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OSMOTIC BEHAVIOUR OF HUMAN RED BLOOD CELLS: AN INTERPRETATION IN TERMS OF NEGATIVE INTRACELLULAR FLUID PRESSURE

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SUMMARY

1. The observation that human red blood cells do not shrink in hypertonic media as much as expected for ideal osmometers has previously been explained in terms of either a marked increase in the osmotic coefficient of the cell contents or an increase in the chloride content of the cells.

2. Changes in suspension pH and haematocrit have been observed when the concentration of the unbuffered NaCl medium was doubled. The small increases in external pH, and the size of the volume decreases, are inconsistent with variations in the Cl content as a significant factor in the non-ideal osmotic responses.

3. Membrane potentials of red cells in buffered media were followed using the fluorescent dye, diS-C_3-(5). On shrinking at pH 7·4, the cells hyperpolarized *ca.* 5 mV as predicted if changes in the osmotic coefficient rather than in Cl content explained the osmotic behaviour.

4. Regarding haemoglobin in concentrated solution as a solute with high osmotic coefficient is formally correct but is little help in understanding the properties of the solution. We have found it useful to consider separately haemoglobin and the rest of the contents of the cell. The haemoglobin then supports part of the total hydrostatic pressure on the cell leaving the crystalloid solution to experience a reduced fluid pressure. In greatly shrunken cells the contents act like a gel with the matrix of haemoglobin under compression and the fluid which fills the spaces within the matrix under tension.

INTRODUCTION

The changes in volume of human red blood cells when they are exposed to hypertonic or hypotonic media are less than expected for cells with constant solute contents ideally dissolved in the total cell water, e.g. when cells are placed in NaCl solution of double the normal concentration they contain *ca.* 60 % as much water as in isotonic NaCl rather than 50 % as expected (for reviews see Lucke & McCutcheon, 1932; Ponder, 1948; Dick, 1965; Hladky & Rink, 1977). Similar findings have been reported for a variety of other animal cells including mouse ascites tumour cells and frog skeletal muscle.

Since there are neither hydrostatic (Rand & Burton, 1964) nor osmotic (Williams, Fordham, Hollander & Welt, 1959) pressure gradients across the human red cell membrane, there are only two possible types of explanation for the observed volume

changes of these cells. Either the osmotic coefficient of the contents increases markedly as their concentration rises or the solute content of the cells does not in fact remain constant as the cells shrink and swell. Most authors (e.g. Dick & Loewenstein, 1958; LeFevre 1964; Miller 19€4; Savitz Sidel & Solomon 1964) have assumed that the cell solutes are constant and have favoured explanations equivalent to postulating variations in the osmotic coefficient even though the coefficients for high concentrations of haemoglobin which could be calculated by extrapolation of Adair's (1929) physical data were too small to account for the changes observed in hypertonic media (Dick 1965). It is now universally accepted that during the time course of shrinking or swelling, haemoglobin, the cations, and the organic phosphate anions do not cross the membrane. The possibility that Cl movements might occur was examined by Cook (1967) who found little change in Cl contents for cells suspended in media of different osmolalities.

Gary-Bobo & Solomon (1968), however, reported substantial Cl shifts when cells were exposed to anisotonic, buffered, media. They discounted Cook's (1967) results and incorporated their own data into a model of red cell volume changes based on variation in the Cl content of the cells and Adair's (1929) osmotic coefficients for haemoglobin. They proposed that the size of the net charge on haemoglobin approaches zero as its concentration increases. Thus they propose that at pH 7·4 where haemoglobin carries a net negative charge, when cell shrinking concentrates the haemoglobin, the net negative charge becomes less negative and Cl enters the cell to preserve electroneutrality (for discussion of such shifts and their effects on cell volume, see Hladky & Rink, 1977). This Cl entry would add to the solute content and in this variable charge model, would account for most of the excess retention of water. Gary-Bobo & Solomon (1971) looked for direct evidence of changes in haemoglobin net charge by examining the ^{42}K distribution across a cellophane dialysis membrane separating 10 mM-KCl, at 4 °C and pH 6·6 from a concentrated solution of haemoglobin. The ^{42}K distribution changed as haemoglobin concentration increased from 3 to 11 m-molal and the results were interpreted as showing a decreasing net charge on the haemoglobin.

