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CHANGING OSMOTIC PROPERTIES OF FOETAL SHEEP ERYTHROCYTES AND THEIR COMPARISON WITH THOSE OF MATERNAL SHEEP ERYTHROCYTES

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The haemolysis of red cells in hypotonic solutions has been extensively studied during the last three decades and many different rates of haemolysis due to species differences have been described (Jacobs, 1932; Jacobs, Glassman & Parpart, 1938). These and many other studies have added greatly to the volume of detailed information available.

Simple experiments demonstrate a remarkable difference in the rate of haemolysis of foetal and maternal sheep red cells when placed in hypotonic saline or distilled water. Species differences in the sense reported hitherto could hardly account for the slow rate of haemolysis of foetal blood as compared with maternal blood in the sheep, and it was therefore decided to investigate this problem as one which might yield additional information on the behaviour of red cells in general, or at least demonstrate some developmental change in red cell properties.

METHOD

A photo-electric method was used to measure the rate of haemolysis. The apparatus consists essentially of two colorimeter glass cells surrounded by a thermostatically controlled water jacket made of Perspex. Light from a 6 V. bulb and condenser is passed through both vessels and falls on a divided barrier-layer photo-cell. The outputs from the two sides of the photo-cell are backed against each other across a potentiometer, the unbalance current being fed into a galvanometer amplifier, the output of which operates a recording milliammeter (3–0–3 mA. range).

One glass vessel (the standard) contains haemolysed cells, the other contains the mixture of test saline and red cells whose rate of haemolysis is being followed. The apparatus is adjusted so that when haemolysis is complete the milliammeter shows zero current. At the commencement of a test (before the cells are injected into the saline) the standard is more opaque and a negative current reading obtained; when the injection of cells is made the galvanometer swings over to a large positive value and then starts to fall off towards zero as haemolysis proceeds.

The initial swing is too rapid for the galvanometer to follow faithfully but serves to mark the time of injection. The overswing is completed within 2 sec., after which time the change in opacity is accurately followed. Two typical traces are shown in Fig. 1 (*a*) and (*b*); the large overswing in the case of maternal cells is due to the extensive haemolysis which occurs even in the 2 sec. period taken for the galvanometer to settle down.

In the case of incomplete haemolysis, it is necessary to allow the apparatus to run for 15–20 min. to determine the final stable degree of opacity. To economize in recording trace, an automatic switch is introduced to cause the paper drive to operate for only 5 sec./min. Such a stepwise trace is shown in Fig. 1(*c*).

The amount of blood to be used in the tests is determined by matching against a red filter; a working dilution is then made in 1% NaCl (buffered to pH 7·4), such that each test injection of 0·5 ml. contains this amount. The matching is merely to ensure that a reasonable deflexion of the galvanometer is obtained in the test runs.

The test solutions are made up so that when 0·5 ml. of cell suspension are added to 5 ml. of test solution the resulting concentrations are 0·090, 0·200, 0·300, 0·400, 0·450, 0·500, 0·525, 0·550, 0·575, 0·600 and 0·625% saline.

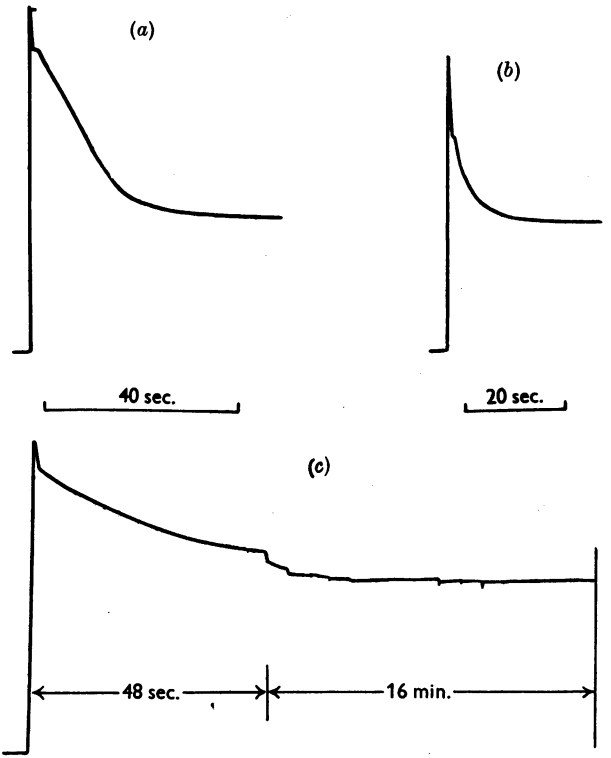

Fig. 1. (*a*) Tracing from foetal erythrocytes in 0·3% NaCl, 37° C. (*b*) Maternal sheep cells, 0·3% NaCl, 37° C. (*c*) Foetal cells, 0·525% NaCl, 37° C., showing incomplete haemolysis.

