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THE ROLE OF UNSTIRRED LAYERS IN CONTROL OF SUGAR MOVEMENTS ACROSS RED CELL MEMBRANES

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SUMMARY

1. [^{14}C]Glucose and [^{14}C]galactose net efflux from human red cells is increased by agitation of the cell suspension at 20°, but the effect of stirring is less at 2° or at 30°.

2. Trans acceleration of [^{14}C]galactose and [^{14}C]glucose efflux is greater at 2° than at higher temperatures and is reduced by agitation.

3. At 30° [^{14}C]galactose net efflux is retarded by external glucose but [^{14}C]glucose net efflux is increased by external galactose.

These results suggest that net sugar flux across red cell membranes is partially determined and that trans acceleration of sugar exchange is likely to be wholly determined by the concentrations and affinities of the sugars in the unstirred layers surrounding the cells.

An appendix shows that K_m (accelerated flux) is a measure of the concentration of labelled sugar in the unstirred layers.

INTRODUCTION

In a persorption exchange model for sugar transport[1] the assumption was made that sugar flux across red cell membranes is partially controlled by an unstirred layer effect. The thickness of stagnant layers surrounding artificial membranes, ion exchangers and toad bladder may be altered by rapid stirring[2–5] so it was considered worthwhile to determine whether agitation of the bulk solution affects net glucose and galactose efflux from red cells and glucose and galactose exchange fluxes across the cell membrane. Also, since the affinity of sugars (K_m net flux) for the cell membrane may be altered by varying the ambient temperature[6,7] the effects of temperature variation on net and exchange sugar flux were measured.

MATERIALS AND METHODS

Materials

Toluene, di-*n*-butyl phthalate, D-galactose, D-glucose, D-mannitol were obtained from British Drug House Ltd., Poole, Dorset, England; Triton X-100 from Lenning Chemicals, London; 2,5-diphenyl oxazole from Koch-Light Ltd., Colnbrooke, Bucks., England.

Radiochemicals

2–4 mC/mM D-[^{14}C]glucose, 2–4 mC/mM D-[^{14}C]galactose and 300 C/mole [^{3}H]inulin, average mol. wt. 5000 were obtained from the Radiochemical Centre, Amersham, England.

All chemicals for Ringer solutions and buffers were Analar grade. Human red cells were obtained from outdated stored blood by kind permission of the Sheffield Regional Hospital Board blood transfusion service.

Measurement of [^{14}C]glucose and [^{14}C]galactose efflux from human red cells

Cold stored human red cells were washed by repeated centrifugation and resuspension in 0.14 M NaCl and 0.02 M Tris–HCl, pH 7.2 (Ringer) at room temperature. The cells were then equilibrated for 3 h at room temperature with Ringer containing [^{14}C]glucose or [^{14}C]galactose at a concentration calculated to bring the equilibrium sugar concentration in the cell water to 120 μmoles/ml.

The cells were then centrifuged at 1500 $\times$ *g* for 10 min and 95 % of the supernatent fluid was removed. [^{3}H]Inulin was added to the cell suspension which was then brought to the temperature required for each experiment. The cells were recentrifuged immediately before use to remove the remaining supernatent fluid which was kept as a zero time reference. 1 ml of packed cells was then added to 50-ml beakers, internal diameter 3.8 cm, containing 20 ml of Ringer with 120 mM non-electrolyte added to maintain osmotic equilibrium. The solutions were stirred continuously with a 1-inch teflon coated magnetic stirrer at speeds calibrated with a stroboscope. During incubation the temperature was held constant by immersion of the beakers in a thermostated water bath.

3 ml of cell suspension were removed from the beakers at intervals and added to ice-cold test tubes containing 5 ml di-*n*-butyl phthalate. The tubes were centrifuged immediately at 1500 $\times$ *g* for 2 min. The red cells, being denser than the di-*n*-butyl phthalate, sediment through the organic phase leaving the cell-free extracellular fluid as an immiscible layer on top of the organic phase[21].

