Extracellular and Metabolic Factors Affecting the Efflux and Influx of Erythrocyte Water

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ABSTRACT Water exchanges between rabbit erythrocytes and extracellular solutions equidistant from intracellular osmolarity were studied by freezing point depression techniques. Water efflux was always less than water influx and both were hypotonic to the intracellular and extracellular fluids. The magnitudes of these water exchanges were not dependent on the presence of extracellular cation. Stimulation of oxygen uptake by the addition of glucose and methylene blue increased water influx and, possibly, decreased water efflux. This could not be accounted for by accumulation of osmotically active intracellular metabolic products. O_2 uptake was markedly decreased during cellular dehydration, was slightly decreased during cellular overhydration, and was maximum at the water content of erythrocytes when suspended in a medium isotonic with plasma.

The concept of the mammalian erythrocyte acting as a simple osmometer changing its water content and shape in response to osmotic gradients has been modified in recent years. Although some question exists as to whether nucleated mammalian cells are isotonic with the extracellular fluid (1), it appears from the work of Williams *et al.* (2) that the mammalian erythrocyte is isotonic with the plasma.

Previous studies by Olmstead (3, 4) have shown that rabbit erythrocytes respond to hypertonic and hypotonic osmotic gradients equidistant from intracellular osmolarity with a greater water influx than water efflux. It was further shown that the magnitudes of these water exchanges are dependent on temperature and pH of the extracellular phase. The present paper reports investigations on the effect of extracellular composition and oxygen uptake on the magnitudes of efflux and influx of rabbit erythrocyte water in vitro.

METHOD

Measurements of exchanges of erythrocyte water with extracellular solutions were determined by a method previously described (3, 4). Briefly, this method is based

on the assumption that erythrocytes suspended in hypotonic or hypertonic solutions containing physiologically impenetrable extracellular solute withdraw water from or add water to the solution and that while no strict chemical partition of extracellular sodium and intracellular potassium is maintained in NaCl solutions, the exchange of these ions across the cell membrane, as summarized by Ponder (5), is reciprocal and equal under the conditions of the experiment. On the basis of this assumption, the change in water content of an extracellular solution is measured by the change in freezing point depression (Δ^t) of this solution after suspension of a measured amount of erythrocytes for a specified period of time.

Heparinized rabbit heart blood was used throughout. After centrifugation for one-half hour at 1400 *g*, the supernatant and buffy coat were removed. The cells were then resuspended in the extracellular solution to be studied with a Δ^t of −0.558°C (approximately isotonic with rabbit serum) and centrifuged for 20 min. The supernatant was again withdrawn and the cells resuspended in the same solution and centrifuged for one-half hour at 1400 *g*. After withdrawal of the supernatant, the surface of the cell column was blotted gently with a cotton applicator. One ml of cells was pipetted into 4 ml of test solutions. The Δ^t of these solutions were equidistant from cellular isotonicity; *i.e.*, −0.446°C and −0.670°C. The suspensions of cells were placed in a water bath at 38°C for 30 min., then centrifuged briefly, and the supernatant decanted for measurement of the Δ^t.

In the study of oxidative metabolism standard Warburg technique was used throughout. Final solutions consisted of 4 ml of the extracellular solution and 1 ml of erythrocytes. Flasks were gassed with oxygen for 5 min. and equilibrated 10 minutes at 38°C before closing stopcocks. Oscillations of the flasks in the water bath were through an arc of 45° at 120 cycles per min. Oxygen uptake was recorded as Q_{O_2} which throughout this study means microliters O_2 per 30 min. per ml of erythrocytes in 4 ml of extracellular solution.

To measure W_E into hypertonic solutions 3½ ml of isotonic test solutions plus 1 ml of erythrocytes were placed in the center wells of Warburg flasks. One-half ml of a concentrated buffered NaCl solution was placed in one side arm. This solution had been previously adjusted with the Fiske osmometer so that when mixed with the solution in the center well the final $\Delta^t = -0.670$°C. Q_{O_2} was measured for 30 min. during which the cells were in the isotonic medium. The flasks were then tilted to mix contents of side arm with center well and Q_{O_2} was measured for 30 min. while cells were in the hypertonic solution. A similar procedure was followed for measuring water influx from hypotonic solutions except that in this case 3 ml of the isotonic test solution was placed in the center well and 1 ml of diluted buffered NaCl in the side arm so that final mixing resulted in $\Delta^t = -0.446$°C.