Gary-Bobo & Solomon (1968) extended the investigation of changes in cell volume with changes in medium osmolality to include pHs other than 7·4. They found that for lower pH the shrinkage caused by increasing osmolality was larger; at pH 6·1 it was even 5 % greater than ideal. If this result were correct it would provide strong evidence for the variable charge model since it would imply a loss of Cl during shrinkage at acid pH. However, more recently J. O. Wieth (personal communication) has found that the volume changes at pH 6·1, while larger than at pH 7·4, are still less than ideal.

Dalmark (1975) measured both the volume changes and the Cl shifts for human red cells when medium pH was changed at constant osmolality. He found, as had Cook (1967) but not Gary-Bobo & Solomon (1968), that after allowing for the chloride movements he could not account for the changes in water content which were still less than expected. Furthermore, Freedman & Hoffman (1977) have considered the pH buffering of the contents of human red cells made permeable to small ions using nystatin. The strength of the buffering did not decrease with increasing haemoglobin concentration (at constant total haemoglobin) in contradiction to the central

premise of the variable charge model. There are, however, differences between the conditions in these experiments and those of Gary-Bobo & Solomon (1968). Thus in Freedman & Hoffman's experiments nystatin was present and neither the ionic strength within the cells nor that within the medium was increased as the cells shrank in response to additions of sucrose to the medium.

We have sought to test the variable charge and variable osmotic coefficient models by finding relatively simple experimental procedures and conditions where the two hypotheses predict quite different behaviour for normal, washed cells suspended in simple media. Our results strongly support the variable osmotic coefficient model. Since the abstractness of this model has been a major barrier to its acceptance, we have also attempted to provide a formally correct description of the volume changes which can be understood in qualitative terms.

METHODS

Blood was freshly drawn from T.J.R. into a syringe containing one drop of heparin (Pularin, Evans Medical Ltd.) and washed 4 times in the standard buffered medium containing 150 mM-NaCl, 2 mM-KCl and 5 mM-HEPES adjusted to pH 7·4 with NaOH. These cells were then loosely packed either for use in membrane potential measurements at pH 7·4 or for further treatment. Cells for the pH measurements at initial pH 7·4 were washed once more in the standard unbuffered medium containing 150 mM-NaCl, 2 mM-KCl. Cells for experiments with initial external pH 6·1 were titrated to pH 6·1 using a citrate buffer solution (140 mM-KCl, 2 mM-NaCl, 15 mM-Na citrate, pH 5·6) and washed twice in unbuffered medium. It should be noted that the only exchangeable anion present in these experiments in osmotically significant quantity is Cl. Traces of HCO_3 (air equilibrium) do, however, remain and are sufficient to allow rapid equilibration of the Cl and OH ratios across the membrane (Jacobs & Stewart, 1942; see also review by Hladky & Rink, 1977).

pH measurements

Packed cells were added to unbuffered medium to give a haematocrit of about 10% in a continuously stirred, thermostatted tube. Suspension pH was recorded continuously using a Pye Unicam 290 pH meter with an E07 40588 electrode. After a steady level was obtained, sufficient 3 M-NaCl (or 3 M-KCl) was added to double the external Cl concentration. The smallest reliably detectable change in pH under these conditions was 0·02 units. External buffering was shown to be negligible compared to that within the cells by noting the initial pH transients after additions of acid or base and the steady levels after equilibration across the membrane.

