CII. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES

IV. THE PERMEABILITY OF "GHOSTS" TO CATIONS

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THE cellular residue, obtained by centrifuging a haemolysed suspension of the red cells of certain species, e.g. the rabbit (the erythrocyte "ghosts"), forms the basis of the present study, which is concerned with the extent to which the erythrocyte is able to maintain its integrity as a cell after it has passed through the phase of permeability to the large molecule of haemoglobin.

The work of Fricke & Curtis [1935], who showed that the red cells of the rabbit etc. may exhibit very similar electrical impedance both before and after haemolysis, suggests the possibility that the normal impermeability to cations, which is the most characteristic property of the red cell, may be maintained, at least in part, after the escape of haemoglobin. The observation of Ponder & Marsland [1935] that during saponin lysis the pigment may escape from the red cell at a rate very much less than that required for diffusion through an aqueous medium, further indicates that during the actual escape of haemoglobin the red cell membrane still presents certain structural impediments to the free passage of this molecule. Further evidence of the at least partial maintenance of the original structure is provided by the microscopical appearance of the ghost after "reversal of haemolysis", for the form is more or less that of the typical bi-concave disk, although it is true that the disk-sphere shape transformation cannot be effected either by enclosure between slide and cover-glass, or by addition of lecithin.²

One of the most striking properties of the erythrocyte is its cation impermeability, which may be used as an index of the degree to which the cell membrane is altered during and after the escape of haemoglobin. In this paper the permeability of the ghost to cations is studied by direct chemical methods.

EXPERIMENTAL

The essential of the procedure is to suspend a quantity of ghosts containing a known amount of K^+ or Na^+ in a given quantity of isotonic NaCl or KCl, and to determine the K^+ or Na^+ in them after centrifuging. The amount of cation found in the ghosts after this treatment is compared with that required for an equal distribution of the cell cation between the ghosts and the external medium. For the K^+ studies the ghosts were formed by addition of rabbit blood to 0.04 *M* KCl, centrifuging and washing the cells in 0.16 *M* KCl; by preparing them in this way one can be sure that they will contain appreciable quantities of K^+ . The washing with 0.16 *M* KCl causes the so-called "reversal" of haemolysis, whereby the ghosts become impermeable to haemoglobin. 0.5 ml.

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² One of us will shortly publish an account of the shape changes which occur in the case of the ghosts produced by hypotonic lysis.

of the concentrated suspension is added to varying amounts of 0.16 *M* NaCl and after the appropriate interval centrifuged down, the supernatant fluid is removed and the K^+ is determined in the cells as described by Davson [1934]. For studies of Na^+ penetration, the haemolysis was brought about in 0.04 *M* NaCl and the subsequent "reversal" produced with 0.16 *M* NaCl; the ghosts were then suspended in 0.16 *M* KCl. By using graduated centrifuge tubes an approximate estimate of the volume of cells, whose cation content is determined, may be obtained. The amount of cation in the original cell suspension is determined separately.

Results

A number of preliminary experiments using a rather simpler technique than that described above gave ample proof that the ghosts are quite permeable to cations, it being quite possible to wash out all the K^+ with two successive washings with NaCl, so that the only problem which remained was to show whether the time required to reach diffusion equilibrium were greater than that necessary for manipulating the cells (6–10 min.) or not. In Table I, Exps. 12, 13 and 15 were performed in the manner described above; they show first that there is a very rapid loss of Na^+ and K^+ and secondly that this loss approximates fairly closely to that required for diffusion equilibrium; the general trend of these experiments and of a number of others not shown, however, appeared to indicate a greater approximation to equal distribution of cation between the two phases after the lapse of 15 min. than when the interval was smaller (by "0 min." is meant the minimum time it was possible to keep the cells in contact with the suspension medium). As the errors in this technique are large compared with those in erythrocyte studies, owing chiefly

Table I

Exp. no.	Dilution. Cells: suspension medium	Initial con- centration	Final con- centration	Theoretical final con- centration	Time min.	Temp. °C.
12 (Na^+)	1 : 5	100	16.5	14.5	0	20
	1 : 9	100	8.9	8.9	0	
	1 : 21	100	4.5	3.3	0	
13 (Na^+)	1 : 21	100	3.0	2.4	0	20
	1 : 21	100	2.0	2.4	15	
	1 : 21	100	2.0	2.4	60	
15 (K^+)	1 : 5	100	12.6	11.1	0	20
	1 : 9	100	7.5	4.9	0	
	1 : 21	100	2.5	2.4	0	
	1 : 5	100	12.0	12.4	15	
	1 : 9	100	7.3	7.7	15	
	1 : 21	100	2.5	3.0	15	
17 (K^+)	1 : 9	—	106	100	0	20
	1 : 9	—	106	100	15	
	1 : 11	—	104	100	0	
	1 : 11	—	108	100	15	
20 (K^+)	1 : 6	—	101	100	0	0
	1 : 6	—	97	100	2	
	1 : 6	—	97	100	10	
	1 : 6	—	102	100	30	
22 (K^+)	1 : 6	—	106	100	0	0
	1 : 6	—	107	100	0	
	1 : 6	—	102	100	20	
	1 : 6	—	105	100	20	