The average dilution of whole blood is 1 part in 25 of the injection. It is thus only 1 part in 275 of the final solution, and the contribution of the cells themselves will be less than 1 in 500. Change in concentration as haemolysis occurs is thus negligible.

The specimens of foetal blood were taken from the umbilical artery and immediately transferred to heparinized and buffered 1% NaCl in rubber covered vaccine bottles. The cells were not centrifuged. Specimens of maternal blood were taken from a cannula in the dorsalis pedis artery. The testing was commenced immediately and continued for the following 6–8 hr.

The test solutions and working stock of blood dilutions were all maintained in a thermostatically controlled water bath; the blood dilutions, in vaccine bottles, being supported on slowly rotating rollers to obviate sedimentation.

Analysis of results

The rate of entry of water into a red cell considered as a simple osmometer was treated by Jacobs (1926) using the relation

$$\frac{dV}{dt} = kA\,(C - C'), \tag{1}$$

together with the assumption that

$$V \times C = V_o \times C_o, \tag{2}$$

since only water enters the cell. (V is volume of cell; k is a permeability constant; A, the area across which diffusion occurs; C, the concentration inside the cell at time t; V_o and C_o are the volume and concentration inside at zero time; C' is the concentration of the outside solution.)

A convenient form of the solution of the differential equation obtained by combining (1) and (2) is

$$\frac{kA}{V_o}t = \frac{1}{C'}\left\{\left(1 - \frac{C_o}{C}\right) + \frac{C_o}{C'}\log_e \frac{1 - C'/C_o}{1 - C'/C}\right\} \tag{3}$$
$$= f(C', C).$$

Since C_o is constant throughout, the function on the R.H.S. of equation (3) depends only on the outside concentration and the inside concentration at time t. If the symbol t_h is used to represent the time required for haemolysis of a particular type of cell and C_h is used to represent the concentration inside the cell when haemolysis takes place, then equation (3) can be rewritten as

$$\frac{kA}{V_o}t_h = f(C', C_h) \tag{4}$$

from which it can be seen that the plot of t_h in sec. against the calculated values of $f(C', C_h)$ should give a straight line passing through the origin and having a slope of kA/V_o.

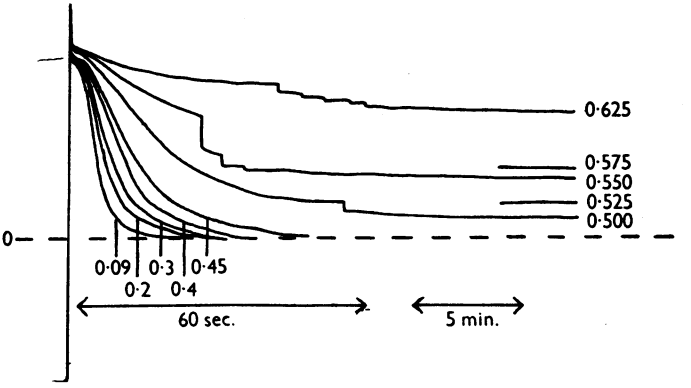

Fig. 2. Series of tracings (superimposed) from foetal erythrocytes in different outside concentrations (see text).

Values of the function for the outside concentrations used and for cells having haemolytic concentrations 0·525, 0·550, 0·575 and 0·625% have been calculated and are given in Table 1.

TABLE 1. Values of $f(C', C_h)$ against which times of haemolysis are plotted

Outside conc. g. %	$C_h = 0{\cdot}525\%$	$C_h = 0{\cdot}550\%$	$C_h = 0{\cdot}575\%$	$C_h = 625\%$
0·09	1·52	1·32	1·17	0·89
0·20	1·89	1·63	1·42	1·07
0·30	2·44	2·07	1·76	1·30
0·40	3·52	2·88	2·40	1·69
0·45	4·65	3·65	2·94	2·00

Typical experimental traces (superimposed for ease of illustration) are given in Fig. 2. The traces of incomplete haemolysis show the opacity remaining when cells of haemolytic concentrations 0·625, 0·575, 0·550 and 0·525% just fail to haemolyse. These opacity levels, which fall on the steep part of the experimental curves, are used to measure the times of haemolysis of the respective types of cells when the outside concentration is 0·09, 0·20, 0·30, 0·40 and 0·45%.