Volume measurements

Cells were suspended in unbuffered medium at a haematocrit of about 23% and external pH 7·3. Packed cell volume was then determined accurately using six tubes in an Adams Autocrit microcentrifuge, spinning for 20 min. The lengths of the columns were measured using vernier callipers. Sufficient 3 M-NaCl was added to double the external NaCl concentration on the assumption of ideal water movement; this addition in fact increases the concentration somewhat more. Haematocrits after the addition of the 3 M salt were corrected for the change (*ca.* 5%) in total suspension volume. Calculation of cell water was based on the assumption that in the initial suspension 30% of the cell volume was occupied by cell solids whose volume is constant (cf. Cook, 1967). No corrections have been made for changes in the trapped extracellular fluid (none would be needed if the percentage trapped in the pellet were the same at the two osmolalities). In order to achieve better than 5% accuracy in the change in water content, conditions were chosen such that the difference in packed cell volumes was more than 5% of the suspension volume.

Potential measurements

Cells were suspended at 0·2 % haematocrit in 2·5 ml. standard buffered medium or a medium containing 150 mM-NaCl, 2 mM-KCl, 4 mM-phosphate, pH 6·1. DiS-C_3-(5) (2·5 μl. of a stock solution in DMSO) was added to give a final concentration of 0·2 μM. The fluorescence of the suspension was monitored with intermittent stirring at excitation 630 nm and emission 665 nm using a Perkin–Elmer MPF 44A spectrofluorimeter. After the fluorescence reached a steady level, 135 μl 3 M-NaCl was added and the new fluorescence level noted. Corrections were made for the dilution of the suspension and the increase in light scattering due to shrinkage of the cells and changes in fluorescence were converted to changes in membrane potential using the calibration curves of Hladky & Rink (1976). The small changes in internal pH expected would require negligible correction of these results, thus none has been made.

In all three types of experiments the tonicity was increased by adding concentrated NaCl (or KCl) so that the Cl ions remained a constant proportion of the osmotically active particles in the external medium. The experimental procedures described here have been chosen for their simplicity rather than for their ability to yield accurate, quantitative data.

Chemicals

DiS-C_3-(5) was kindly given by Dr A. Waggoner (Amherst, Mass.). HEPES was obtained from Sigma Chemical Company. All other reagents were A.R. grade.

THEORY

Prediction of the models

pH changes

If the solute content of red cells and the net charge on haemoglobin remain nearly constant during osmotic shrinking, then doubling the concentration of an unbuffered NaCl medium will not double the internal Cl concentration, $[Cl]_i$. For instance at pH 7·4 only about 80 % of the expected water loss occurs and $[Cl]_i$ will increase about 1·7 times and the Cl ratio $[Cl]_i/[Cl]_o$ will decrease by a factor of 0·85. Since the OH ratio equals the Cl ratio and, on the osmotic coefficient model, the buffered internal pH remains nearly constant, this model predicts that the unbuffered external pH should increase by about 0·07 pH units.

From the data of Gary-Bobo & Solomon (1968), when the osmotic pressure is doubled at $pH_o = 7{\cdot}4$, the variable charge model predicts that the net negative charge of haemoglobin decreases releasing at least 30 m-mole OH per litre cell water. These OH ions are buffered by the cell contents which have a buffer capacity of *ca.* 70 m-mole OH per litre cell water per pH unit (Harris & Maizels, 1952). Thus on doubling the osmotic pressure the internal pH should rise by about 0·5 pH units and the external pH should then rise by more than $0{\cdot}5 - 0{\cdot}07 = 0{\cdot}43$ pH units.

For initial $pH_o = 6{\cdot}1$ as the cells shrink the variable charge model predicts the loss of net positive charge on haemoglobin and via the arguments above a decrease in pH_o of the order of 0·5 units. The osmotic coefficient model now predicts an increase between 0 and 0·07 units.

Volume changes

The variable charge model explains the excess retention of water within shrunken cells partly as a result of the increase in osmotic coefficient of haemoglobin but primarily as a result of Cl uptake. In unbuffered solutions this uptake is not possible since there is nothing which can replace the Cl in the external solution in order to

preserve electroneutrality, and thus the change in the net charge of haemoglobin should appear as the change in pH discussed above rather than as a movement of Cl. For cells at high haematocrit in unbuffered media the variable charge model predicts a decrease in water content on shrinking which can be calculated from extrapolations of Adair's osmotic coefficients. On this basis, from the figures given by Dick (1965), the change in water content for a doubling of the NaCl concentration should be more than 90 % of the ideal.

