by spreading a film of the purest stearic acid (Eastman Kodak), dissolved in redistilled petrol ether, on a solution of $\text{M/10,000 \ Ca (HCO}_3\text{)}_2$ at pH 6.6, the film being compressed to 30 dynes/cm. by a secondary film of oleic acid. The slides were placed beneath the surface of the solution before the film was spread, and were slowly pulled up through the film so as to coat them with a monolayer of the stearate; they were then dried at 40° C.

When both slide and coverslip are covered with monolayers of Ca stearate, the hydrophobic hydrocarbon chains directed outward from the surfaces, washed human red cells placed between them remain discoidal, even if the coated surfaces are only 50 μ apart. The coating of the surfaces with a hydrophobic film accordingly prevents the disk-sphere transformation, which agrees with Ponder's (1929) observation that coating with paraffin prevents the shape change. ¹

We were surprised to find, however, that the cells begin to haemolyse after about 3 min. under these experimental conditions (Fig. 1B), and it should be emphasized that lysis between two glass surfaces is never observed until hours have elapsed, except in the case of a very few cells. Between the two Ca stearate-coated surfaces, on the other hand, lysis is almost complete in 60 min., ² cells in succession changing from disk to sphere, and then disappearing from view. The spherical forms which the disks assume before lysis by any simple lysin, and these "prolytic spheres" are quite different from those seen between glass slide and coverslip; there is no difference in volume, but the prolytic sphere has a peculiar homogeneous appearance, and the diffraction bands at its edges are characteristic. Between Ca stearate-coated surfaces it fades slowly, the fading time being about 10–15 sec.

The interesting point about this progressive lysis is that we have found it to occur even when the cell suspension is made so concentrated that the total surface

¹ Waller's observation that red cells do not become spherical between slides and cover-glasses which have been freshly cleaned with ether is possibly accounted for by the fact that washing with ether is very apt to leave a thin film of hydrophobic material on the glass, unless the ether is exceedingly pure.

² If the number of cells which haemolyse from minute to minute, expressed as a percentage of the total, is plotted against time, excellent sigmoid curves result from these experiments. We have never observed anything which would support Kesten's claim (Kesten, 1929) that the percentage haemolysis curve is concave to the time axis instead of sigmoid, when it is based on observations of the rate of lysis of individual erythrocytes.
of the cells between the opposed Ca stearate-coated slide and coverslip is several times greater than the surfaces of the slide and coverslip themselves. Since the glass surfaces are covered with monolayers only, it will be clear that even if all the stearate molecules were to leave the glass surfaces under these conditions (and they are usually regarded as being very firmly attached to the glass), there would not be enough molecules to form a monolayer on the surface of the cells of the preparation.

Exps. 3 and 4. Coating either the slide or the cover-glass, but not both, with a monolayer of Ca stearate does not prevent the disk-sphere transformation, which is in agreement with Ponder's observation that coating only one of the surfaces with paraffin does not prevent the shape change. When only one surface is covered with the monolayer, however, lysis does not occur as it does when both surfaces are coated; with both surfaces coated, lysis is usually complete within an hour, but when only one surface is coated at least 95% of the cells are intact at the end of that time. It is immaterial whether the coated surface is the upper or the lower one (Fig. 1C, D).

Exp. 5. The fact that lysis occurs between two stearate-coated surfaces, but not when one only is coated, is puzzling at first sight. It is true that the cells are exposed to only half the number of stearate molecules in the latter case, but this alone cannot account for the absence of lysis. A cell suspension containing $10^6$ cells/cc. will undergo complete lysis between two coated surfaces, but one containing $10^7$ cells/cc. will remain unhaemolysed if only one surface is coated; if the concentration of stearate molecules were the determining factor, the decrease by a factor of 100 in the cell number would surely offset the decrease by a factor of 2 in the number of stearate molecules available from the one surface only. Any explanation of this sort, moreover, tacitly supposes that the stearate molecules leave the glass surfaces to react with the cells, which they almost certainly do not, and it can be shown that even if they did, they would exist in a concentration far too small to be lytic. If all the stearate molecules were to leave the glass and become distributed in the volume of fluid between the surfaces (about 0.05 c.c. in our experiments), the lysin dilution would be of the order of 1 in $10^8$.

