THE OSMOTIC PROPERTIES OF THE NORMAL HUMAN ERYTHROCYTE

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WHEN erythrocytes are suspended in a hypotonic medium containing solutes which do not penetrate the cell membrane, the cells swell by imbibing water until the osmotic equilibrium on the two sides of the cell membrane is re-established. The determination of the degree of cell swelling may be calculated from the known laws of physical chemistry, and it may also be determined experimentally. If the calculated value is the same as the observed value, then the erythrocyte obeys these laws of physical chemistry and behaves as a perfect osmometer. If, on the other hand, the two values do not agree, then the erythrocyte is, for some unknown reason, an imperfect osmometer and either obeys some chemical laws which are as yet undiscovered or is a law unto itself.

Theoretically, the degree of cell swelling may be calculated from the equation (Ponder, 1948) :—

$$V = W\left(\frac{p-pT}{pT+1}\right) + 1$$

when the volume of the medium surrounding the cells is limited. In this equation, the initial volume of the cell is taken as unity; V is the relative volume of the cell at equilibrium; W is the fraction of water in the cell, commonly taken as 0·70; T is the tonicity of the medium, *i.e.* the ratio of the osmotic pressure of the diluted extracellular fluid to the osmotic pressure of plasma; and p is the ratio of the initial volume of the extracellular medium to the initial volume of the cell water. The equation is mathematically correct and of the four unknown quantities, three may be determined by direct measurements.

Many attempts have been made to determine the relation between the degree of cell swelling and the extracellular tonicity, and these have been tabulated by Ponder (1948). The observed degree of cell swelling seldom agrees with the calculated value and the discrepancy may be considerable. Most observers report values markedly smaller than the theoretical, and in order to reconcile experiment with theory, a factor R has been introduced into the equation which now becomes

$$V = R \cdot W\left(\frac{p-pT}{pT+1}\right) + 1$$

It will be noted that the factor R governs the degree of swelling of the cell—not the final equilibrium volume. If the erythrocyte obeys the laws of physical chemistry and behaves as a perfect osmometer, the value of R should be unity. Different observers have found values of

R ranging from 0·5 to 1·0 (Ponder, 1948) and if these values are accepted, the only conclusion which can be drawn is that the cell is *not* a perfect osmometer.

Certain doubts arise on this point. There would appear to be no obvious reason why the red cell does not behave as a perfect osmometer and the values of R recorded in the literature are rather unexpected. The considerable variation in the observed values of R seem to be more than could legitimately be accounted for by differences of technique, and finally, the " constant " R has never been interpreted in any way other than as an expression of the extent of the deviation of the cell from its predicted behaviour.

Two previous publications have some bearing on this problem. They deal, not with the actual cell swelling, but with the end-point of osmotic swelling—hæmolysis.

The degree of hæmolysis in hypotonic sodium chloride solution depends on the temperature; at constant concentration, the greater the temperature the less the degree of hæmolysis, and *vice versa*. The van't Hoff-Mariotte equation ($\Pi . V = n . R . T$) correlates osmotic pressure with temperature and it has been shown that when solutions of constant concentration are replaced by solutions of constant osmotic pressure, the degree of hæmolysis is independent of the temperature within the range 4-36° C. (Hendry, 1949). Proof of this fact entailed the use of the van't Hoff-Mariotte equation and therefore assumed that the red cell was a perfect osmometer. In other words, it showed that the degree of hæmolysis depended not on temperature but on the osmotic pressure of the surrounding medium. Now, if the erythrocyte behaves as a perfect osmometer under conditions of varying temperature, there would seem to be no valid reason why it should not behave as a perfect osmometer under conditions of varying extracellular tonicity.

The other publication referred to is less definite but is also suggestive. It deals with the degree of hæmolysis when whole blood is added to hypotonic solutions of various alkali halides under certain specified conditions. The osmotic pressure (in atmospheres) of solutions of alkali halides which would produce 50 per cent. hæmolysis was found to be :—

Lithium chloride	2·67
Sodium chloride	2·81
Sodium bromide	2·78
Sodium iodide	2·72
Potassium chloride	3·01
Potassium bromide	2·88
Potassium iodide	2·80
Rubidium chloride	2·92
Cæsium chloride	2·77
Mean	2·82

(The value for sodium fluoride has here been omitted as this salt appears to have some intrinsic hæmolytic action.)

These figures were obtained by distributing normal blood into a series of dilutions of each electrolyte and taking the mean of a group of fragility curves for each salt from which the average osmotic pressure corresponding to 50 per cent. hæmolysis could be calculated (Hendry, 1948). The values are therefore subject to the errors of biological variation of the different blood samples, but in spite of this, the results are very close together. The greatest difference from the mean is 6·8 per cent. (in the case of potassium chloride) and in no fewer than seven of the nine salts, the difference from the mean is 3·5 per cent. or less. This suggests that the red cell is indifferent to its surroundings so long as the solute is an alkali halide, for it hæmolyses almost to the same extent in all cases. In other words, the cell is a perfect (or almost perfect) osmometer under conditions of varying electrolyte in the extracellular medium. This point is further examined in the present work.