The osmotic coefficient model predicts shrinking which is independent of the buffering of the medium, in other words about 80 % of the ideal (Dick, 1965). At $pH_o = 6{\cdot}1$ where the initial volume is larger each model predicts that the shrinkage in unbuffered media will be more nearly ideal than predicted by that model at $pH_o = 7{\cdot}4$.

Potential changes

With low haematocrit suspensions in buffered media, substantial Cl movement into and out of the cells is possible in exchange for the buffered external OH. The variable charge model predicts that shrinkage at pH 7·4 which occurs when the external NaCl is doubled should promote Cl entry which accounts for the excess water retention. Since Cl would then be a larger fraction of the total solutes within the cell, its concentration would have more than doubled and $[Cl]_i/[Cl]_o$ would increase. The membrane potential, related to $[Cl]_i/[Cl]_o$ by the Nernst equation, would then become less negative by some 5 mV. On the osmotic coefficient model the Cl ratio should decrease on shrinking as outlined above and the membrane potential should become more negative by some 4 mV. At $pH_o = 6{\cdot}1$ both models predict a small negative shift.

RESULTS

pH changes

In all these experiments, when the NaCl concentration was doubled, pH_o increased slightly, typically 0·05 units. The range was between 0·00 and 0·08 in different trials. This was true at 25 and 37 °C and at pH_o 7·4 and 6·1. The shifts were completed in a few seconds and pH_o was then stable for many minutes.

Volume changes

At pH 7·32 and 37 °C the measured cell volume changes when the external NaCl concentration was doubled were 27·2 and 28·5 % in two experiments compared with 35 % predicted from ideality. The changes in water contents were thus 78 and 81 % of those expected from ideality. At pH 6·1, where more accurate data would be required to distinguish between the models, the present techniques are not adequate. The changes observed in preliminary experiments were slightly less than ideal.

Potential changes

In all instances, on doubling the NaCl concentration there was a small decrease in fluorescence from the suspension containing the potential indicating dye diS-C_3-(5). At pH 7·4 at both 25 and 37 °C, the fall in potential estimated by comparison with the data of Hladky & Rink (1976) was *ca.* 5 mV. At pH 6·1 there were smaller

decreases in suspension fluorescence such that the scattering and dilution corrections rendered the change in potential uncertain. These results confirm a preliminary report of similar experiments by Freedman & Hoffman (1977).

DISCUSSION

These results, obtained by simple experimental procedures, clearly support the hypothesis that the non-ideal osmotic responses result primarily from changes in the osmotic coefficients of the cell contents. They are not consistent with significant changes in the net charge of haemoglobin during cell shrinkage. Thus at pH 6·1 the observed small increase in pH_o on shrinkage contrasts with the large fall predicted by the variable charge model. Similarly the observation of volume changes about 80 % of ideal in unbuffered media conflicts with the greater than 90% changes predicted by the variable charge theory for these conditions. Furthermore, at pH 7·4 the observed negative shift in potential (this report and Freedman & Hoffman, 1977) contrasts with the positive shift predicted by the variable charge model. The pH results at 7·4 confirm the finding of a small external alkaline shift on cell shrinkage in unbuffered media noted by Cook (1967).

These findings support Cook's (1967) conclusion, based on direct determination of Cl, that Cl movements do not account for the water movements when red cells shrink. Gary-Bobo & Solomon (1968), having obtained results quite different from those of Cook, criticized his method, stating that he had 'precipitated the red cell proteins, a process which irreversibly alters the state of charge of the haemoglobin molecule. Hence such experiments cannot reveal the change in z (the net charge per molecule of haemoglobin) that is associated with changes of the haemoglobin concentration in the red cell.' However, it is clear from Cook's description of his method that the precipitation step is used only to obtain the total Cl content of a known volume of cell suspension. The distribution of Cl between cells and medium was then calculated from the haematocrit, known external Cl concentration and suspended volume, all determined before the precipitation. Since there is no reason to question Cook's data, and since none of the predictions of the variable charge model for human red cells can be verified, one can only conclude that the Cl and volume measurements of Gary-Bobo & Solomon were incorrect.