1 ml of the extracellular fluid was added to 1 ml of 0.5 M perchloric acid to remove any protein present in this solution, haemolysis did not exceed 1 % even with the most vigorous stirring, and 0.5 ml of the centrifuged perchloric acid extract was then added to vials containing scintillation fluid which had the following composition; 6 g 2,5-diphenyl oxazole dissolved in 750 ml toluene and 250 ml Triton X-100[8]. The samples were then counted in a Packard scintillation counter. Quench corrections for ^{14}C counts carried over to the ^{3}H channel and for efficiency loss were made on all samples using the Packard external standard which was calibrated against samples of known quench values.

[^{14}C]Glucose and [^{14}C]galactose effluxes at 20° and at 30° were calculated from the fractional change in the ^{14}C/^{3}H ratio (equilibrium measured after 1 h) in the first 15 sec of efflux. At 2° efflux was calculated from the change in ^{14}C/^{3}H ratio during the first minute of efflux and the equilibrium ratio was measured after 3 h incubation.

The initial concentration of labelled sugar in the bulk solution, which was calculated from the [^{3}H]inulin dilution factor, never exceeded 1 mM, hence was not an important factor determining sugar flux.

Some of the fluxes obtained by the above method were about 15 % faster than those reported by MILLER[9]. The reason for this may be that fluxes here were calculated

over a shorter time interval. This was done because it was found that labelled sugar exchange efflux decays rapidly after the first 30 sec as unlabelled sugar accumulates within the cells.

RESULTS

Effects of stirring at varying temperatures on [^{14}C]glucose efflux from red cells into Ringer containing mannitol

Red cells loaded with [^{14}C]glucose, 120 μmole/ml cell water were added to Ringer solutions with 120 mM mannitol added to maintain osmotic equilibrium. The solutions were stirred at varying speeds as indicated in Fig. 1 and Table I. Agitation increases [^{14}C]glucose efflux at 20°, at both 2° and at 30° the effect of stirring on net efflux is less than at 20°.

Effects of stirring at varying temperatures on [^{14}C]glucose efflux from red cells into Ringer containing glucose

The method adopted was similar to that described above, except that varying glucose concentrations were added to the Ringer and mannitol was added to bring the total non-electrolyte concentration to 120 mM. Increasing the external concentration of glucose increases the rate of [^{14}C]glucose efflux from red cells up to a maximum level (Fig. 2). As has frequently been observed[9–11], efflux is a rectangular hyperbolic function of external glucose concentration. Agitation also increases [^{14}C]glucose into solutions containing glucose but to a lesser extent than it increases net [^{14}C]glucose efflux into glucose-free solutions; agitation thereby reduces the maximal trans acceleration of glucose exchange. This is found at all temperatures tested. Additionally, as temperature is raised above 2° trans acceleration of [^{14}C]glucose efflux is reduced (Table I).