The experiments have been carried out as a routine at 37 and 27° C., and the opacities appropriate to the critical concentrations determined at each temperature. This is essential, since fewer cells are stable at the lower temperature. This phenomenom, first reported by Jarisch (1921), has been attributed by Jacobs & Parpart (1931) to a volume effect due to a change in the base combining power of haemoglobin.

RESULTS

General osmotic behaviour of foetal cells

(i) 50–120 *days*. The results obtained by analysing the times of haemolysis for erythrocytes of sheep foetuses from 50 to 120 days conform to the theoretical relationship up to values of t of 30 sec. and outside concentration 0·450% NaCl. A typical result is shown in Fig. 3. A remarkable feature of these results is that the plot of the points for the four different populations of cells (whose $V_{haem.}/V_o$ ratios vary from 1·6 to 1·9) fall on the same straight line within experimental error.

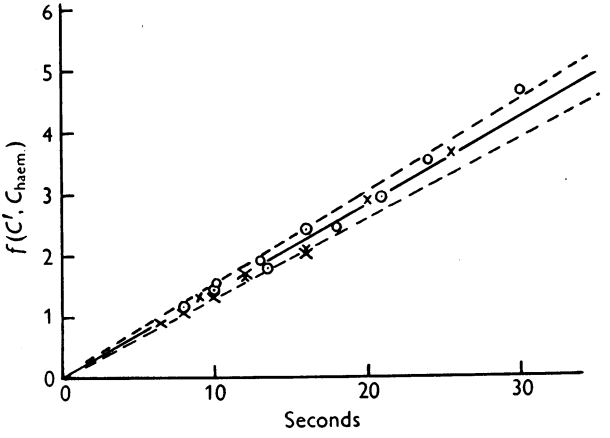

Fig. 3. Foetus, 86 days foetal age. 37° C. Times of haemolysis plotted against $f(C', C_h)$ for cells of haemolytic concentration 0·525, 0·550, 0·575 and 0·625% NaCl. Slope $kA/V_o = 0{\cdot}14$. Dotted lines indicate ±5%. 0·525% = ○, 0·550% = ×, 0·575% = ⊙, 0·625% = ×.

This fact has been used to determine the best value for the slopes of the line by averaging the ordinate and abscissa values of all twenty results (from four types of cells at five different concentrations), and drawing the line through the origin and the point of greatest density. The broken lines at either side are ±5%. The slopes from different bloods lie in the range from 0·105 to 0·195 and this aspect is considered more fully below. The linear relationship is maintained at the lower temperature to over 40 sec. but only to the same concentration 0·450%.

(ii) 120 *days to term.* The determination of slopes for cells from foetuses of 120–145 days is not so satisfactory in that the stable values are difficult to define. Of nine experiments, four gave results in which the slopes of all four types of cells were reasonably co-linear and in the range of 0·18–0·195. Two others (in which the blood was drawn at the end of an unrelated experiment) gave values of 0·205 and 0·22 respectively. The remaining three experiments gave values in which the points for the four types of cells did not fall on straight lines through the origin. The approximate lines varied in a manner similar to that which makes adult cells unsatisfactory. In this range of ages the determinations at 27° C. were unsatisfactory for similar reasons even in cases which were well defined at 37° C.

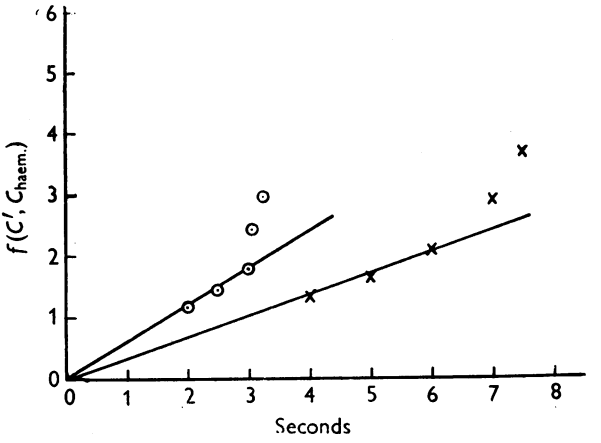

Fig. 4. Maternal sheep cells 37° C. Times of haemolysis plotted against $f(C', C_h)$ for cells of haemolytic concentrations of 0·55 and 0·575% NaCl. 0·550% = ×, 0·575% = ⊙.