These experiments, of course, cannot be accepted as proof of the cell's behaviour as a perfect osmometer at different extracellular tonicities, but they are in direct opposition to the conclusions which have been reached as the result of determinations of the degree of cell swelling, and to the validity of the factor R. The present investigation is directed towards an explanation of this discrepancy.

Experimental

Material and Methods.—In the following work, normal human blood has been used in all except one minor experiment. Coagulation was prevented by the addition of a trace of heparin powder. Immediately after withdrawal, the blood was oxygenated for thirty minutes by exposure to a current of moist oxygen with frequent gentle mixing. Thereafter, the experiment was started without delay.

The various salts used were of the highest purity and were dried to constant weight *in vacuo*, or, in the case of hydrated salts, at 120° C. Concentrations throughout are expressed in terms of the anhydrous salts only. The degree of hæmolysis was determined by estimating the liberated hæmoglobin (Hendry, 1947). *p*H was measured by the Marconi glass-electrode *p*H-meter which has a sensitivity of about 0·02 unit.

Whole blood was used in preference to washed cells partly to avoid delay, but mainly to avoid any possibility of damage to the red cells by repeated washing and centrifuging. Allowance must therefore be made at appropriate points for the volume of plasma which is present.

The following values have been taken for the apparent " degree of dissociation " at 18-20° C. of the salts used and they have been calculated from the data given in the International Critical Tables (1929) for electrical conductivity and depression of the freezing point: sodium chloride, 0·89; sodium bromide, 0·89; potassium chloride, 0·88; potassium bromide, 0·87; and potassium iodide, 0·89.

Total protein was estimated by the method of Fine (1935).

Hæmolysis in Solutions of the Alkali Halides.—It has been mentioned above, that the degree of hæmolysis was found to depend to some extent on the nature of the alkali halide in the extracellular fluid. The matter is here briefly re-examined by experiments which eliminate the variation in the blood samples as a cause of the variation in the degree of hæmolysis.

The alkali halides selected were sodium chloride and bromide, and potassium chloride, bromide and iodide. A solution of each salt was prepared the concentration being so adjusted, by means of the van't Hoff-Mariotte equation, that all five solutions had the same osmotic pressure at 18° C. The oxygenated blood was added to give a final dilution of 1 in 20. After mixing, the tubes were allowed to stand for five minutes in a thermostat and were then centrifuged at 1500 r.p.m. for two minutes. The degree of hæmolysis was calculated from the amount of liberated hæmoglobin.

These conditions differ in two major and in two minor respects from the experiments which were carried out in 1948. The same blood is now distributed into a series of solutions of different salts so that biological variation from one sample of blood to another is eliminated. More important, hæmolysis is allowed to proceed for only five minutes (instead of two hours) and the degree of hæmolysis is thereby considerably reduced. The minor differences are the replacement of oxalate by heparin and a more rigid control of the temperature.

The results obtained are shown in Table I.

TABLE I

The Hæmolysis of Erythrocytes in Solutions of the Alkali Halides

Blood.	Osmotic Pressure (atmos.).	Percentage Hæmolysis.				
		NaBr.	KI.	NaCl.	KBr.	KCl.
1	2·82	1	1	1	1	2
2	2·82	2	4	4	4	6
3	2·82	1	4	3	4	6
4	2·82	1	1	2	2	4
5	2·75	4	7	8	7	14
6	2·75	10	18	21	18	36
7	2·75	14	28	33	29	51
8	2·75	49	70	73	75	84
9	2·60	57	67	70	70	76
10	2·60	35	67	57	66	76
11	2·60	31	57	53	65	76
12	2·60	34	56	59	55	70

The figures in Table I must not be taken strictly at their face value. With all these salts, the osmotic fragility curve is sigmoid so that when the degree of hæmolysis is in the region of 1-5 per cent.

the results are embarrassingly constant; it requires a relatively large change in osmotic pressure to alter the degree of hæmolysis on this part of the curve. Nevertheless, it must be conceded that hæmolysis "begins" at very much the same osmotic pressure in all cases. Conversely, in the region of 30-70 per cent. hæmolysis, the system is maximally sensitive to any alteration in the external conditions or to any error of manipulation. The results presented here are in good agreement with the results which were previously published; the degree of hæmolysis is always greatest in potassium chloride and is always smallest in sodium bromide, the other three salts being intermediate. Since biological variation in the blood samples has been eliminated the differences must lie in the extracellular electrolyte and the reason for these differences remains to be discovered. The differences between the effects of the different alkali halides are unquestionably real, but in the majority of cases they are small (consider, for example, the osmotic pressures of sodium chloride, sodium bromide, potassium bromide, potassium iodide, and cæsium chloride required to produce 50 per cent. hæmolysis) and whatever be the explanation, it seems reasonable to conclude that the red cell is dependent only to a small extent on the nature of the alkali halide in the extracellular medium and that in this respect it behaves almost (but not quite) as a perfect osmometer. We now turn to direct measurements of the cell volume.