Once it is accepted that the cell contents are effectively constant for cells exposed to different osmolalities, one can obtain an empirical relation between the osmotic pressure and the concentration of the solutes. If it is assumed that only the haemoglobin behaves non-ideally (or corrections are made for the non-idealities of the other components) it is possible to interpret these data in terms of the osmotic coefficient of haemoglobin within the cells. McConaghey & Maizels (1961) found that the osmotic coefficient could be as high as 12 when the haemoglobin was at 16·5 m-mole/kg cell water (*ca.* 50 % haemoglobin by volume at this concentration), i.e. at this concentration a mole of haemoglobin would hold 12 times as much water within the cells as would a mole of ideal solute.

A number of authors have sought to explain the large amount of water held within the shrunken cells in terms of water binding to haemoglobin (Le Fevre, 1964; Savitz *et al.* 1964; Levin, Cravalho & Huggins, 1976) but these attempts have

always suffered from the lack of a convincing explanation of how such water of hydration could also be solvent water for small molecules such as glucose (Mackay, 1932; Miller, 1964), ethylene glycol (Macleod & Ponder, 1936), short-chain alcohols (Hutchinson, 1952; Gary-Bobo, 1967) and Cl (Cook, 1967). Dick (1965) emphasizes that selective water binding is only one of a number of factors affecting the osmotic coefficient of haemoglobin. Large values of the osmotic coefficient can be a consequence of high volume concentration of a protein even in the absence of water binding. In simplified terms when haemoglobin is packed into a small volume (Dick discusses volume fractions near 10%) there are spaces between the haemoglobin molecules which are too small for another haemoglobin and are therefore filled with components of the crystalloid solution contained in the cell. As a result only a fraction of the water occupies space which could be occupied instead by haemoglobin and thus only a portion of the water is 'free' to leave the cell. For low volume concentrations the osmotic coefficient of haemoglobin can be calculated from statistical theories of the entropy of mixing for molecules of vastly different sizes and assumptions about selective binding of water and solutes (discussed by Dick, 1965).

It is, however, instructive to take a more primitive approach and consider separately the haemoglobin and the crystalloid solution in the cell. The haemoglobin then supports part of the hydrostatic pressure acting on the cell, the crystalloid solution supports the rest. Thus while the solution has a lower osmotic pressure than the external medium, the hydrostatic pressure on it is also less and the water equilibrium across the membrane is preserved. As the crystalloid deficit inside a normal red cell suspended in 150 mM-NaCl is *ca.* 20 m-osmole/l. cell water, the haemoglobin would be supporting a pressure of *ca.* 0·45 atm, and the pressure on the crystalloid solution would be 0·45 atm less than that on the external medium.

It should be emphasized that there is no contradiction between this proposal and the experimental findings that there is neither a gradient of total hydrostatic pressure across the membrane nor a gradient of total osmotic pressure as measured by freezing point. Thus the inside of the cell membrane (or any ice formed during a freezing point determination) experiences the sum of the pressures supported by the haemoglobin and the crystalloid solution and this pressure is equal to the external hydrostatic pressure. Similarly at constant total hydrostatic pressure the total osmotic pressure of the cell contents is a measure of the chemical potential of water within the cell which determines properties such as the freezing point. If haemoglobin is regarded as contributing osmotically active particles to the solution, it reduces the chemical potential of the water by diluting the 'free' water. Alternatively if the haemoglobin is regarded as being separate then it lowers the chemical potential of the water in the solution by reducing the pressure on the solution by an amount (with respect to the total pressure) equal to the osmotic pressure formerly ascribed to the haemoglobin.

If the haemoglobin molecules were much smaller and behaved in a more ideal manner, it would be appropriate to adopt the usual convention that they are dissolved in the water and dilute it. On the other hand, if they were so large (or long) as to span a large fraction of the cell it would be only reasonable to regard them as part of a lattice structure within the cell which resists compression of the cell and thus reduces the pressure acting on the solution. While both views are thermo-

dynamically permissible, we propose that the latter is a more accurate description of the inside of a red blood cell. In this view as a red cell shrinks, the pressure on the haemoglobin increases rapidly and the corresponding decrease in pressure on the solution causes less water to leave the cell than expected from ideality.