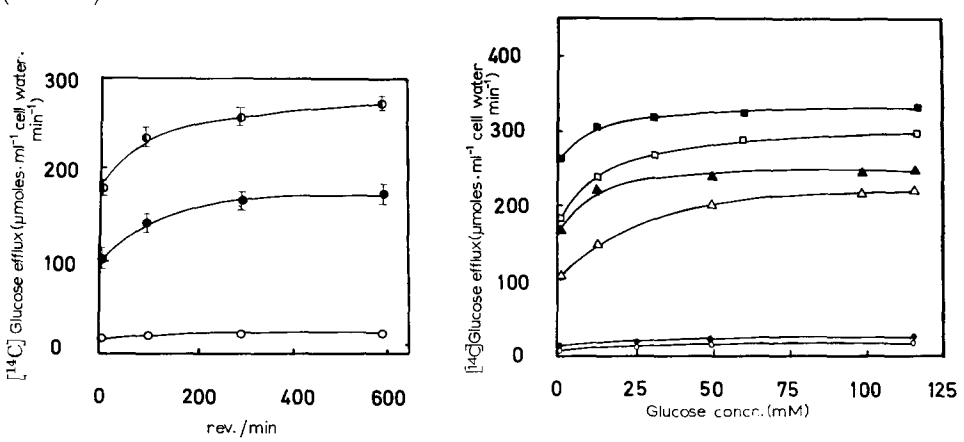

Fig. 1. Effect of stirring at different speeds on the efflux of [^{14}C]glucose from red cells. ○—○, 2°; ●—●, 20°; ◐—◐, 30°. Each point is the mean of at least 6 determinations ± S.D. is shown at 20° and 30°. The deviation at 2° was too small to represent on this scale.

Fig. 2. Effect of stirring at different temperatures on [^{14}C]glucose efflux into solution containing varying concentrations of glucose. Filled symbols, stirring rate 600 rev./min; empty symbols, stirring rate 15–20 rev./min. □—□, ■—■, 30°; △—△, ▲—▲, 20°; ○—○, ●—●, 2°. Each symbol is mean of at least 6 determinations.

TABLE I

GLUCOSE EFFLUX FROM HUMAN RED CELLS

Each figure is the mean ± S.D. Figures in parentheses are the number of determinations of each value.

	Glucose efflux μmoles per ml cell water per min					
	2°		*20°*		*30°*	
	Stirring speed (rev./min)		*Stirring speed (rev./min)*		*Stirring speed (rev./min)*	
	0	*600*	*0*	*600*	*0*	*600*
[^{14}C]glucose efflux into 120 mM mannitol	7.19 ± 0.79 (8)	9.72 ± 1.03 (8)	100 ± 8.5 (12)	175 ± 7.2 (12)	187 ± 10 (8)	268 ± 10.5 (8)
Acceleration due to stirring	1.35		1.75		1.44	
[^{14}C]glucose flux into 120 mM glucose	19.55 ± 1.7 (8)	23.18 ± 1.52 (8)	210 ± 7.6 (12)	235 ± 9.5 (12)	298 ± 9.1 (8)	332 ± 8.1 (8)
Acceleration glucose/mannitol flux	2.72	2.49	2.1	1.34	1.59	1.24
[^{14}C]glucose flux into 120 mM galactose	19.26 ± 1.96 (8)	24.1 ± 2.9 (8)	275 ± 8.4 (12)	290 ± 10.3 (12)	375 ± 26.4 (8)	400 ± 18.5 (8)
Acceleration galactose/mannitol flux	2.69	2.47	2.75	1.66	2.0	1.49
K_m for [^{14}C]glucose acceleration by glucose	15 mM	15 mM	25 mM	10 mM	15 mM	7.5 mM

TABLE II

GALACTOSE EFFLUX FROM HUMAN RED CELLS

Each figure is the mean ± S.D. Figures in parentheses are the number of determinations of each value.

	Galactose efflux μmoles per ml cell water per min					
	2°		*20°*		*30°*	
	Stirring speed (rev./min)		*Stirring speed (rev./min)*		*Stirring speed (rev./min)*	
	0	*600*	*0*	*600*	*0*	*600*
[^{14}C]galactose efflux into 120 mM mannitol	7.6 ± 0.54 (6)	10.2 ± 0.83 (6)	141 ± 10.9 (9)	176 ± 7.7 (9)	265 ± 13.3 (6)	259 ± 13.7 (6)
Acceleration due to stirring	1.32		1.25		1.0	
[^{14}C]galactose flux into 120 mM galactose	17.2 ± 3.6 (6)	26.5 ± 3.0 (6)	223 ± 8.0 (9)	244 ± 8.6 (9)	265 ± 15 (6)	257 ± 7 (6)
Acceleration galactose/mannitol flux	2.26	2.6	1.58	1.39	1.0	1.0
[^{14}C]galactose efflux into 120 mM glucose	17 ± 1.8 (6)	22.4 ± 1.67 (6)	150 ± 7 (9)	165 ± 8.7 (9)	185 ± 10 (6)	168 ± 9.8 (6)
Acceleration glucose/mannitol flux	2.25	2.2	1.06	0.94	0.7	0.64

Effects of stirring at varying temperatures on [^{14}C]glucose efflux from red cells into Ringer containing galactose

[^{14}C]Glucose efflux from red cells into external solutions containing varying concentrations of galactose with mannitol added to bring the total non-electrolyte concentration to 120 mM was measured as described above.

