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The Physical Instability of Human Red Blood Cells*

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It is well established that the internal contents of the red cell are not in thermodynamic equilibrium with its environment, but are maintained at a steady level by active processes. While some of the structural components, and the haemoglobin are metabolically inert (Muir, Neuberger & Perrone, 1952), it has been shown that the cell lipids are in a condition of rapid metabolic turnover (Muir, Perrone & Popják, 1951; Altman, 1953). The exchange of cholesterol between the cells and their plasma by diffusion has also been reported (Gould, 1951). The integrity of the cell depends upon the presence of the lipids; if it is assumed that these substances are free to diffuse away, then the cell itself can be considered as a steady-state system maintained intact by metabolic activity. This notion receives support from the observed changes in red cells during cold storage.

Lovelock (1954) reported that cells stored at temperatures between 0 and −79° may lose a considerable proportion of their membrane lipids, and that this loss precedes their haemolysis. Cold storage at −20° or lower is an effective means of arresting metabolic activity, but diffusion processes can still continue at low temperatures although at a reduced rate.

The investigation of the dissolution of red cells at temperatures as low as −79° is necessarily slow, and complicated by the presence of ice and the high viscosity of the suspending medium, which always includes glycerol. This paper reports experiments on the diffusion of lipid components from the red cell and its ensuing dissolution at temperatures above 0°. The loss of components was accelerated by suspending the cells in media maintained unsaturated with these components. This was achieved either by repeated washing or by including a neutral adsorbent substance, namely alumina. The progress of the dissolution of the cells is described and the structure and state of the cell membrane discussed in the light of the experimental observations.

* The experimental data recorded in this paper formed the basis of a dissertation which gained a Ciba Foundation Award (1954–5) for a paper descriptive of Basic Research Relevant to the Problems of Ageing.

METHODS

Red blood cells. Human blood to which anticoagulant had been added was stored at 4° until required for use. The anticoagulant solution was composed of trisodium citrate, 0·073 M; citric acid, 0·035 M; and glucose, 0·01 M. It was added to fresh blood in the proportion of 15 ml. of anticoagulant to 100 ml. of blood. Blood older than 10 days was not used.

Alumina. Commercial chromatographic alumina was used; the particles of this substance were approximately 100 μ. in diameter.

Procedure for repeatedly washing red cells. Stored blood was centrifuged at 3000 *g* for 20 min. The supernatant plasma and the top layer of cells were then pipetted off. The cells were then suspended in an equal volume of lightly buffered solution (NaCl, 0·15 M; Na_2HPO_4, 0·05 M; KH_2PO_4, 0·05 M; and glucose, 0·01 M) centrifuged, and the supernatant and top layer of cells pipetted off. This procedure was used in order to remove as much as possible of the plasma with the minimum of washing. The volume of supernatant trapped with the cells at each of the above stages was less than 10%, so that the final concentration of the original plasma remaining with the packed cells was less than 1·0%. Packed cells (10 ml.) were then transferred to graduated centrifuge tubes and 11 ml. of the buffered glucose NaCl solution added to suspend them. The suspension was left for 10 min. in a water bath at 37°, then centrifuged for 5 min. at 3000 *g*, and 10 ml. of the supernatant were removed for analysis and the volume of the cells observed. A further 10 ml. of sodium chloride solution at 37° were then added and the suspension was returned to the water bath for a further 10 min. at 37°. This procedure was repeated 20 times.

Exposure to alumina. Powdered alumina was weighed into test tubes and 9·0 ml. of sodium chloride solution (NaCl, 0·15 M; Na_2HPO_4, 0·05 M; glucose, 0·01 M) added. After standing for 15 min. during which the suspension was gently agitated sufficient 0·1 M-KH_2PO_4 solution was added to lower the pH to 7·0. The final phosphate ion con-

centration was usually 0·015M. Red blood cells (1·0 ml.) packed and washed according to the method described in the previous section were then added, and the suspension mixed by inverting the tube twice. The suspension was kept in a water bath at the required temperature and gently mixed by inverting the tube once every minute. After various intervals the supernatant red-cell suspension was pipetted off for analysis. The alumina was then washed twice with 0·16M-NaCl solution to remove trapped red cells, and the washings added to the remainder of the red-cell suspension. The washed alumina was analysed separately for the lipid components adsorbed upon it. In experiments at temperatures below 0°, 2·0M glycerol was included in the suspending medium to prevent freezing.