Oxygenation Effects.—As in studying fragility, so in determining cell volumes the blood should first be fully oxygenated in order to eliminate changes in volume due to loss of carbon dioxide during manipulation. Oxygenation alone has an appreciable effect on the cell volume. In six experiments in which the packed cell volume was determined by the hæmatocrit directly on the venous blood and then on the same blood after oxygenation, it was found that the cells decreased in volume by 3·9-5·5 per cent. (mean 4·5 per cent.) of their original volume in the venous blood. Hæmoglobin concentrations, determined simultaneously, were identical in the venous and oxygenated bloods thus ruling out the possibility that the cell volume change was due to uptake of water from the moist oxygen. Oxygenation (*i.e.* the conversion of reduced hæmoglobin to oxyhæmoglobin) has probably no effect on cell volume, but the process removes carbon dioxide and increases the pH of the blood by about 0·5 unit. Such a change in pH has been shown by Hampson and Maizels (1926-27) to decrease the cell volume. Failure to oxygenate at the start of the experiment may account partly for the irregularity of the reported values of R, but such an omission is not a fundamental source of error for the values of R obtained with oxygenated blood are no better than those obtained with venous blood.

The Observed Value of R.—In the experiments described in the literature, the degree of cell swelling (and hence the value of R) is usually determined by hæmatocrit methods. In a series of similar

experiments cell swelling was determined (*a*) by accurately diluting normal blood with two or three volumes of hypotonic sodium chloride solution and determining the packed cell volume in straight 100 mm. Hawksley hæmatocrit tubes closed with rubber caps, and (*b*) by diluting with hypotonic sodium chloride solution in Van Allan hæmatocrit tubes. (The Van Allan tube consists of a graduated capillary stem surmounted by a bulb about 1 c.c. in volume so that the packed cell volume of relatively high dilutions of blood in saline may be accurately determined.) In both types of experiment, the tubes were centrifuged at 3000 r.p.m. for thirty minutes. In brief, the experiments followed the usual pattern. So did the results. In 34 determinations, the value of R lay within the limits 0·67-0·79 (mean = 0·74). Unless some explanation can be found for these values of R the only conclusion to be drawn is that the red cell is not a perfect osmometer.

Possible Sources of Error.—Some of the possible sources of error are not easily susceptible of investigation. Even in stating the problem, it has been assumed that the alkali halides do not penetrate the cell, and that osmotically active material does not escape from the cell during the determination of the cell volume. It has long been known that when red cells are suspended in hypotonic saline, potassium escapes, and it has been suggested that this escape of intracellular material is the cause of the failure of the red cell to behave as a perfect osmometer. But the theory has been rejected by all who have examined it closely. Under these conditions, different observers record different rates of escape of potassium from the cell, but these differences can be satisfactorily attributed to differences in experimental detail. All, however, are now agreed that the escape of potassium is a very slow process compared with the speed of swelling of the cell when it is placed in hypotonic saline. Were further evidence required, the following simple experiment might be added.

If potassium leaves the cell and no ion or molecule enters to replace it (*i.e.* the conditions required by the low experimental values of R) then water will also leave the cell in order to maintain osmotic equilibrium. The cell volume should therefore decrease when erythrocytes are placed in hypotonic saline. In actual fact, the cell volume *increases* by about 1 per cent. per hour. This increase does not contradict the established fact that potassium leaves the cell under such conditions. It certainly does. But presumably sodium or some other osmotically active material enters in an amount osmotically slightly greater than the amount of potassium which leaves the cell. There may be other explanations, but such an experiment is further proof that the *rapid* anomalously small swelling of the erythrocyte is not due to the very *slow* transfer of potassium across the cell membrane.

The Fraction of Water in the Cell.—The accepted value of 0·70 for the cell water (W in the equations) comes under suspicion. It is usually determined by drying a mass of packed cells to constant weight at 60-80° C. and there is the possibility that the figure of 0·70 might

include some water of constitution driven off at this temperature. If the value of $\dot{W}$ could legitimately be reduced to 0·52, the average value of R would automatically rise from 0·74 to unity.

To test this possibility, heparinised blood was centrifuged in very narrow tubes (to effect as complete separation as possible of the cells and plasma) at 3500 r.p.m. for forty-five minutes. After removal of the plasma, white cell layer, and the top of the packed red cell mass, the lower part of the red cell layer was transferred by pipette to a silica boat, weighed, and dried to constant weight (ten to twelve days) over calcium chloride at room temperature (20° C.) and atmospheric pressure.

In three experiments, the average water content of the packed cells was found to be 0·692 gm. per c.c. The dried cells were then transferred to a special drier and further exposed to the influence of sulphuric acid at 60° C. at atmospheric pressure until constant weight was again reached. The average water content rose to 0·699 gm. per c.c.

Since the calculation of the extracellular tonicity (T), and the ratio of extracellular to intracellular water (p) present no technical difficulties, the low values of R must be either correct or must be due to some error in the determination of the final equilibrium volume (V). As has been mentioned above, the final equilibrium volume is usually determined by hæmatocrit measurements and if error exists, the hæmatocrit method must, by a process of exclusion, be incriminated as the cause of the trouble.