Osmotic properties of maternal cells

The results for foetal cells contrast sharply with the results obtained with maternal sheep erythrocytes. A large percentage of maternal cells haemolyse before the apparatus has recovered from its initial overswing, but it is, nevertheless, possible to follow cells whose critical concentrations are 0·550 and 0·575% NaCl, although the stable values are not well defined. The times for haemolysis of these cells which have been plotted against the appropriate values of $f(C', C_h)$ in Fig. 4 do not form a straight line, although the first three points suggest reasonable linearity. There is also a considerable difference in slope between these two types of cells whose V_h/V_o ratios are only 1·82 and 1·74 respectively. It will be noted that the apparently higher kA/V_o value is associated with the cells of lower V_h/V_o ratio.

Variation of kA/V_0 for foetal cells with foetal age

The results of experiments on the erythrocytes of eleven foetuses varying in age from 50 to 120 days and in weight from 16 g. to 1·5 kg. are given in Fig. 5. The kA/V_0 values have been obtained in each case by the graphic method described above and plotted against foetal age. The foetal age was calculated from the date of 'tupping' where known, and where this was in doubt, the age was estimated from weight and length measurements (Huggett, unpublished data). The correlation coefficient of increase in kA/V_0 with age is 0·93 (significance $P < 1/1{,}000$). A similar correlation is obtained with log kA/V_0 and log foetal weight ($r = 0{\cdot}92$).

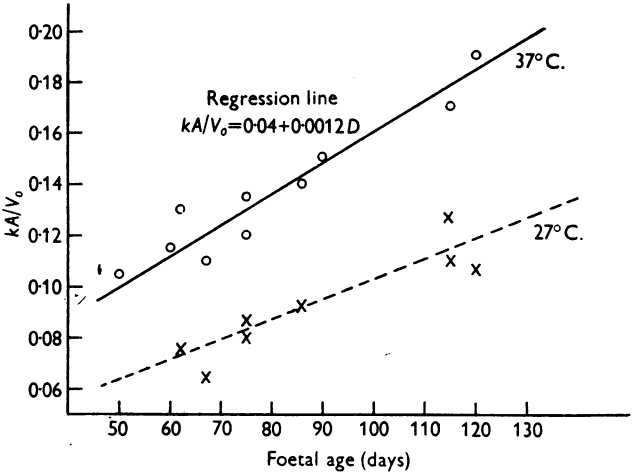

Fig. 5. Variation of kA/V_0 values of foetal cells with foetal age.
O = values at 37° C. × = values at 27° C.

Percentage haemolysis of stable values

The calibration of the apparatus in terms of percentage haemolysis was carried out by removing portions of the suspension in 1% saline and replacing by equivalent volumes of saline containing fully haemolysed cells.

The results of six cases where no further adjustment to the apparatus was needed during the haemolysis tests are given in Fig. 6. The percentage of cells stable at concentrations of 0·625, 0·575 and 0·525% are shown for bloods whose kA/V_0 values vary from 0·110 to 0·195. The higher kA/V_0 values are associated with a larger proportion of cells stable at 0·625% but the number of stable cells at 0·525% shows little significant change.

Apparent energy of activation of diffusion process

Since diffusion and viscosity are rate processes (Glasstone, Laidler & Eyring, 1941), and the cell membrane may be accepted as offering a potential energy barrier to diffusion, as postulated by Danielli (1941), it appears justifiable to

use the values of kA/V_0 at 37° and 27° C. for the purpose of calculating the experimental activation energy. The mean value obtained is $E = 7{\cdot}6 \pm 1{\cdot}6$ kg. cal./mol. and the line in Fig. 4 through the points for 27° C. has been drawn on this basis.