At 20° and 30° [^{14}C]glucose efflux is accelerated more by 120 mM galactose than by 120 mM glucose (Table I). This confirms the findings made previously by MILLER[9]. However, at 2° no significant difference is observed between the trans acceleration of [^{14}C]glucose efflux produced by galactose or glucose. [^{14}C]Glucose efflux into galactose does not plateau like the efflux into glucose but continues to increase as the external galactose concentration is raised to 120 mM (Fig. 3). Consequently the extra acceleration of [^{14}C]glucose efflux seen at 20° and at 30° is only seen at high external galactose concentrations. Agitation also increases glucose–galactose exchange flux and decreases the acceleration of [^{14}C]glucose efflux on adding 120 mM galactose to the external solution.

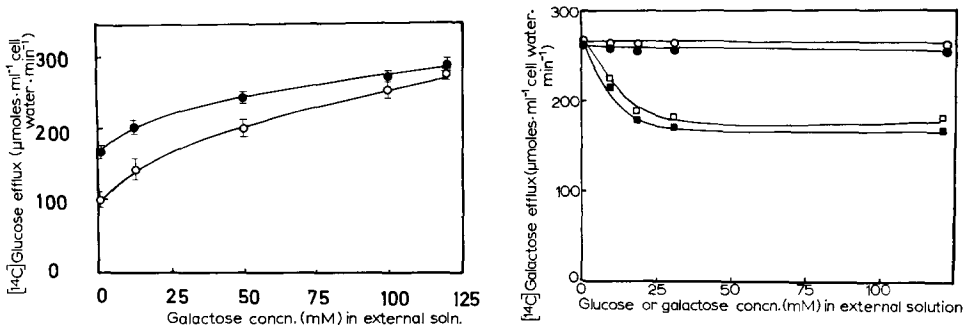

Fig. 3. Effect of stirring and of addition of varying concentrations of galactose to the bulk solution to [^{14}C]glucose efflux from red cells at 20°. ●, circles stirring at 600 rev./min; ○, stirring at 15–20 rev./min. Each point is the mean of at least 6 determinations ± S.D.

Fig. 4. Effect of agitation and of addition of glucose and galactose to the bulk solution at 30° on [^{14}C]galactose efflux from human red cells. Filled symbols, stirring at 600 rev./min; empty symbols stirring at 15–20 rev./min; ○—○, [^{14}C]galactose efflux into solution containing varying concentrations of galactose; □—□, [^{14}C]galactose efflux into solutions containing varying concentrations of glucose. Each point is mean of at least 6 determinations.

Effects of stirring at varying temperatures on [^{14}C]galactose efflux from red cells into Ringer containing mannitol, glucose or galactose

Red cells loaded with [^{14}C]galactose 120 μmoles/ml cell water were added to Ringer solutions containing 120 mM mannitol, or 120 mM glucose or 120 mM galactose or mixtures of either mannitol and glucose or mannitol and galactose to make a final non-electrolyte concentration of 120 mM. Efflux of [^{14}C]galactose was determined as has already been described for [^{14}C]glucose efflux.