The Hæmatocrit Method.—In the hæmatocrit method of determining absolute or relative cell volumes, the completeness of cell packing is determined by the applied centrifugal force and the time for which that force is applied. It is a notorious fact that different observers quote a variety of speeds and durations of centrifuging but seldom mention the effective radius of the centrifuge—which is required for the calculation of the relative centrifugal force. Ponder (1948, p. 56) gives some illuminating examples of this. The relative centrifugal forces under which the values of R have been determined must vary widely, and it is just such a wide variation that is found in the experimental values of R which have been recorded in the literature.

The question of plasma trapped in the mass of red cells has frequently been examined and the general conclusion is that, under the usual conditions of determining packed cell volumes, namely centrifuging at 3000 r.p.m. for thirty minutes on the ordinary laboratory centrifuges, some 2 per cent. of plasma remains in the cell layer (Vazquez, Newerly, Yalow and Berson (1952), Chaplin and Mollison (1952)). In such cases the relative centrifugal force is of the order of 2000 . g. Errors due to this source are comparatively small and are not sufficiently large to account for the low experimental values of R. But the question of distortion and compression of the red cell, especially an *artificially swollen* red cell, under forces of the order of 1000 to 3000 . g has

received little attention, and it is difficult to believe that swollen erythrocytes subjected to such centrifugal forces do not suffer some degree of compression. Ponder (1949) has drawn attention to the variation in the cell volume and in the value of R when different centrifugal forces are employed, and he has been able to reproduce values of R approximating to unity only at relatively low rates of centrifuging.

The indictment of the hæmatocrit method of determining *relative* cell volumes is completed by a straightforward experiment of the following type. One volume of oxygenated blood is added to two volumes of 0·380 per cent. sodium chloride solution and the mixture is stored at 20° C. during the experiment. After thorough mixing, samples are withdrawn, transferred to 100 mm. hæmatocrit tubes, and centrifuged at various speeds for thirty minutes. Two identical centrifuges were used so that determinations could be made every fifteen minutes. The effective radius of each centrifuge was 12·4 cm. The blood used had a packed cell volume of 45·8 per cent. cells determined by centrifuging at 2200 · g for thirty minutes, and the initial volume of the cells in the blood-saline system was taken as one-third of this (15·3 per cent. cells). The tonicity of the plasma is taken as unity and is assumed to be the same as that of a solution containing 0·87 gm. sodium chloride per 100 c.c. The value 0·70 is used for W, and by calculation the equilibrium volume of the cells in the blood-saline system is found to be 1·45 times their initial volume in the plasma. Results are shown in Table II.

TABLE II

The Dependence of R on the Relative Centrifugal Force

Relative Cell Volume (calc.) = 1·45 times the initial volume

Relative Centrifugal Force. ×g.	Packed Cell Volume (observed). Per Cent. Cells.	Relative Cell Volume.	R.
150	27·0	1·76	1·69
375	23·7	1·55	1·22
500	23·3	1·52	1·15
890	21·9	1·43	0·96
1285	21·1	1·38	0·84
2440	21·0	1·37	0·82

When the relative centrifugal force is plotted against R, these six results form a tolerably smooth curve and a value of R equal to unity is obtainable only under a centrifugal force of about 750 · g. applied for thirty minutes. Furthermore, the duration of centrifuging is as important as the relative centrifugal force, for the value of R can be increased or decreased by shortening or prolonging, respectively, the time of centrifuging. At a centrifugal force of 150 · g the value of R was reduced from 1·76 to 1·29 by increasing the time of spinning

from thirty to sixty minutes, and at a centrifugal force of 500 · g the value of R was increased from 1·15 to 1·32 by reducing the time of spinning from thirty to fifteen minutes. Exactly similar results were obtained when the red cells were suspended in a hypotonic solution of potassium chloride.

There can be no doubt that the high values of R obtained under low centrifugal forces are due to the presence of plasma trapped in the red cell mass, and few would cavil at the conclusion that the low values of R obtained under high centrifugal forces are due to compression of the cells. There is no reason for attaching any special significance to the combination of a force of 750 · g and a time interval of thirty minutes, and in all probability it represents a balance between the errors of residual plasma in the cell mass and of cell compression.

The dependence of the packed cell volume of whole blood on the applied centrifugal force is not an original observation; it was the subject of a classical paper by W. G. Millar in 1925 and his observations have been repeatedly confirmed by other workers. But the extent to which differences in the applied centrifugal force and the duration of centrifuging can alter the degree of swelling (and hence the value of R) does not appear to have been generally realised. The experiments described above make it abundantly clear that the erythrocyte can always be persuaded to function as a perfect osmometer (or as an imperfect osmometer) merely by a little judicious manipulation of the centrifuge controls and hence the hæmatocrit method of determining relative cell volumes must be abandoned and all results obtained by it must be rejected.

These arguments apply only to the determination by the hæmatocrit of the relative volume of swollen red cells which are apparently very sensitive to compressive forces. They do not apply to the determination of the packed cell volume of whole blood. It will be shown later that the cells in whole blood are not compressed to any measurable extent by the routine method of determining the packed cell volume.