to the difficulty in sedimenting the ghosts satisfactorily and the small amounts of cation left in the cells for analysis, it must be conceded that a systematic error in the direction of a decreasing concentration of cation in the cells with time might easily be involved. To make quite sure of this point a set of experiments was carried out in which the K^+ contents of both the suspension medium and the cells, equal amounts of which being measured out in micro-pipettes, were deter-

mined directly. This proved to be an improvement on the technique described earlier chiefly because the necessity for centrifuging the cells down completely is not so great, and the use of micro-pipettes gives an accurate estimate of the volume of material used for analysis.

The results of some of the experiments done in this manner are shown in the same table (Exps. 17, 20 and 22). The value of the concentration of K^+ in the supernatant fluid is put at 100 in the "Theoretical column" and the amount of K^+ in an equal volume of cells is shown in the "Final concentration" column as a percentage of the theoretical. It must be concluded from these results that the time taken to achieve diffusion equilibrium is less than that required for the manipulation of the cells, even when the temperature is kept as low as 0° . The failure to obtain equal quantities of K^+ in cells and supernatant fluid, manifest particularly in Exps. 17 and 22, is most probably due to the presence of unhaemolysed cells in the ghost preparation, a difficulty it has been found almost impossible to avoid. It should perhaps be mentioned that in one experiment at 0° values for the cell concentration of K^+ of 157, 130, 114 and 118 after intervals of 0, 2, 5 and 15 min. respectively were obtained. As it has been found impossible to repeat such a result, it must be concluded either that the ghost preparation was unusually resistant to the migration of cations or that it contained an unusually large number of unhaemolysed cells, and that the decrease in apparent K^+ concentration with time represented fortuitous variations in the quantities of these cells sucked up in the pipettes.

DISCUSSION

1. *Electrical properties.* The experimental section of this paper indicates that the erythrocyte ghost is rapidly permeable to cations; the rate of migration may be, nevertheless, very much less than that which would occur were the cation diffusing through an aqueous medium. The fact that the ghosts retain the electrical properties of their parent erythrocytes, although somewhat modified, is not necessarily at variance with this result because of the way in which the conductivity data are obtained. In the conductivity method [Fricke & Curtis, 1935] the resistances of the suspension of ghosts and of the suspension medium are measured, and the conductivity of the ghosts can then be calculated provided that their volume concentration is known. The volume concentration may be found, somewhat roughly, by use of the high speed haematocrite, or it may be assumed to have a certain value, as in Fricke & Curtis' investigation; in either case, the figure for the volume concentration is subject to some error. If this error is 10%, say, then the calculation will give the ghosts a conductivity amounting to at least 10% of that of the suspension medium. All that Fricke & Curtis showed was that the conductivity of the ghosts could not be greater than 10% of that of the surrounding fluid, and that it was probably very much less; indeed, their experimental results were compatible with its being zero, when all possible sources of error were taken into account. But the results are also compatible with the ghosts' having a small conductivity, of the order of a few % of that of the suspension medium corresponding to a rate of cationic diffusion which might be very much greater than that observed here. Thus the present paper serves to emphasize what Curtis [1936] seems willing to admit, namely that the electrical properties of cell suspensions determined by present methods are a poor index to the actual permeability of the cell membrane.

2. *Reversal of haemolysis.* The problem of the essential difference between the intact erythrocyte and the haemolysed ghost has now to be discussed, and at first the discussion will be confined to the ghost formed after hypotonic haemolysis. If the erythrocyte in hypotonic saline swells up so that its envelope is subjected to strain, the envelope allows the passage of haemoglobin and of course of the cations Na^+ and K^+ ; in consequence of this interchange further strain on the membrane is avoided, and if the latter is not elastic, it will tend to

remain in its stretched state.¹ Washing the ghost with further quantities of hypotonic saline will then wash out further quantities of haemoglobin; if, on the other hand, a stronger saline is used, e.g. 0.16 *M* NaCl, the contraction of the ghost due to the osmosis of water is sufficient to restore the ghost to a haemoglobin-impermeable cell, and further washing with the stronger saline causes no loss of the haemoglobin remaining inside. The results of the present work show that the ghost in this restored state is still permeable to cations, although the rate of exchange may be slow compared with what it was during the actual escape of haemoglobin.