Measurement of haemolysis. After exposure to the various experimental conditions the red cells remaining undamaged were removed by centrifugation. The supernatant solution was then diluted with 0·1% Na_2CO_3, shaken to convert the haemoglobin to oxyhaemoglobin, and the concentration of the latter measured in terms of the absorption of light at 5200 Å.

The analysis of lipids and lipoprotein. For the analysis of cholesterol, phospholipid, lipoprotein, or for the dry weight of lipids, a common extraction procedure was used as follows: 1 vol. of material for analysis (red cells, suspending media, or alumina) was added to 25 vol. of 2:1 (v/v) ethanol–ether mixture. This was heated to boiling for 5 min. and then left for 2 hr. at room temperature. The ether–ethanol solution was filtered and divided into three portions which were then evaporated to dryness in a water bath at 50° under reduced pressure from a water pump. The dry weight of material extracted was obtained by direct weighing and the lipids analysed as follows: *Cholesterol.* This was estimated by the method of Bloor (1916). No attempt was made to analyse the cholesterol esters separately. The cholesterol in these experiments originated almost entirely from the cells and the proportion of cholesterol esters present is likely to have been small. *Phospholipid.* The lipid phosphorus was estimated by the method of Youngberg & Youngberg (1930).

Lipoprotein. The total lipid nitrogen was estimated by the method of Koch & McMeekin (1924). The lipoprotein nitrogen was obtained from the total nitrogen by subtracting from it the nitrogen contributed by the phospholipid. The accuracy of the lipoprotein estimations depended principally upon solubility in the 2:1 ethanol–ether mixture. The analysis of red cell suspensions by the methods just described for both the total dry weight of membrane lipids and for lipid nitrogen gave values in good agreement with those quoted in a monograph on the red cell (Ponder, 1948). The solubility of lipoprotein is nevertheless greatly dependent upon small changes in the physical environment, and the possibility remains that some of the figures given for the dry weight of cell lipids and lipoprotein nitrogen are less than the true values.

RESULTS

The effects of repeated washing. Fig. 1 shows the progressive removal of lipid components from the red cell during twenty successive resuspensions in 0·16M sodium chloride. The loss of volume and haemolysis during the experiment are also shown. The cells used in this experiment had been stored for 10 days at +4°. The loss of components from freshly drawn cells is between one-half and one-third as much, but proceeds in a similar manner.

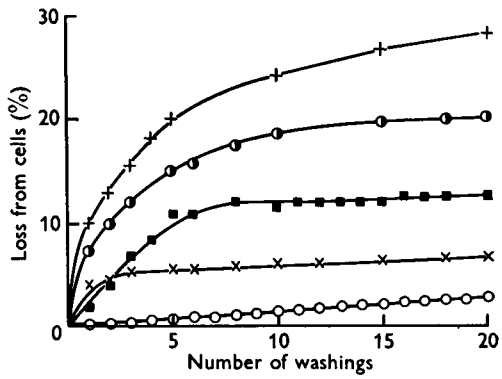

Fig. 1. The removal of components from the red cell by repeated washing. The cells were suspended in 0·16M-NaCl for 10 min. at 37°, separated by centrifuging and resuspended in fresh 0·16M-NaCl. This was repeated 20 times. +, Lipoprotein; ■, volume; ◑, cholesterol; ×, phospholipid; ○, haemoglobin.