Alternative Methods of determining Cell Volume.—Of all the possibilities, the most attractive alternative is the determination of cell volume changes by measuring the change in concentration of some extracellular substance when water moves from the medium to the intracellular space.

When the cell is placed in a hypotonic medium, cell swelling occurs by movement of water from the extracellular medium to the cell interior. The concentration of any extracellular solute will simultaneously increase provided that that extracellular solute cannot penetrate the cell membrane (*e.g.* protein). The degree of cell swelling can be calculated from the change in concentration of the selected extracellular solute.

This method was first used by G. N. Stewart in 1899 and has the great advantage that prolonged high-speed centrifuging is unnecessary. The method was later used by Ponder and Saslow (1930)

who employed the animal's own hæmoglobin as the external "indicator," the change in the concentration of which was used to calculate the movement of water across the cell membrane. A further improvement was introduced by Ponder (1943-44) who used Evan's Blue as the extracellular "indicator" and obtained satisfactory results with it, but only when heparin was used as the anticoagulant.

The determination of cell volume *in vitro* by the Evan's Blue method has certain disadvantages which have led to its being abandoned in the present investigations. The available commercial samples of the dye were badly contaminated with inorganic chloride and such an impurity is obviously highly objectionable in these experiments. Aqueous solutions of the dye obey the Beer-Lambert law up to very high concentrations, but the extinction of a dilute solution is most seriously affected by the addition of sodium chloride. Thus when 1 c.c. of an aqueous solution of the dye was diluted to 10 c.c. with distilled water the extinction was found to be 0·520, but when diluted to 10 c.c. with 0·5 per cent. sodium chloride solution, the extinction was only 0·476. In the systems under consideration, the bulk of the extracellular osmotic pressure is due to sodium chloride and as measurement of the concentration of the dye varies with the sodium chloride concentration, serious complications are introduced.

After some preliminary investigation, it was decided to measure the changes in cell volume by measuring the changes in the extracellular total plasma protein concentration. The method has certain obvious advantages: no extraneous matter other than saline or distilled water is added to the blood; should any hæmolysis occur, it is detectable immediately and the specimen is discarded; it is certain that no measurable amount of protein crosses the cell membrane in either direction; and finally, small changes in protein concentration can be measured with a fair degree of accuracy. The method has certain disadvantages: small errors in the determination of the extracellular protein concentration lead to considerably magnified errors in the value of R (a fault common to all experiments of this type); dilution of the plasma may precipitate part of the plasma globulin; and it is still necessary to centrifuge the systems containing artificially swollen red cells, but only for a short time under a low centrifugal force.

Experience has shown that of these three disadvantages, the first is the only one of importance. In a short series of preliminary experiments, protein was estimated by the micro-Kjeldahl method but the time required for replicate analyses (which are essential) and the corrections which have to be made for the non-protein nitrogen make this a most unwieldy method for the present purposes. In preference, the biuret method of Fine (1935) has been used. This method has the advantages that quadruplicate analyses may be carried out with ease, that no correction need be applied for non-protein nitrogen, and that the method gives results which are highly reproducible. The method

is standardised by determination of protein nitrogen by the micro-Kjeldahl method.

Principle of the Method used.—Blood is diluted with saline of such concentration that the red cells do not change in volume. The same blood is also diluted with water which produces a predictable degree of cell swelling. The protein concentrations in the extracellular fluid from each of these systems are determined, and from the results, the degree of cell swelling may be calculated.

Isoplethechontic Saline.—The term " isoplethechontic " is used by Ponder to describe the concentration of a solution in which the volume of the normal erythrocytes is maintained. It produces neither swelling nor shrinkage of the red cells. If the above principle is to be followed, it is first necessary to tackle the problem of determining the concentration of sodium chloride which is isoplethechontic with the plasma from normal oxygenated blood. The results of previous investigators are not encouraging—Ponder (1949) points out that the values quoted in the literature range from 0·85 to 1·10 gm. sodium chloride per 100 c.c. Nor can the often-quoted figure of Kirk, Sorensen, Trier and Warburg (1941) be accepted, for they state (p. 327) that " The calculations are carried out on the supposition that erythrocytes have the same volume in an 0·15*N* NaCl solution (0·86 gm. NaCl per 100 c.c.) as in the Christensen-Warburg solution." The chief source of error in determinations of this type is the continuous loss of carbon dioxide during the manipulations involved and the consequent changes in *p*H. It has been mentioned above that a change in *p*H has a marked effect on the red cell volume. This trouble is largely, if not wholly, avoided by the use of blood which has previously been fully oxygenated. The following method has been used :—

The packed cell volume of heparinised oxygenated blood is determined in duplicate by centrifuging in sealed 100 mm. hæmatocrit tubes at 2200 . g for thirty minutes.

Into each of five tubes is placed 2·00 c.c. of the same blood and to each tube is added 2·00 c.c. of saline—the saline concentrations used being 1·00, 0·95, 0·90, 0·85 and 0·80 gm. per 100 c.c. for each experiment. After thorough mixing, the packed cell volume of each blood-saline mixture is determined in duplicate under the conditions described for the determination of the packed cell volume of the whole blood.