The addition of water by micropipette to 4 ml of the test solutions results in a linear change of the Δ^t of these solutions which makes possible the determination of the amount of water added to (water efflux) or the amount of water subtracted from (water influx) the above solutions by erythrocytes. Four equations may be derived:

in buffered NaCl

$$W_E = \frac{0.668 - Y}{0.151} \qquad W_I = 0.40 - \frac{(0.485 - Y)}{(0.098)}$$

in sucrose solutions

$$W_E = \frac{0.668 - Y}{0.172} \qquad W_I = 0.279 - \frac{(0.484 - Y)}{(0.136)}$$

where W_E and W_I are water efflux and water influx, respectively, and Y is the final Δ^t of the extracellular solutions (see Appendix).

Effect of Varying Extracellular Content on W_E/W_I

Varying the content of the extracellular solution as shown in Table I resulted in a variation of W_E/W_I of 0.48 to 0.55. Although the osmolarities of the extracellular solutions were identical at the beginning of each experiment, the

TABLE I

EFFLUX AND INFLUX OF ERYTHROCYTE WATER

Extracellular solution	Water efflux (W_E)	Water influx (W_I)	W_E/W_I
	ml/100 ml RBC	*ml/100 ml RBC*	
Buffered NaCl pH 7.4	6.98 (0.12)*	14.55 (0.39)	0.48
Sucrose	6.70 (0.42)	12.77 (0.24)	0.52
Buffered NaCl + vasopressin	9.46 (0.55)	17.29 (0.22)	0.55
Sucrose + vasopressin	8.26 (0.24)	17.13 (0.56)	0.48

* = Standard error of the mean of 10 determinations listed in parentheses.
Vasopressin = 100 milliunits/ml.

ratios were not strictly comparable as the pH was controlled only in the case of the buffered NaCl solutions.

Present evidence suggests that water transport across nucleated cell membranes is isoosmotic (6, 7). The above studies with sucrose alone in the extracellular phase suggest that the movement of water in both directions across the rabbit erythrocyte membrane is hypotonic to the extracellular and intracellular fluids. The water movement occurs in the absence of extracellular cation and the ratio of W_E/W_I in the sucrose solution is not markedly different from that in the buffered NaCl solution. This is in contrast to the studies of Rixon and Stevenson (8) on rat diaphragms and Deyrup (9) on kidney slices which showed no change in tissue water when sucrose was used as the main osmotic constituent of extracellular solutions.

Addition of vasopressin, 100 milliunits per ml, to the extracellular NaCl and sucrose solutions resulted in a significant increase in W_E and W_I when compared with erythrocytes in buffered NaCl and sucrose solutions alone. Vasopressin has been reported to increase the passive transport of water across tissue membranes (10, 11) apparently by increasing membrane pore size or by increasing number of pores available for water transport. The present studies suggest that vasopressin also acts on the rabbit erythrocyte to increase

water transport in both directions across the cell membrane in response to osmotic gradients.

Relationship of W_E/W_I to O_2 Uptake

Water exchanges by cells placed in a Warburg vessel oscillating through an arc of 45° at a rate of 120 oscillations per min. are significantly different from water exchanges occurring when cells are placed in stationary test tubes in a water bath. The main difference consists in a reduction of the water efflux and

TABLE II

RELATIONSHIP OF OXYGEN UPTAKE TO W_E AND W_I

Extracellular solution	W_E	Q_{O_2} 30 min.	W_I	Q_{O_2} 30 min.	W_E/W_I
	ml/100 ml RBC	*μl*	*ml/100 ml RBC*	*μl*	
Buffered NaCl	3.36 (0.57)	0	19.60 (0.70)	0	0.17
Buffered NaCl + glucose	4.12 (0.37)	6.1 (1.4)	20.20 (0.71)	8.9 (1.6)	0.20
Buffered NaCl + glucose = NaCN	5.13 (0.29)	0	20.60 (0.53)	2.4 (0.30)	0.25
Buffered NaCl + glucose + MB	2.60 (0.42)	18.0 (1.9)	26.80 (0.73)	39.1 (3.2)	0.11
Buffered NaCl + glucose + MB + NaCN	5.79 (0.32)	26.2 (2.9)	25.00 (0.40)	16.0 (2.7)	0.22