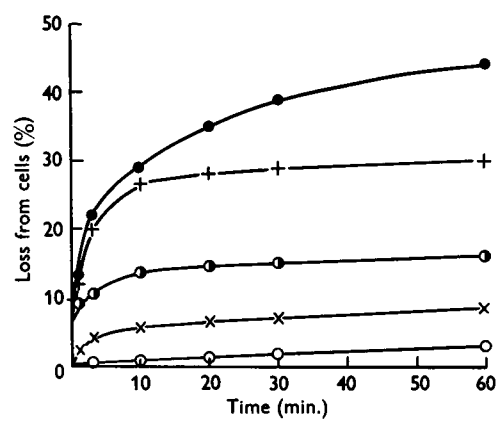

Fig. 2. The rate of removal of components from the red cell by exposure to alumina. Cells suspended in 0·16M-NaCl were exposed to powdered alumina (500 mg. Al_2O_3/ml. of cells) at 37°. ●, Dry weight of lipids; +, lipoprotein; ◑, cholesterol; ×, phospholipid; ○, haemoglobin.

The effects of exposure to alumina. Fig. 2 shows the effects of exposing red cells suspended in 0·16M-NaCl to 500 mg. of alumina/ml. of cells at 37°.

Fig. 3 shows the effects of varying the proportion of alumina to red cells in experiments similar to that shown in Fig. 2.

Fig. 4 illustrates the effect of temperature on the loss of components from red cells exposed to 2·0 g. of alumina per ml. of cells, for 15 min.

Fig. 5 shows the relationship between the logarithm of the rate of haemolysis and the reciprocal of the absolute temperature.

In all the experiments using alumina the red cells had been stored for 3 or 4 days at +4°. The removal of all components by the treatments just described is expressed in the figures as percentages of the composition of the test cells before treatment.

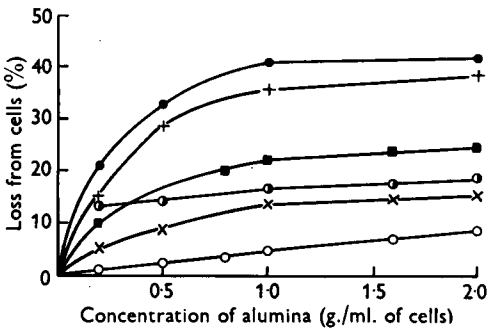

Fig. 3. The removal of components from the red cell by various concentrations of powdered alumina. Cells suspended in 0·16 M-NaCl were exposed for 15 min. at 37° to concentrations of alumina from 0·2 to 2·0 g./ml. of cells. ●, Dry weight of lipids; +, lipoprotein; ■, volume; ◑, cholesterol; ×, phospholipid; ○, haemoglobin.

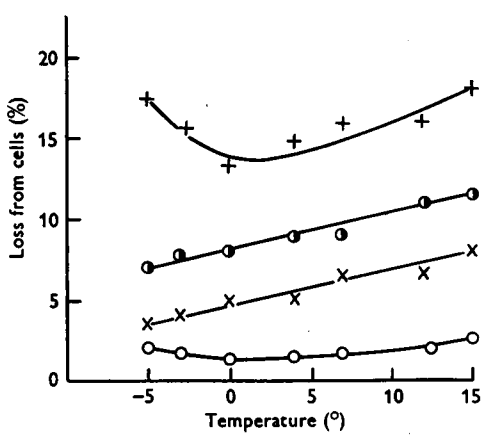

Fig. 4. The removal of components from the red cell by alumina at various temperatures. Cells suspended in 0·16 M-NaCl were exposed to 1·0 g. of alumina/ml. of cells for 15 min. at temperatures between 15° and −5°. +, Lipoprotein; ◑, cholesterol; ×, phospholipid; ○, haemoglobin.

DISCUSSION

The suspension of human red cells in 0·16 M-NaCl is followed by a loss of lipid components of the cell membrane and to a lesser extent haemolysis (Lovelock, 1954). The loss of lipids is rapid at first, but after about 3 min. a steady level is reached, and the rate of loss falls to a low value. The steady concentration of lipids does not appear to represent a simple saturation of the medium with these substances, but depends upon the previous treatment of the cells. It increases with the length of time the cells have been stored *in vitro* and decreases with the number of times they have been washed.