When the packed cell volumes of the blood-saline mixtures are plotted against the corresponding concentrations of sodium chloride, the five points lie on a smooth curve. The concentration of sodium chloride on this graph which corresponds to a packed cell volume equal to one-half of the packed cell volume of the original blood is taken to be the concentration which is isoplethechontic with normal plasma.

This technique eliminates errors due to the loss of carbon dioxide since the preliminary oxygenation removes the carbon dioxide at the

start of the experiment. There is also sufficient plasma in the blood-saline mixture to prevent any serious change in *p*H during the several manipulations. And finally, the cells, even when the blood is mixed with a solution containing 0·80 gm. sodium chloride per 100 c.c., retain the form of a biconcave disc so that we are not subjecting swollen erythrocytes to high-speed centrifugation.

In ten such experiments, using ten samples of blood from different normal subjects, the concentration of isoplethechontic sodium chloride was found to lie between 0·850 and 0·895 gm. per 100 c.c. (mean = 0·871 gm. per 100 c.c.). The difference between the extremes of the range is to be expected for no precautions were taken to ensure that all subjects were in the same state of hydration although all blood samples were collected at 10 a.m.

In one other experiment blood was obtained from a case which was moderately dehydrated (a case of hypertensive encephalopathy) and the concentration of sodium chloride found to be isoplethechontic with the plasma was 0·972 gm. per 100 c.c. The method might therefore be developed as a means of estimating dehydration quantitatively.

There is no reason to believe that the variations found in the ten experiments with normal blood are anything other than the normal biological variation. The mean value (0·871 gm. per 100 c.c.) is rather lower than most values quoted and this is due to the fact that most of the blood bicarbonate has been removed by oxygenation, thus lowering the total osmotic pressure of the blood.

The Determination of R.—The procedure is as follows :—

(1) Two c.c. of heparinised oxygenated blood is centrifuged at about 3000 r.p.m. for twenty minutes. A specimen of the plasma is removed and the total protein concentration is determined.

(2) To 30·0 c.c. of the blood is added 2·00 c.c. of isoplethechontic sodium chloride containing 0·871 gm. NaCl per 100 c.c. After thorough mixing and centrifuging at 1000 . g for three minutes, a specimen of the supernatant fluid is removed and the total protein concentration is determined.

(3) 30·0 c.c. of the blood is centrifuged at about 2000 r.p.m. for five minutes. To the plasma is carefully added 2·00 c.c. of distilled water which is first mixed carefully with the plasma (the red cells are protected by the buffy layer of white cells) and the diluted plasma is then mixed rapidly and thoroughly with the red cell mass. The mixture is then centrifuged for three minutes at a centrifugal force *not greater than* 500 . g and a specimen of the supernatant fluid is removed for determination of the total protein concentration.

The low centrifugal force employed and the short duration of centrifuging reduce any compression of the swollen cells to a minimum.

In the third stage, the amount of water which is added to the blood is sufficient to cause the cells to increase in volume by about 50 per cent., and this degree of swelling does not normally produce hæmolysis. Should any hæmolysis occur, the whole experiment would have to be abandoned, but in practice, this never occurred.

The three protein analysis are each carried out in quadruplicate and every care is taken to avoid fortuitous errors, for on the accuracy of these determinations the success of the whole experiment depends. If any single analysis differed from the mean by more than 1 per cent., that analysis was discarded and a further analysis was carried out, but this happened very seldom. Only one of seven experiments had to be abandoned and this, for the reason that the protein in the three supernatants could not be completely precipitated by 10 per cent. trichloracetic acid ; even after prolonged centrifuging, the trichloracetic acid supernatant remained cloudy. The cause of this was never discovered.

When the protein concentrations of the plasma and the plasma-saline supernatant have been determined, the percentage of cells in the whole blood (the " packed cell volume ") may be calculated on the assumption that no water moves across the cell membrane in the blood-saline system, *i.e.* that a solution containing 0·871 gm. sodium chloride per 100 c.c. is truly isoplethechontic with the plasma of that particular experiment. Knowing the percentage volume of the cells in the whole blood, the theoretical relative cell volume at equilibrium in the blood-water system may be calculated from the equation taking 0·70 as the value of W. Having determined the protein concentration in the supernatant fluid from the blood-water system, the volume of this supernatant may be calculated on the assumption that no protein moves across the cell membrane in this system. From this figure, the volume of the swollen cells, their relative volume, and finally R, may be calculated.

The results of six experiments using the blood from different normal subjects, are shown in Table III.

TABLE III

The Determination of R

Experiment	1	2	3	4	5	6
Total plasma protein (mgm. per c.c.)	58·4	72·0	59·0	64·85	60·1	66·4
Blood-saline supernatant protein (mgm. per c.c.)	26·25	33·1	27·65	29·6	28·8	31·2
Blood-water supernatant protein (mgm. per c.c.)	32·6	40·7	33·2	36·8	33·9	37·55
Percentage cells	45·5	43·3	41·2	44·0	38·7	40·9
Relative cell volume (calculated)	1·54	1·54	1·53	1·54	1·53	1·53
Relative cell volume (observed)	1·52	1·53	1·51	1·55	1·50	1·52
R	0·96	0·98	0·96	1·02	0·94	0·98

Mean value of R = 0·97.