Glucose = 100 mg per cent
MB = methylene blue 10^{-5} molar
NaCN = 10^{-3} molar
Standard error of the mean of 8 determinations listed in parentheses.

an increase in water influx (Table II). The resulting ratio $W_E/W_I = 0.17$ as compared with 0.48 when cells are in stationary tubes. The reason for this is not clear. Greater exposure of cell surface to the extracellular phase may be one possibility. In view of the mathematical relationships involved in determining the two parameters (see Appendix), the difference could also be due to an asymmetrical shift of intracellular components to the extracellular solution under the mechanical stress of the Warburg technique. However, cells suspended in isotonic solutions of buffered NaCl with glucose or glucose and methylene blue and placed in the Warburg apparatus under the same experimental conditions as recorded in Table II showed no significant change in the Δ^t of the extracellular solution after treatment for as long as 75 min. Nevertheless, the data in Table II should be regarded as comparative only and not necessarily representing the absolute magnitudes of the water fluxes under investigation.

Mammalian erythrocytes have a small but measurable oxidative metabolism (12, 13). In the present studies oxygenated rabbit erythrocytes in buffered isotonic NaCl containing 100 mg per cent glucose had a mean O_2 uptake of 11.1 ± 0.13 μl per 30 min. in solutions of 1 ml RBC plus 4 ml of extracellular solution in 28 determinations.

Harrop and Barron (14) first demonstrated that erythrocyte oxidative metabolism can be markedly increased by addition of small amounts of methylene blue to the substrate containing glucose. The details of the early studies of this mechanism were summarized by Ponder (15). Recent extensions of this work by Brin and Yonemoto (16) and Huennekens *et al* (17) demonstrate that human erythrocytes in the presence of methylene blue metabolize glucose *via* the hexose monophosphate shunt. These methods were utilized in the present study to increase the O_2 uptake of rabbit erythrocytes and to study the relationship of increased oxygen uptake of W_E/W_I. Addition of 10^{-5} molar methylene blue in an isotonic extracellular solution containing glucose increased the oxygen uptake of 1 ml of rabbit RBC to 45.9 ± 0.70 μl per 30 min. in 32 determinations—an increase of approximately 450 per cent over the oxygen uptake of the RBC with glucose alone.

The magnitudes of W_E and W_I are not significantly affected by the presence or absence of glucose in the extracellular solution (see Table II). With a threefold increase in O_2 uptake upon addition of methylene blue the W_E is reduced to 2.60 ml H_2O per 100 ml RBC as compared with 4.12 ml per 100 ml of RBC in the NaCl solution containing glucose alone. With an approximate fourfold increase in O_2 consumption due to the addition of methylene blue W_I is increased to 26.8 ml H_2O per 100 ml RBC as compared with 20.2 ml H_2O per 100 ml RBC in NaCl solutions containing glucose alone. The resulting ratio W_E/W_I is 0.11 in the presence of methylene blue and glucose and 0.20 in the presence of glucose alone. Although W_I is increased significantly ($P < 0.01$) in the presence of methylene blue, the differences in magnitudes of W_E are small and must be interpreted with caution.

NaCN 10^{-3} molar was added to the glucose and the glucose and methylene blue solutions to test its effect on W_E/W_I. W_E was increased into both solutions (Table II) although O_2 uptake was 0 in the glucose solution and 26.2 μl in the solution containing glucose and methylene blue. It would appear that W_E in the presence of NaCN is independent of O_2 uptake under these experimental conditions.