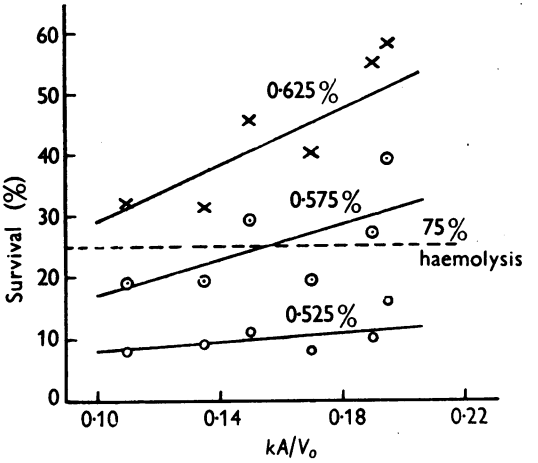

Fig. 6. Change in percentage of cells surviving in haemolytic concentrations 0·525, 0·575 and 0·625% NaCl as kA/V_0 values increase.

Theoretical consideration of effect of co-existence of cells of varying kA/V_0

The results for foetal cells show that kA/V_0 values for cells having haemolytic concentrations 0·525–0·625% are equal in a given blood but vary from one blood to another. It is important to consider the consequence of cells of different kA/V_0 being present in the same blood. This can most readily be seen from consideration of Fig. 7. The $f(C', C_h)$ is the ordinate as usual and horizontal lines have been drawn indicating the ordinate appropriate to the cells haemolysing at 0·525, 0·550 and 0·575% when the outside concentration is 0·90% NaCl.

The time for any of these cells to haemolyse depends upon the appropriate kA/V_0 slope. Thus, in a mixture of cells of slopes (*a*) and (*b*) the order of haemolysis would be different from a mixture (*b*) and (*c*), as indicated in Table 2.

It is seen that where cells of different kA/V_0 coexist in the same blood one cannot argue that a certain degree of opacity is due to the cells which would just maintain stability at the critical haemolytic concentrations. By considering the position at other outside concentrations in the same way it can readily be shown that there will be a very considerable interchange of order of haemolysis due to the interaction of the two variables—haemolytic concentrations and kA/V_0 slopes.

It is indeed clear that a good fit to the theoretical line can only be expected if the variation in slopes for any haemolytic concentration is small and for a

given small variation in slopes the best fits will be obtained when the angle which the line makes to the time axis is acute.

These considerations give an interpretation to the general character of the results obtained with foetal and maternal cells which are summarized in Table 3.

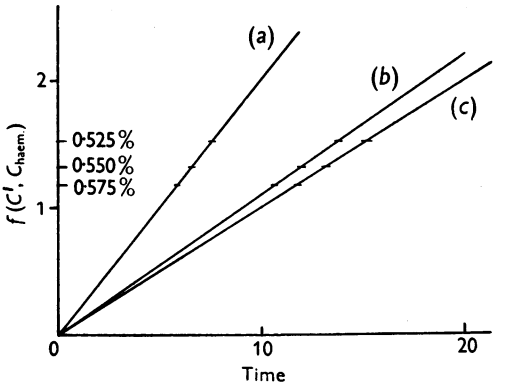

Fig. 7. Effect upon order of haemolysis if cells of different kA/V_o values co-existed in the same blood (see text).

TABLE 2. Order of haemolysis of mixtures of cells of different kA/V_o slope shown in Fig. 7

Mixture (*a*) and (*b*)	Mixture (*b*) and (*c*)	Order of critical concentrations
0·575 *a* 0·550 *a*	0·575 *b* 0·575 *c*	0·575
0·525 *a* 0·575 *b*	0·550 *b* 0·550 *c*	0·550
0·550 *b* 0·525 *b*	0·525 *b* 0·525 *c*	0·525

TABLE 3. Different character of results of osmotic haemolysis of foetal and maternal sheep cells

Foetal cells (50–120 days)	Maternal cells
1. Very small variation in kA/V_o values of cells	Considerable apparent variation in kA/V_o values of cells
2. Lines more acute to time axis, i.e. kA/V_o small	All kA/V_o lines steeper to time axis
3. Good fit to theoretical equation	Poor fit to theoretical equation

DISCUSSION

Variation of kA/V_o value for foetal cells with foetal age

The cause of the variation of kA/V_o for foetal cells as the age of the foetus increases might, at first sight, be considered to be due to the changing red cell population. It is known, for instance, that in early foetal life the erythrocytes are chiefly produced in the liver, whereas later, the normal adult centres (the bone marrow) assume greater importance. Alternatively, the average age of the individual red cells might be considered to have a bearing, although perhaps

with less justification, since, due to rapid growth and increase in blood volume, there must always be a large proportion of relatively young cells.