One important practical point remains to be mentioned. In these six experiments, the packed cell volume of the whole blood was also determined by the hæmatocrit. Duplicate samples of the oxygenated bloods were centrifuged at 2000 . g for thirty minutes in 100 mm. hæmatocrit tubes of internal diameter 3 mm. The observed packed cell volumes were :—45·7, 42·9, 40·9, 44·2, 38·2 and 41·4 per cent. cells. (These figures should be compared with the corresponding figures for the percentage cells given in Table III.) Were no error involved, the values obtained by the hæmatocrit method should be consistently about 2 per cent. greater than the corresponding values calculated from protein concentrations, due to the former including about 2 per cent. of trapped plasma in the cell mass. No such agreement is found. In three cases the hæmatocrit packed cell volume is greater, and in three cases it is less than the percentage cell volume calculated from protein analyses. In no case, however, is the difference between the results obtained by the two methods very great. The discrepancy may be partly due to technical errors, but it must also be partly due to the fact that in no case is the plasma necessarily exactly isoplethechontic with a solution containing 0·871 gm. sodium chloride per 100 c.c. since this value is the mean of a different series of experiments.

Discussion

The results set out in Table II leave no doubt that gross errors occur when the volume of swollen erythrocytes is determined by the ordinary hæmatocrit method. The fact that at a given centrifugal force applied for a given time, the erythrocyte appears to function as a perfect osmometer (R = 1·00) solves no problems, for the selection of the given centrifugal force and the duration of its application are quite arbitrary. One is therefore forced to discard all values of R which have been determined by the hæmatocrit, and to adopt some method which does not involve high-speed centrifuging.

The results obtained by the method which has been described in the text of calculating the cell volume change from changes in the extracellular protein concentration, agree with the findings of Ponder (1943-44) in those experiments where he determined the value of R using Evan's Blue as the external indicator of volume change with heparinised, but not with oxalated, blood. Ponder regards the Evan's Blue method as far more satisfactory than any other method for determining relative cell volumes. The method which has been described here is based on G. N. Stewart's original idea which, in common with the Evan's Blue method, obviates the necessity of subjecting swollen erythrocytes to high centrifugal forces, and I would agree that this method is the most satisfactory type of method of determining relative cell volumes. Indeed, it is the only type of method which is not open to the most serious objections. The results which have been obtained with it are a marked improvement on those obtained by

hæmatocrit methods, not only in the better agreement of observation with theory, but also in the consistency of the results amongst themselves.

While the results shown in Table II are unequivocal, they refer only to artificially swollen red cells which are suspended in a hypotonic medium, and it may be argued that the packed cell volume of whole blood is similarly dependent on the applied centrifugal force. The packed cell volume of whole blood is unquestionably dependent on the centrifugal force and the time for which it is applied, but in this case, a limiting centrifugal force is reached at about 2200 · g (applied for thirty minutes) where less than 2 per cent. of plasma remains in the cell mass. Under these conditions, the biconcave cells of whole blood do not appear to suffer any demonstrable compression, for the difference between the packed cell volume determined by the hæmatocrit, and the percentage cell volume calculated from changes in extracellular protein concentration, is very small (see above). With swollen erythrocytes suspended in a hypotonic medium, a limiting centrifugal force, beyond which no further packing occurs, is also reached and in all probability a certain amount of plasma is again trapped in the cell mass. But in this case, considerable compression of the cells must have occurred for there is no other way of explaining the low values of R which are consistently obtained when swollen erythrocytes are subjected to high-speed centrifuging. On the other hand, when a method is used which does not involve high-speed centrifuging the observed results are in good agreement with those calculated from the theory. In addition, at the centrifugal force at which R is unity (750 · g applied for thirty minutes) it cannot be questioned that a good deal more than 2 per cent. of the supernatant fluid is still present in the cell mass, and the only possible reason why values of R considerably greater than unity are not obtained, must be that the cells have undergone some degree of compression. There is no other possible explanation of the facts.

All arguments and observations, therefore, point to the hæmatocrit as the sole source of error. Discussing the value of the hæmatocrit in determining the packed cell volume of whole blood, Ponder and Saslow (1930) concluded that the packed cell volume recorded was always a balance between the two errors of intracellular plasma and cell compression, and that an absolute hæmatocrit value was unattainable. This conclusion is certainly true, but whereas the hæmatocrit method gives results very close to the true cell volume when one is dealing with whole blood, the results are wildly erroneous when the percentage volume of swollen red cells is determined.

The method which has been described above for the determination of R is theoretically the simplest of all modifications of the original method of G. N. Stewart. The weak point is the assumption that all specimens of plasma are isoplethechontic with a solution containing 0·871 gm. sodium chloride per 100 c.c. To determine that concentration of sodium chloride which is isoplethechontic with each individual

specimen of plasma, would require very large samples of blood and, what is probably more important, would involve delay in starting the experiments which lead directly to the determination of R. Although this remains a source of error, there is no evidence that this assumption leads to any wrong conclusions.