The ghost in the restored state after osmotic haemolysis is certainly an unstable system, since it still contains some haemoglobin, and is separated from an aqueous salt solution by a salt-permeable membrane. This point may be demonstrated in a variety of ways, one of which is by measuring the conductivity of ghost suspensions after reversal of haemolysis. To 1 ml. of washed rabbit cells, contained in a small conductivity cell, are added 4 ml. of water, and a few minutes later 1 ml. of 5% NaCl (this produces the reversal of haemolysis), and conductivity measurements at 1000 cycles are made minute by minute (Table II). The slow increase in resistance of the cell suspension is best accounted for on the basis of a slow increase in volume of the cells, due to the entry of water. This contributes to the re-haemolysis which follows the reversal after a time, and which is characterized by the suspension becoming more and more translucent. Bayliss [1924] explains the re-haemolysis on the basis of the cells being permeable to salts and slowly swelling, to which we would add that the translucence is contributed to by some agglutination.

Table II

Time after reversal, min.	Resistance, ohms
1	102.0
2	104.8
3	106.1
5	108.4
10	111.2
20	112.1
30	113.4

3. *A possible dual mechanism for haemolysis.* While searching for an explanation of the only partial nature of the reversal of haemolysis by hypertonic solutions the following hypothesis was examined, namely that haemolysis occurs as a result of two processes: (*a*) the stretching and rupture of the lipid envelope producing permeability to cations, and (*b*) the stretching of a protein mesh-work (the stroma protein Boehm [1935]) resulting in permeability to haemoglobin. In the case of osmotic haemolysis, both of these processes occur simultaneously, and we can explain the reversal of haemolysis by the contraction of the protein mesh-work with resulting impermeability to haemoglobin, while permeability to cations remains, as the lipid layers are irreparably damaged. Where lysis is brought about by lipid solvents, which presumably would not attack the

¹ It is better to speak of a distorted state instead of a stretched state, for when red cells swell in hypotonic systems, the surface area does not increase measurably, and the cell attains its greater volume by changing its shape from disk to sphere, preserving its surface area unchanged. This has been shown simultaneously and independently by Ponder [1937] and Castle [1937]. One may, however, postulate a very small amount of stretching as a final process, although the amount cannot be measured.

stroma protein, the condition (b) is only fulfilled as the result of a secondary process of swelling consequent upon the free permeability to cations caused by the destruction of the lipid envelope. If this hypothesis is true, then the fading time, i.e. the time required for 90% of the pigment to leave the individual haemolysing cells, of erythrocytes undergoing lysis by chloroform should be of the same order as that for a cell undergoing hypotonic lysis.

Simple visual observation of cells haemolysing in systems containing chloroform, ether, amyl alcohol and other lipid solvents showed clearly that the fading time was in the neighbourhood of 5 sec., very much longer than the 0.6–0.8 sec. given by Ponder & Marsland [1935] for osmotic haemolysis.

This contradiction would disappear, however, if it could be shown that the permeability of the red cell to haemoglobin produced by hypotonicity were not the "all-or-none" process it has hitherto been considered to be [v. e.g. Ponder & Marsland, 1935], but rather that varying states of permeability may be induced by different degrees of stretching. Thus if this were true the fading times obtained by Ponder & Marsland for osmotic haemolysis would be minimal ones, since their hypotonic systems were prepared by micro-injection of very hypotonic solutions, or even of water itself, into droplets of red cell suspensions; the resulting tonicity was accordingly very low, and the swelling of the cells very rapid. Under the conditions of a Donnan swelling the process would necessarily be much slower and so experiments were carried out in which the osmotic pressure differences were controlled so that the rate of swelling was slow. To do this, use was made of the fact that the osmotic pressure within the red cell decreases with increase of temperature, so that cells which are swollen almost to their critical volume at 37° swell further when the temperature is lowered to 25°, say, and, reaching their critical volume, haemolyse. By trial, a tonicity of NaCl was found in which a rabbit red cell suspension would remain intact on a warm microscope stage at 37° but would undergo partial lysis at room temperature, and the temperature of the warm stage was allowed to drop while the cells were being observed. Under these conditions of very slow swelling, the fading times were found to be very much larger than in Ponder & Marsland's systems, and to average from 5 to 10 sec.¹

Thus the interesting fact that haemoglobin may escape at different rates from the red cell during hypotonic swelling according as the conditions of swelling are varied is brought to light by this investigation; consequently hypotonic haemolysis must not necessarily be considered as a complete rupture of the erythrocyte membrane, but must be provisionally regarded as a state of permeability to haemoglobin, due to a mechanical deformation of the membrane.