O_2 uptake was reduced to 2.4 μl in the presence of NaCN and glucose in the experiments on W_I. W_I under these conditions was not significantly different from W_I in the presence of glucose alone. However, W_I was significantly ($P < 0.01$) greater from solutions of NaCN with glucose and methylene blue than from solutions of glucose and NaCN alone and this W_I occurred in association with an O_2 uptake of 16.0 μl and was similar in magnitude to that oc-

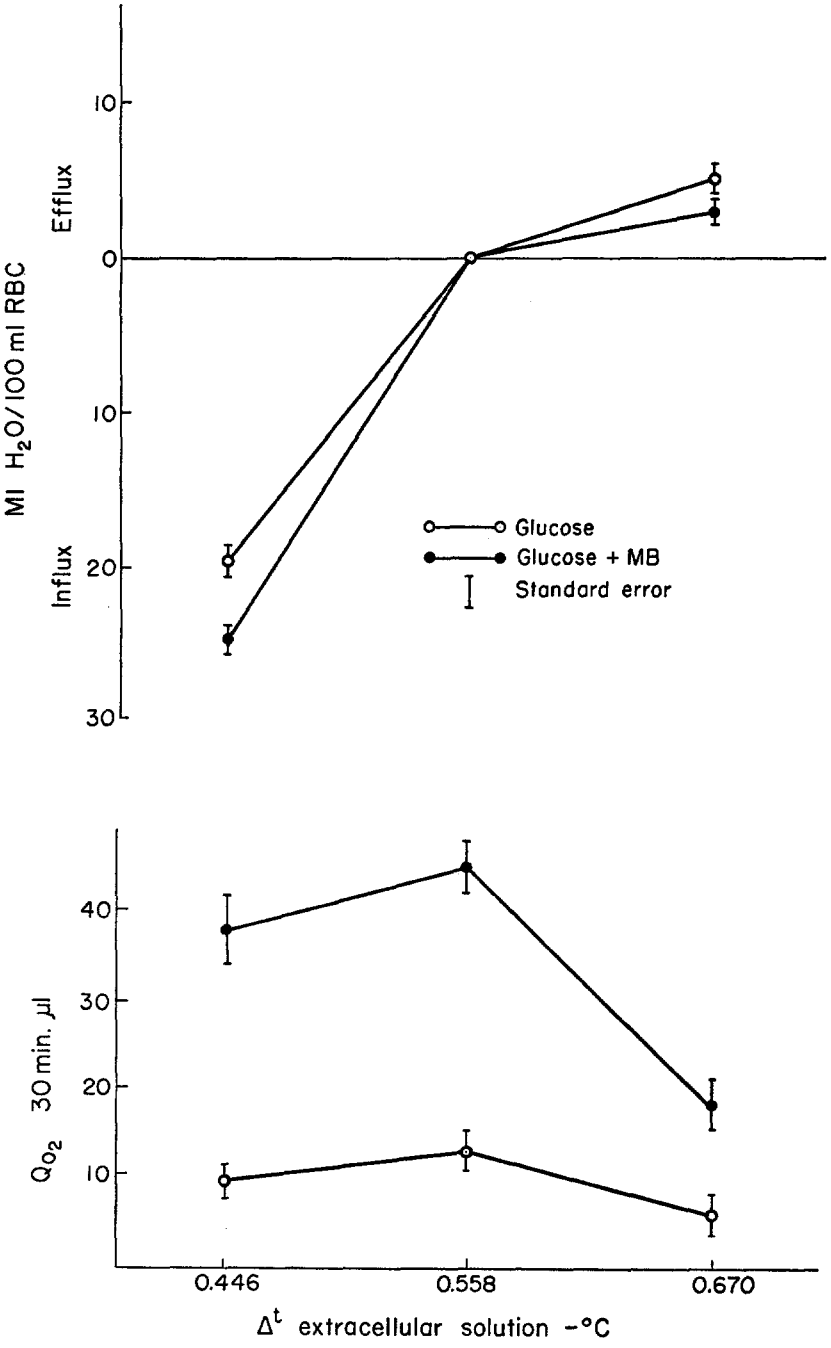

FIGURE 1. Relationship W_E/W_I to O_2 uptake. Glucose = 100 mg per cent, MB = methylene blue 10^{-5} molar.

curring with glucose and methylene blue in the absence of NaCN. It appears that an increase in O_2 uptake by rabbit erythrocytes in the presence of methylene blue is associated with an increased W_I and that this phenomenon is not affected by the presence of NaCN.