The accurate determination of small differences in protein concentrations has been simplified by the use of the biuret method of analysis. Of the many modifications, that of Fine was selected partly because of its simplicity, but mainly because the results obtained by it are reproducible to a very high degree when used in conjunction with a good photoelectric colorimeter, and whatever the failings of the biuret method as an absolute method of determining protein concentrations, all observers stress the reproducibility of the method. The standard graph was calibrated by micro-Kjeldahl nitrogen analyses using the conversion factor 6·25, but the accuracy of the standardisation and the assumption of the factor 6·25 are of little moment since all that is required is a series of comparative readings of three protein solutions all derived from the same plasma in any given experiment. Nor are we concerned with differences in the ratio albumin : globulin : fibrinogen from one blood sample to another. The important technical point is the initial standardisation of the conditions under which the colour is developed and the rigid adherence to these conditions. Quadruplicate analyses are very easy to carry out with the consequent reduction in the unavoidable errors of analysis.

The method which has been used for the determination of R is satisfactory only when the degree of swelling of the cells is fairly large so that the difference in protein concentration between the supernatants of the blood-saline and the blood-water systems is of the order of 20 per cent. This occurs when the external tonicity is reduced to about 0·46. If the degree of swelling is less than this, the determination of R is correspondingly less accurate ; if it is greater than this, there is the danger of hæmolysis in the cell-water system. It was for these reasons that the ratio of blood to saline (or water) equal to 3 : 2 was chosen. If the erythrocyte behaves as a perfect osmometer at an external tonicity of 0·46, it may reasonably be assumed that it will function as a perfect osmometer at any other tonicity short of one low enough to produce hæmolysis.

The possible occurrence in the erythrocyte of water which is not osmotically active (" bound " water) has also been invoked to explain the necessity for the factor R. But the concensus of opinion favours the conclusion that its amount in the red cell is negligible, if it exists at all. The finding of values of R approximating to unity by a method which cannot cause cell compression offers further indirect evidence of the absence of bound water from the erythrocyte. The deviation of the observed mean value (0·97) from unity would be better explained in terms of experimental error.

Sodium Oxalate as an Anticoagulant.—In the literature on cell

swelling and osmotic hæmolysis, much has been written on the discrepancies in the properties of the red cell due to the use of sodium (or potassium) oxalate as the anticoagulant. In all cases where whole blood (in contradistinction to washed red cells) has been used, the trouble can be traced to the use of a vast excess of the salt which is thenceforth present, and exerting an osmotic pressure, in the system.

The calcium of blood is entirely confined to the plasma and its concentration in health is reasonably constant. Theoretically, 10 c.c. of blood requires 2 mgm. of sodium oxalate to precipitate the whole of the calcium, and if this amount is used, the osmotic equilibrium of the blood is altered only by the substitution of two sodium ions for one calcium ion. It is however, a common practice to use 20 mgm. of sodium oxalate to prevent the clotting of 10 c.c. of blood, and the excess oxalate has no effect other than to increase the tonicity of the plasma from unity to about 1·2. In experiments on osmotic hæmolysis, where blood is distributed into a relatively large volume of hypotonic solution, the effect of even a moderate excess of sodium oxalate is small, and it has been my experience that the fragility of oxalated and heparinised blood is identical, at a dilution of 1 in 20, provided that the anticoagulants have been used in minimal amount. In experiments on cell swelling where the blood is only slightly diluted with the hypotonic medium, the use of even a moderate excess of sodium oxalate is ruinous—for obvious reasons. The only acceptable anticoagulant in such cases is a trace of heparin powder.

The determination of the value of R by a method which does not involve high-speed centrifuging of swollen cells, combined with the observations that the cell behaves as a perfect osmometer under conditions of varying temperature, and as an almost perfect osmometer in presence of different alkali halides in the external medium, removes all criticism of the erythrocyte's ability to behave as a perfect osmometer. While the experiments offered here need not take us back in history to the concept of the red cell as a simple balloon-like structure which can be inflated or deflated merely by juggling with the external conditions, they do return us to the idea of a cell which conforms to the known laws of physical chemistry without the necessity of introducing debatable correction factors. This need have no reference either to the nature or function of the cell membrane; it simply represents agreement between predicted and observed behaviour under certain specified conditions.

Summary

It has been confirmed that normal erythrocytes exhibit slightly different fragilities in solutions of the various alkali halides.

It has been shown that the hæmatocrit method of determining the relative cell volume of swollen erythrocytes in hypotonic media is quite unreliable due to compression of the cells under high centrifugal forces.

A new modification of the method of determining cell volume by measuring changes in concentration of extracellular material has been used. It is based on the measurement of changes in the extracellular plasma protein concentration and does not therefore involve high-speed centrifuging of swollen cells. The results which have been obtained indicate that the normal erythrocyte behaves as a perfect osmometer.

This method of determining the percentage cell volume of whole blood gives results which are in reasonable agreement with those obtained by the usual hæmatocrit method.

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