The dual theory of haemolysis put forward above, is thus not at variance with the facts so far described. That it is nevertheless probably wrong is shown by the following experiment. A suspension of erythrocytes is haemolysed with chloroform, and the ghosts are centrifuged down; after removing the supernatant fluid the ghosts are washed with hypertonic saline and again centrifuged down. If the factor responsible for the impermeability to haemoglobin were merely a contraction of the protein mesh-work, then in these circumstances the ghosts should be impermeable to haemoglobin, and should appear as a bright red layer at the bottom of the centrifuge tube. Such was not the case; washing with

¹ We are not sure whether or not there is a concentration dependence of the fading time in the case of lipid solvents as with saponin. If these act by dissolving the lipid layers in an all-or-none fashion, one would expect there to be none. Nevertheless, more concentrated solutions of chloroform, ether etc. seem to give shorter fading times than less concentrated ones. The very laborious cinematographic technique would be required to settle the question unequivocally.

hypertonic saline merely caused the removal of more haemoglobin from the ghosts, so that they appeared as a white layer. Since it is fairly safe to assume that chloroform is haemolytic because it attacks the lipid constituents of the membrane, the failure to bring about the reversal of haemolysis must be attributed to a partial dissolution of this constituent, and one may state further that the presence of lipid in the membrane is a necessary condition for impermeability to haemoglobin, and that a shrinkage of the stroma protein is not sufficient of itself to bring about this state. (This argument is not absolutely conclusive, however, as it may be that the osmosis of water from the cell on washing with hypertonic saline was not rapid enough to cause the shrinkage of the stroma protein.)

The facts established above, taken in conjunction with earlier work by Davson [1937] on the loss of K^+ during swelling, show that there is a continuous process of change occurring in the red cell membrane during osmotic haemolysis. Before the critical volume is reached there is a reversible stage of slow cation permeability. Then there comes a stage at which haemoglobin just begins to leak out, with a fading time of about 10 sec., which corresponds to a rate of diffusion from the cell less than 1/250th of that expected on the basis of free diffusion. Finally in very hypotonic solutions, further stretching of the membrane causes a very rapid loss of haemoglobin with a fading time of less than 1 sec., which corresponds to a rate in the neighbourhood of 1/10th of that required for free diffusion through an aqueous medium.

There is an interesting consequence to the observation that the process of lysis occurring in hypotonic solutions may take as long as 10 sec. The cell, while permeable to haemoglobin must necessarily be so permeable to Na^+ and K^+ as to offer no restriction to their migration, owing to the smaller volumes of these ions, so that a Donnan equilibrium appropriate to the diffusibility of both cations and anions should be established within about 1/50th of a sec.¹ from the beginning of the escape of haemoglobin. If this equilibrium is established, a difference of osmotic pressure will be brought into play corresponding to a concentration difference of about 0.01 *M* NaCl. This difference will be accompanied by a rapid diffusion of water into the cell producing further stretching and consequent acceleration of the rate of escape of haemoglobin. It thus seems that such long fading times would be excluded by these considerations; the difficulty can be got over by ascribing the leakage of haemoglobin in the first instance, not to a uniform stretching of pores over the whole surface but rather to the formation of one or more holes, large enough to permit the unrestricted passage of many haemoglobin molecules simultaneously, so that the restraint to the penetration of haemoglobin consists merely in the limited area of these holes in comparison with that of the rest of the surface of the membrane, and not in the energy required for an individual molecule to pass through any particular pore of a size comparable with its own. In this event, the rate of achievement of a Donnan equilibrium will be considerably restricted: thus if the area of the hole is 1/250th of the area of the membrane, the time required for this process will be 250 times greater than if free diffusion were possible all over the surface.

Thus, whereas the evidence from the fading times of cells undergoing hypotonic lysis appeared to indicate that the escape of haemoglobin should be attributed to a permeability to haemoglobin in contradistinction to a complete

¹ This value is obtained by use of Fricke's equation [1934] using a value for *D* for NaCl of 1×10^{-5} cm.²/sec. It is obviously only approximate.

collapse of the cell membrane, further consideration leads to a conclusion midway between these two extremes, i.e. that the escape of haemoglobin occurs through one or more holes whose total area may vary from 1/10th to 1/250th of that of the area of the cell. If this is true, the diffusion coefficients appropriate to the different fading times of cells undergoing lysis calculated by Fricke [1934] should be interpreted as estimates of the effective areas available for diffusion.

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