This increase in W_I and possible decrease in W_E under conditions of increased oxygen uptake cannot be accounted for by the accumulation of intracellular metabolites incident to accelerated oxygen metabolism. This is demonstrated by the fact that there is no change in the Δ^t of an isotonic extracellular solution containing glucose and methylene blue after incubation of erythrocytes in an oxygen atmosphere for as long as 75 minutes.

Effect of Cellular Hydration on Oxidative Metabolism

Fig. 1 shows the relationship of O_2 uptake to W_E/W_I. Oxidative metabolism in the presence of glucose or glucose and methylene blue is highest at isotonicity (−0.558°C). Oxidative metabolism is markedly reduced in both cases in the presence of a hypertonic extracellular solution.

These findings are similar to those of Hunter (18) on chicken erythrocytes. Robinson (19) also noted a reduction of O_2 consumption of liver cells in solutions below 200 and above 450 milliosmoles.

DISCUSSION

The present studies indicate that water fluxes in rabbit erythrocytes can be modified in magnitude by changes in the content of extracellular solutions without changes in the osmolarity of these extracellular solutions. Extracellular cation is not necessary for water exchange across the red cell membrane. Water exchanges under experimental conditions reported here are hypotonic to the extracellular and intracellular fluid in contradistinction to isoosmotic water exchanges occurring in response to osmotic gradents in nucleated cells.

Among the factors which distinguish nucleated mammalian cells from mammalian erythrocytes is the presence of an active oxidative metabolism in the former. If this oxidative metabolism is reduced by physical or chemical means, nucleated mammalian cells imbibe water. This imbibition of water can be largely prevented by increasing oxygen uptake *in vitro*. Rabbit erythrocytes are isotonic with solutions of the same osmolarity as the plasma whether their oxygen uptake is zero or as high as 45 μl per 30 min. in the presence of glucose and methylene blue. However, when subjected to osmotic gradients equidistant from intracellular osmolarity in the presence of an active oxygen uptake induced by glucose and methylene blue, rabbit erythrocytes show an increased water influx and, possibly, a decreased water efflux when compared

with erythrocytes under identical conditions respiring at a lower rate in the presence of glucose alone. These changes are not due to the accumulation of osmotically active metabolic products during accelerated glucose metabolism.

The relationship of cellular hydration to oxygen uptake needs special comment for a decreased oxygen consumption by erythrocytes with decreased

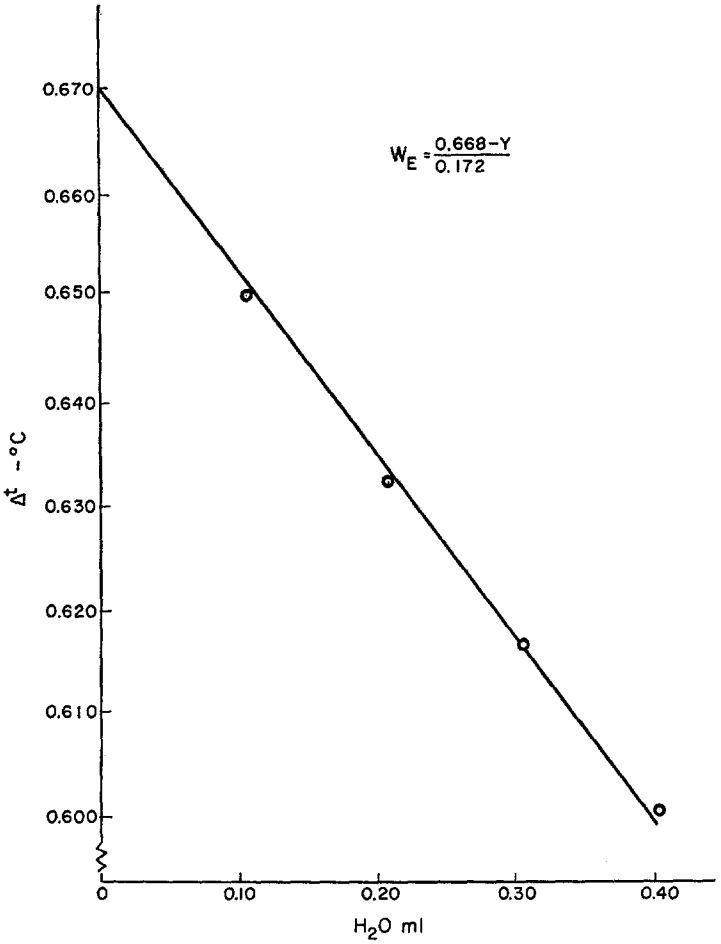

FIGURE 2. Relationship of addition of H_2O to Δ^t of hypertonic sucrose solution.