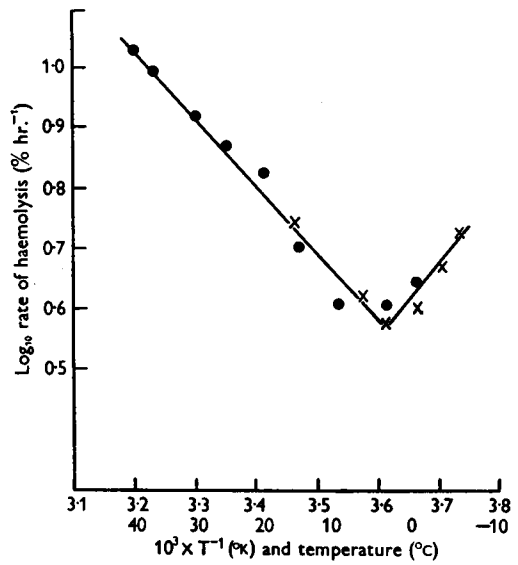

Fig. 5. The haemolysis of red cells in the presence of alumina at temperatures between 40° and −5°. Cells suspended in 0·16 M-NaCl were exposed to 2·0 g. of alumina/ml. of cells for 15 min. For temperatures below 0°, 2·0 M glycerol was included in the suspending medium to prevent freezing. Where glycerol was used the experiment is indicated (×), otherwise (●). The results are expressed as the log. rate of haemolysis and the reciprocal of the absolute temperature.

The effect of repeated suspensions in 0·16 M-NaCl is shown in Fig. 1. Even after twenty washes only 6% of the cells had haemolysed, and the proportion haemolysing was constant for each washing. During the first seven washes, however, a considerable amount of the cell membrane was dispersed in the medium and shrinkage of the cells took place. Thereafter the losses of cell components proceeded at a rate equivalent to the loss of cells by haemolysis.

Figs. 2 and 3 show that the effects of exposure to alumina are closely similar to those of repeated washing. The haemolysis of the cells is directly related to the time of exposure and concentration of alumina. The loss of lipids, however, bears a direct relationship to the time and intensity of exposure only after a considerable proportion of the membrane has been removed. This proportion appears to be more or less independent of the concentration of alumina.

The results suggest that the dissolution takes place in two steps. First, there is a rapid loss of superficial or surplus material which is not immediately harmful. Thereafter the rate of loss of membrane components is closely proportional to the rate of haemolysis. This suggests that any further loss of material from individual cells leads to their rapid and complete destruction.

Figs. 4 and 5 show the effect of temperature upon the rate of loss of lipids and the haemolysis of cells exposed to alumina. Between 40 and 5° the rate of haemolysis proceeds in a manner consistent with a process governed by simple diffusion—that is, with an activation energy of 5500 cal. Below + 5° the rate of haemolysis and loss of lipoprotein begins to increase, but the loss of cholesterol and phospholipid continues to fall.

alumina. The portion detached does not appear to be a defined superficial layer or capsule, since the proportion lost varies with temperature and duration of cold storage. The removal of this layer is not immediately harmful, and cells so treated do not haemolyse more rapidly than normal cells when suspended in 0·16 M-NaCl. The loss of components is, however, accompanied by a loss of volume. This could result from changes in the internal composition of the cell due to alterations of its permeability. If, however, the lost volume is simply that which was occupied by the detached components it is possible to calculate the concentration of these components in the detached layer. The results, in particular the dry weight of the membrane components of the cell before and after treatment with alumina, suggest that the concentration of the removable layer is equivalent to that of a 2 % lipoprotein gel. This agrees well with the value suggested by Mitchison (1953) based on birefringence measurements for the concentration of lipoprotein in the whole membrane. Ponder (1954), however, suggests that the membrane is much less hydrated than this and gives an estimate of 33 % for the concentration of membrane material from observations of the volume of fragmented ghosts.

Table 1. *The loss of components from the human red blood cell when exposed to alumina*

Conditions	Quantities of membrane components from 1 ml. of red blood cells remaining after various treatments (units: mg. and ml.)					
	Cholesterol	Phospholipid	Lipid N	Volume	Haemoglobin	Dry wt. of lipids
Fresh cells	1·21	2·7	0·24	1·0	345	12
After 15 min. with 1 g. alumina/ml. of cells at 37°	1·09	2·15	0·15	0·75	340	7
After 1 hr. at 37° in plasma	1·13	2·20	0·15	0·75	340	7
Lost from cells	0·12	0·55	0·09	0·25	5	5
Recovered from alumina	0·12	0·30	0·08	—	0	4·5

Table 1, which is reproduced from a previous paper (Lovelock, 1954), shows that the loss of components from the red cell is not reversible in a simple manner. Cells which have been denuded of membrane components regain very little of their lost material after 1 hr. in fresh plasma at 37°.