Haemoglobin within the cell can be imagined to support part of the hydrostatic pressure in either of two ways, as if it were a gas or as if it were the matrix of a gel. By a small extension of the analogy between particles in solutions and molecules in a gas (attributed to van't Hoff, 1886, see Glasstone, 1946, pp. 662 et seq.) haemoglobin may be regarded as a gas which is contained within the volume of the cell, V, and which supports a pressure

$$p_{Hb} = nRT/(V - V_{exc}), \tag{1}$$

where n is the number of moles of haemoglobin within a cell and V_{exc} is the volume within the cell excluded to haemoglobin molecules by the presence of other haemoglobin molecules. The excluded volume varies from $4n$ times the (hydrated) molar volume of haemoglobin, $\bar{V}_{Hb}$, in dilute solutions (see Glasstone, 1946, p. 294) to presumably slightly more than n times $\bar{V}_{Hb}$ in close packing. Since at normal tonicity, haemoglobin occupies about 30% of the cell volume, V_{exc} may be almost as large as the entire volume of the cell, V, a point noted by Ponder (1948, p. 140). Since the osmotic coefficient for haemoglobin (for concentrations in mole/l. cell water), Φ_{Hb}, can be defined by the equation

$$p_{Hb} = \Phi_{Hb} nRT/V_{H_2O} \tag{2}$$

it follows that

$$\Phi_{Hb} = \frac{V_{H_2O}}{V - V_{exc}}, \tag{3}$$

which can easily become very large indeed. (The expressions for the osmotic coefficient given by Dick (1965), eqs. (3.50) and (3.51), are for haemoglobin concentrations in moles per litre of cells). Eq. (3) may be regarded in another light as specifying the value which must be assigned to V_{exc} if the model is to agree with the thermodynamically correct description in terms of osmotic coefficients. The calculation of V_{exc} from first principles is equivalent to the calculation of the entropy of mixing.

Many experiments on changes in red cell volume have been interpreted using either Ponder's equation or equivalently Lucke & McCutcheon's (1932) more conventional equation (rewritten in the present notation),

$$\pi(V - b) = n_T RT, \tag{4}$$

where π is the total osmotic pressure, b is the 'non-solvent' volume, and n_T is the total number of moles of solute particles within the cell. Eq. (4) can now be seen to be inadequate since it assigns the same value to the excluded volume for haemoglobin and the other solutes. In terms of the present model (4) should be replaced by

$$\pi = \frac{nRT}{V - V_{exc}} + \frac{(n_T - n)\,RT}{\bar{V}_{H_2O}}. \tag{5}$$

More refined versions of the model would allow for non-idealities of the other solutes and specific interactions between water, haemoglobin and the other solutes.

The gas model assumes that the molecules are in contact with each other for only a small proportion of the time. Since at pH 7·4 the volume concentration of haemoglobin is 30% even before shrinking, the gas model is not obviously applicable. The haemoglobin is better described as forming an irregular lattice which resists compression by forces between the molecules in the regions of contact. For red cells suspended in 300 mM-NaCl where the crystalloid deficit in the cell is about 130 m-osmole/l. cell water, the pressure on the gel would be about 3 atm, and there would be a tension on the crystalloid solution amounting to about 2 atm which is the same as a negative intracellular fluid pressure of 3 atm.

The concept of a negative fluid pressure within red cells is the converse of that of swelling pressure well known from studies of ion-exchange resins (Helfferich, 1962)

and is closely analogous to the concept of negative interstitial fluid pressure (Guyton, Granger & Taylor, 1971). It provides a particularly direct explanation of how water is held within the red cell. Considering the intracellular fluid to be under a smaller pressure than that exerted at the surface of the cell may be even more appropriate for other types of cells where the cytoplasm is actually a gel.

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