It finally occurred to us that an important difference between the condition in Fig. 1B, in which lysis occurs between the two coated surfaces, and that in Fig. 1C, in which no lysis occurs when only the slide is coated, is that in the former case the cell is a disk, and presents a considerable surface to the Ca stearate monolayer when it settles on to the slide; in the latter case it is a sphere, and so presents only a very small surface of contact. (The condition in Fig. 1D, may now be left out of consideration, for here the cell is a sphere, and settles on to a glass surface; there is no lysis, nor would any be expected.) We accordingly modified the condition in Fig. 1C, so as to ensure that the cells would remain discoidal. This is done by cementing four strips of glass, about 1 mm. thick, to a slide, so as to form a square chamber, closed except for a few mm. at one corner. The upper surfaces of the strips are lightly vaselined, and a Ca stearate-coated coverslip pressed down on them; the slide is then inverted so that the coverslip forms the floor of the chamber, which is filled with the red cell suspension with a capillary pipette.
Within about 5 min., the cells of the suspension sediment out on to the lower (coated) surface of the chamber, and can be seen to be discoidal, the distance between the floor and the roof of the chamber (1 mm.) being too great to bring about sphere formation (Fig. 1 E). After from 5 to 10 min., however, the haemolytic process begins exactly as in Exp. 2; one by one, the cells become prolytic spheres and haemolyse, so that lysis is complete in about an hour. The difference between this result and that obtained in Exp. 3, in which there was no lysis, appears to be that in this experiment the cells were allowed to come into contact with the stearate monolayers while discoidal, thus presenting a considerable surface to the monolayer; in Exp. 3, on the other hand, the small distance between slide and cover-glass brought about sphere formation, with a very small area of contact with the stearate-coated surface.

**DISCUSSION**

These experiments show that a red cell can haemolyse when less than half of its surface is in contact with a monolayer of a lytic substance, and when there are not enough lysin molecules to cover the whole cell surface with a monolayer. Since the Ca-stearate monolayer in contact with which the cell haemolyses has its hydrophobic groups directed away from the glass and towards the cell, the hydrophobic groups in the cell membrane may be strongly attracted to the monolayer, and the result may be a special case of "contact haemolysis".

An interesting point lies in the length of the "fading time" of the cells which haemolyse in contact with these monolayers (Fricke, 1934). The fading time is between 10 and 15 sec., and is about the same as that observed in the most dilute saponin systems which are lytic (12 sec., Ponder and Marsland, 1935). It has been suggested that in the latter systems the amount of lysin present is just enough to cover the cell surfaces with a monolayer (Gorter, 1937); if the fading time is dependent on the concentration of lysin, as it seems to be (Ponder and Marsland, 1935), the fading time in dilute saponin systems would accordingly be what one would expect on Gorter's hypothesis.

From the fading time of the cells in contact with the monolayers can be calculated either the permeability of the entire membrane to haemoglobin, \( \mu_H \), or \( N_d \), the number of holes of diameter \( d \) (in \( \mu \)) through which pigment would escape if the membrane were to break down in spots. For a fading time of 12 sec., \( \mu_H = 0.186 \times 10^{-4} \) cm./sec., i.e. the rate of pigment diffusion is less than 1/1000th of that which would occur if the membrane were completely permeable. Alternatively \( N_d = 0.25 \mu \), which corresponds to one hole of diameter 2500 A., ten holes of diameter 250 A., 100 holes of diameter 25 A., and so on. Presumably there is more than one hole, and less than 100, for the former case is scarcely compatible with the results for the capacity and frequency dependence of the red cell membrane (Fricke and Curtis, 1935), and a diameter of 25 A. is small compared with the diameter of a haemoglobin molecule; ten to twenty holes of 250-125 A. diameter would therefore meet requirements, but it must be noticed that their combined
area would be very small, and less than 1/10,000th of the surface of the membrane exposed to the monolayer. The existence of such "key spots" has been already suggested by Abramson (1934) on the basis of the constancy of the $\zeta$-potential before and after lysis.

It should, of course, be emphasized that because lysis can occur when only such a small fraction of the total surface is involved, it is not to be thought that lysins in greater concentration do not involve much larger areas. Thus with 1 in 100 saponin, the fading time is about 0.23 sec., to account for which we have to postulate 1600 "permeable patches" each 250 A. in diameter, which amounts to about 1% of the cell surface; if there were fewer holes of greater diameter, the fraction of the cell surface involved would, of course, be greater.

Unfortunately these two possibilities are not mutually exclusive, for we can have $N$ areas of diameter $d$ and of finite permeability $\mu_H$ instead of $N$ completely permeable holes or instead of a permeability $\mu_H$ of the entire membrane. Under such circumstances, the product $Nd/\mu_H$ is a constant for any value of $t$ except extreme values, i.e. a fading time of 12 sec. might approximately correspond to a permeability $\mu_H = 0.186 \times 10^{-4}$ cm./sec. over the whole surface, to $\mu_H = 18.6 \times 10^{-4}$ cm./sec. over 1% of the surface, or to $\mu_H = 186.0 \times 10^{-4}$ cm./sec. over the 0.01% of the surface occupied by the ten "holes" of 250 A. diameter. The whole question of a "key spot" or local action must be decided on independent grounds.

The experiments do not throw much further light on the causes underlying the disk-sphere transformation between slide and cover-glass, except that they show very clearly that the transformation does not occur if either of the opposing surfaces is covered with a hydrophobic layer.

**REFERENCES**