The experiments just described were carried out using cells stored at 4° in 'acid–citrate–dextrose' media, for between 3 and 10 days. During this period of storage there is little or no change in the viability of the cells as judged by their survival on transfusion (Loutit, Mollison & Young, 1943). It was noted, however, that the deleterious effects of both repeated washing and exposure to alumina increased during this period of storage.

The experimental evidence shows that a considerable proportion of the red-cell membrane is easily detached by washing or by exposure to

It seems worth considering the possibility that the concentration of membrane components is not constant throughout its thickness but increases radially inwards from the surface. This could explain the ease of detachment of the tenuous surface layers, and to some extent resolve the discrepancies between the estimates of the membrane thickness and composition.

The classical view of the structure of the red blood cell membrane (Ponder, 1948) implies a framework of stroma protein surrounded by a bimolecular layer of lipids, with a sprinkling of antigenic protein at the surface. It seems unlikely that a cell possessing such a structure could lose a substantial part of its membrane and still remain intact. Among the more recent views upon the architecture of the red blood cell, that of Moskowitz & Calvin (1952) agrees best with the experimental

results above. They envisage a membrane composed principally of fibrils of a lipoprotein, elenin, lying parallel to the cell surface and cemented together by ether-soluble lipids.

If the structure of the red-cell membrane suggested by Moskowitz & Calvin is accepted, then the physical dissolution of red cells might proceed as follows: when the cells are suspended in a fresh saline medium the surface lipids will dissolve or disperse in the medium, the lipoprotein which was held in position by the lipids will then become detached and diffuse away. In a medium maintained continuously unsaturated with respect to the lipid components of the cell membrane this process will continue until so much of the lipoprotein has unravelled that the intact existence of the cell is no longer possible. The increase in the rate of haemolysis below 5° could result from a hardening of the cementing lipids, if the repair of small breaches in the membrane depended on their ability to flow. Recent investigations of the effects of thermal shock on red cells suggest that such a hardening of the lipids does in fact occur (Lovelock, 1955).

The free energy of the lipid components of a highly organized structure such as the red-cell membrane may be higher than that of the same components in their saturated solution. It follows that the red-cell membrane is probably unstable in a physical sense and its intact existence may well depend upon the continuous synthesis of lipid components, and upon the presence of a considerable reserve of membrane material. The observed slow deterioration of living cells stored at −79° where all but diffusion processes are slowed to a negligible level is in accord with this notion.

SUMMARY

1. The effects on human red cells of repeated washing and of exposure to a neutral adsorbent substance, alumina, has been observed. Both of these treatments were found to remove considerable quantities of the lipid components of the cell membrane, and to cause a reduction of the cell volume.

2. The losses sustained by the cells during these treatments could not be reversed, but were not immediately harmful. Only slight haemolysis took place during and after detachment of up to 30% of their membrane materials.

3. The physical stability and architecture of the red-cell membrane is discussed in the light of these observations.

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The Determination of Glutathione in Blood and Tissues

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Several methods have been suggested for determining glutathione, in the reduced or sulphydryl form (GSH), and among those applied to biological materials are: iodine titration (Tunnicliffe, 1925; Woodward & Fry, 1932; Fujita & Numata, 1938*a*), silver nitrate titration (Benesch & Benesch, 1950), colour development with sodium nitroprusside (Fujita & Numata, 1938*c*; Grunert & Phillips, 1951), arsenophosphotungstic acid (Benedict & Gottschall, 1933) or phospho-18-tungstic acid (Potter & Franke, 1935), reduction of potassium ferricyanide (Mason, 1930), and coenzyme activity in the glyoxalase system (Woodward, 1935). Although all these methods can give accurate results with glutathione in pure aqueous solution, only the glyoxalase method can be regarded as reliable in biological materials. The others are all subject, in varying degree, to inter-