cellular water as would occur in hypertonic solutions is paradoxical. Enzymatic reactions, being chemical in nature, should be increased in rate with increased concentration of intracellular enzyme and substrate as would occur in cellular dehydration. The fact that this is not the case suggests that O_2 uptake in erythrocytes occurs at a maximum rate at an optimum intracellular water content which is obtained in this case when erythrocytes are suspended in extracellular solutions isotonic with plasma.

APPENDIX

The derivation of equations for magnitude of W_E and W_I is similar for each set of solutions. The derivations for sucrose solutions serve as an example. Five tubes are set up containing 4 ml of a sucrose solution of $\Delta^t = -0.670°C$. By means of a micropipette, 0.10, 0.20, 0.30, 0.40 ml of H_2O are added to tubes 2, 3, 4, and 5 respectively. The tubes are swirled to mix solutions and Δ^t determined on each.

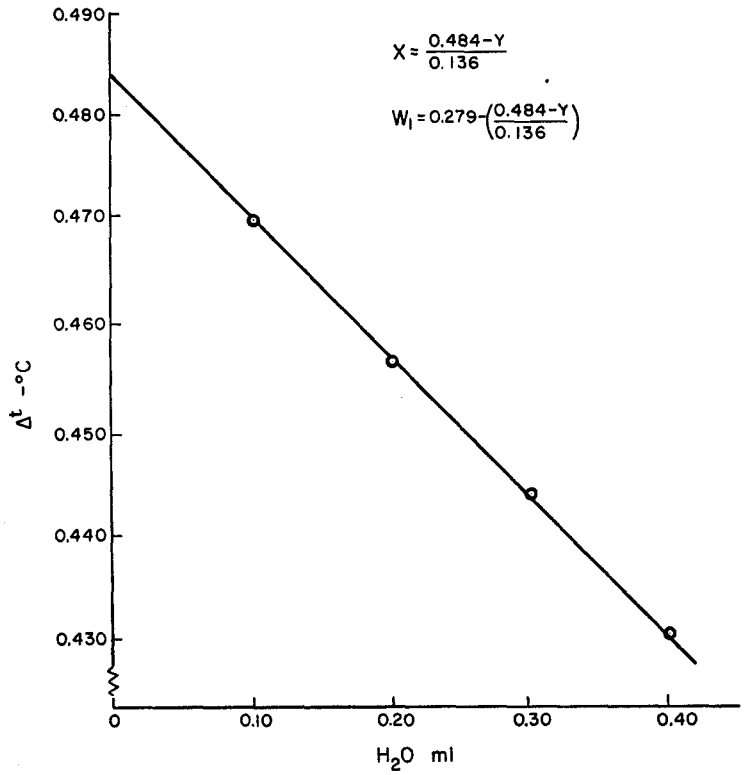

FIGURE 3. Relationship of addition of H_2O to Δ^t of hypotonic sucrose solution.

Plotting Δ^t against milliliters of H_2O added to each tube gives a linear relationship (Fig. 2). This equation derived by the method of least squares is

$$X = \frac{0.668 - Y}{0.172} = W_E \tag{1}$$

where X is the amount of water added and Y is the final Δ^t.

Similarly, additions of water are made to 5 tubes containing 4 ml of sucrose solution of $\Delta^t = -0.484°C$. Δ^t of the final solutions shows a linear relationship to the amount of water added (Fig. 3).

In this case

$$X = \frac{0.484 - Y}{0.136} \tag{2}$$

However, it is desired to know what X, the amount of water added, would be when Y is -0.446°C (equidistant from intracellular osmolarity). By solving equation (2) $X = 0.279$ ml H_2O when $Y = 0.446$°C; therefore, H_2O withdrawn from the sucrose solution of $\Delta^t = -0.446$°C is

$$W_I = 0.279 - \frac{0.484 - Y}{0.136} \tag{3}$$

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