Both these possibilities, however, would meet with a serious difficulty in explaining the experimental facts. That is because in both cases cells of different kA/V_o values would be in the circulation at one and the same time, and this is made improbable by the good fit to the theoretical equation and by the co-linearity of the points for cells whose haemolytic concentrations vary from 0·525 to 0·625%.

The results can only be reconciled with the view that as the foetal age increases the kA/V_o values of the *cells as a whole* (young and old alike) are changed by some *intravascular process.*

Although this view is forced upon one, it immediately prompts the question as to how such a change can be brought about in such a way that the kA/V_o values all change in step and the population as a whole is kept uniform in so far as this value is concerned.

The relation of V_h/V_o *and* kA/V_o. The ratio V_h/V_o, where V_h is the volume at which haemolysis occurs, is obtained from the haemolytic concentration by applying equation (2). Since all cells are initially equilibrated in 1% buffered saline, the V_h/V_o ratios for the cells of haemolytic concentrations 0·525–0·626% lie in the range 1·6–1·9. The values of V_h and V_o cannot necessarily be identified with cell volumes as estimated by the usual means, since not all the cell volume is osmotically effective. The point is fully reviewed by Ponder (1948), who considers that only 70–90% of the cell volume is osmotically effective.

The red cell being a biconcave disk can effect a considerable increase in volume without change in surface by assuming the spherical form, and the general view has been that very little swelling beyond the spherical form (which must be accompanied by surface expansion) can occur before haemolysis takes place. On this view, different V_h/V_o ratios which are found in all bloods are due to variations in the surface/volume ratio in the initial state. This is equivalent to postulating that all V_h values are reasonably constant. If this were so, then in the range of foetal cells examined, the initial V_o values must vary by 13–17%, and it would be expected that this would be reflected in the values of kA/V_o. As already seen, however, these values are the same in foetal blood to within $\pm 5\%$ and any effect in this direction must be smaller than would be necessary to account for the full range of V_h/V_o ratios found.

Microscopical examination also demonstrates the presence of some larger cells among those stable at any critical concentration, and the initial surface/volume ratio cannot be instrumental in determining the variety of different sizes and shapes reached before haemolysis occurs. Ponder (1948) has described haemolysis occurring in some cases before the sphere of equal surface is attained.

In the case of maternal cells, where different kA/V_o values are associated with different V_h/V_o ratios, the change is the reverse to what might be expected,

since if the lower V_h/V_o value is associated with a greater initial volume, then one would expect to find kA/V_o smaller but, in fact, one finds the opposite. At 37° C. no maternal cells are stable in 0·500% NaCl and at 27° C. no cells are stable in 0·525% NaCl. Some foetal cells are stable in 0·500% NaCl at both temperatures although the number stable at the lower temperature is somewhat reduced.

Variation in V_o for whole population of cells. Although as considered above, a variation in the initial volume of the cells does not satisfactorily account for the range in haemolytic concentrations and V_h/V_o ratios, a change in V_o for the whole population of cells would have an effect upon the kA/V_o ratios as between one blood and another. If the range of haemolytic volumes show little change, then the proportion of cells stable at any given V_h/V_o ratio would also change with change in V_o.

Variations of V_o for the whole population of cells are not only possible but likely to occur for small changes of internal pH, of temperature or degree of oxygenation at the time the specimens were taken into the buffered saline. Such volume changes have been treated theoretically by Jacobs & Stewart (1947).

A decrease in V_o (relative to the initial V_o and without surface change) would produce an increase in the kA/V_o ratio and also an increased stability at a given critical concentration. This is qualitatively the same direction as the change shown in Fig. 6, but quantitatively the change which would account for the greater stability of even the 0·625% cells would only represent a 10% change in V_o and kA/V_o.

Progressive changes in cell constituents. Karvonen (1949) has shown that foetal sheep haemoglobin is much less soluble than that of adult sheep haemoglobin. The adult type first appears in foetuses of about 100 days and thereafter its proportion rapidly increases. It is difficult to see that this difference in solubility can be instrumental in causing the different rates of haemolysis unless the interior of the cell is in the state of a gel. Nor can it be seen how a progressive change with age affecting all the cells could be accounted for on such grounds.

Change in cell membrane as a factor. Although one cannot completely rule out an effect due to changes in the body of the cells, there are several considerations which point to the results being due to membrane changes. First, Jacobs's treatment of the diffusion process is based on the rate-determining step being penetration of the membrane, and the results for foetal cells give a good fit to this theory. Secondly, the changes which occur in the kA/V_o value with age must affect all cells alike whether recently released to the circulation or of older origin. The possible change in initial volumes due to internal pH or other effects could only account for a 10% change in kA/V_o, leaving the major change to be accounted for by some other mechanism.

Viewing the results from the concept of a membrane change, it is necessary to consider further the interpretation to be placed on k and A in the ratio kA/V_o. The rate-determining step for the penetration of water as postulated by Danielli is that from the water phase to the lipid membrane; this process may be considered to be akin to evaporation, since the mutual association of water molecules by hydrogen bonds must be broken down. There is some confirmation for this view in the value of the apparent experimental activation energy, although assessment of the true free energy of activation would require, in addition, knowledge of the entropy change.

That monolayers can retard evaporation was described by Rideal (1925) and more recently Sebba & Briscoe (1940) have shown that quantitatively the effect depends upon the surface pressure. If a similar property could be ascribed to the bimolecular lipid layer of the cell membrane, a physical interpretation of the change of kA/V_o with age would be a progressive change in surface pressure of the cell membranes.

The constant value of kA/V_o for cells in a given blood might thus be suggestive of a surface pressure equilibrium among all the cells of the circulation. Although there is no direct experimental evidence for this speculative possibility, it appears to be a line which merits further study. Regarding the cell membrane as having pressure-area relationships similar to those of mixed lipid monolayers, expansion or change of shape of the surface would normally occur by the system descending the Force-Area curve until a zone of heterogeneity was reached, when a continuous water channel connecting inside and out would start haemolysis. With partial rigidity of the membrane imposed by lipoprotein complexes, the underlying stromatin or other causes, local zones of heterogeneity might develop at an earlier stage in the surface change. On this view, the distribution of cells in various V_h/V_o ratios would be a statistical probability of cells surviving a given degree of expansion and surface rearrangement rather than postulating that they start with different surface-volume ratios.

Cells, such as maternal cells with a high kA/V_o value, are indicative of lower surface pressure. They may be considered to start farther down the Force-Area curve and thus would not be able to complete the full range of expansions shown by foetal cells; also the approach to the flatter part of the Force-Area curve would tend to make the definition of the stable values unsatisfactory and so produce a bad fit to the equation.

In this respect it is interesting to consider the effect of temperature. It is a property of liquid expanded films (Adam, 1941) that at lower temperatures they reach the zone of heterogeneity at smaller areas per molecule. This would cause the system to reach the flatter part of the Force-Area curve earlier at a low temperature than at a higher temperature. If an unsatisfactory fit to the equation can be attributed to reaching this zone it would explain why the older

foetal cells are unsatisfactory at 27° C., although six cases out of nine give a reasonable fit to the equation at 37° C. Any intracellular haemolysin released during partial haemolysis would also distort the stable values and produce a bad fit to the equation, but such a cause would be expected to operate also at 37° C.

SUMMARY

1. A study of the different rates of haemolysis of erythrocytes of foetal and maternal sheep has been made using a photoelectric method. The results have been analysed by the use of relationships advanced by Jacobs (1926) and a measure of the permeability constant for water obtained.

2. The results for foetal cells show that there is a progressive change in this permeability factor (kA/V_o) with foetal age. The value is approximately doubled between the 50th and 120th day of pregnancy and the change gives an approximate regression line

$$kA/V_o = 0{\cdot}04 + 0{\cdot}0012d$$

where d is the foetal age in days.

3. The results also show that, in a given foetal blood, cells of different haemolytic concentrations (having V_h/V_o ratios from 1·6 to 1·9) have the same kA/V_o value within experimental error. From this and other theoretical considerations it appears that foetal blood cannot contain cells of very different permeability properties at one and the same time, and the progressive change with foetal age is therefore attributed to an intravascular change in the permeability properties of the cells.

4. Some of the implications of this change in permeability are discussed in terms of a membrane effect but there is no direct evidence how the change is brought about.

The author wishes to express his great indebtedness to Professor A. St G. Huggett for stimulating his interest in foetal physiology as a means of obtaining new angles of evidence on problems of general physiology; for providing him with maternal and foetal blood samples as a first priority from animals being experimented upon in connexion with other research problems; and for much advice and guidance in the preparation of this paper, as well as for the use of unpublished data referred to in the text.

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