At 2° and at 20° both rapid stirring and addition of galactose to the external medium increase [^{14}C]galactose efflux, however, at 30° neither agitation nor addition of galactose to the external solution accelerate [^{14}C]galactose efflux from the cells (Fig. 4). This finding, which has not been previously observed, indicates that at 30° the affinity of galactose for the cell membrane is so low that there is no significant unstirred layer effect retarding efflux. At 2° and 20° [^{14}C]galactose efflux is a rectan-

gular hyperbolic function of external galactose concentration (R. J. NAFTALIN, unpublished results), and, as with [^{14}C]glucose efflux, both agitation and raising the temperature from 0° to 20° reduces the maximal trans acceleration (Table II). At 30° [^{14}C]galactose net efflux is actually retarded by external glucose (Fig. 4). Rapid stirring slows [^{14}C]galactose flux into solutions containing glucose still further (Table II and Fig. 4). At 20° [^{14}C]galactose efflux is virtually unaffected by external glucose and at 2° it is accelerated by external glucose.

The retardation of [^{14}C]galactose efflux at 30° by glucose is the converse of MILLER's [^{14}C]glucose–galactose exchange finding[9] and like MILLER's finding is irreconcilable with a mobile carrier model for sugar transport.

DISCUSSION

The results show that trans acceleration of labelled sugar exchange across red cell membranes is maximal at 2° and falls towards zero as either temperature is raised or the degree of agitation of the bulk solution is increased. The temperature sensitivity of the fall in trans acceleration is dependent on the affinity of the sugar for the cell membrane; with galactose, trans acceleration is absent at 30° whereas with glucose it is still present. In additional, agitation increases net sugar efflux from the cells, the extent of this increase is a function both of temperature and the sugar affinity for the membrane. Finally, at 30° [^{14}C]galactose net efflux is retarded by glucose in the external solution, whereas [^{14}C]glucose net efflux is accelerated by external galactose. The acceleration of [^{14}C]glucose by external galactose is dissimilar to both trans acceleration of glucose exchange and galactose exchange, in that [^{14}C]glucose efflux continues to increase as the external galactose concentration is increased to 120 mM whereas [^{14}C]glucose and [^{14}C]galactose exchange rates plateau at external sugar concentrations between 25–50 mM.

An interpretation of these findings is that net sugar flux across red cell membranes is partially controlled by an unstirred layer effect more precisely termed 'partial film diffusion control'[4]. The extent of this control depends on the membrane affinity and concentration of sugar in the film surrounding the membrane. Trans acceleration as previously described[1] depends on competitive displacement of labelled sugar from the outer membrane surface by unlabelled sugar from the bulk solution. Consequently, reduction of film diffusion control of labelled sugar efflux reduces the amount of labelled sugar bound to the outer membrane surface and so reduces trans acceleration of exchange efflux. Since galactose has a lower affinity for the red cell membrane than glucose[7,12] and the membrane affinity for sugars is reduced as temperature is raised[6,7] and the results show that agitation, raising the temperature, or use of sugar with a low membrane affinity reduce trans acceleration; it is likely that the assumption that trans acceleration is dependent on the unstirred layer effect is valid.

Further, in the absence of film diffusion control sugar efflux is controlled by the rate of sugar diffusion across the membrane. HELFFERICH[13] has indicated that coupled interdiffusion of two molecular species is not confined to ion exchange where coupling is enforced by the electroneutrality restriction, but also occurs with non electrolytes when coupling is enforced by a constant volume restriction[14]. Coupled non-electrolyte interdiffusion is also enforced during sorption through a membrane with a restricted

number of adsorption sites. The interdiffusion coefficient $\bar{D}_{AB}$ for non-electrolytes A and B diffusing within a sorbing membrane may therefore be expressed as follows:

$$\bar{D}_{AB} = \frac{\bar{D}_A \bar{D}_B(\bar{x}_A + \bar{x}_B)}{\bar{x}_A \bar{D}_A + \bar{x}_B \bar{D}_B} \quad (1)$$

(*cf.* ref. 13, equation 6–39). See Diagram I.

$\bar{x}_i$ and $\bar{D}_i$ are the partial mole fraction and the diffusion coefficients of species i within the membrane respectively.

Eqn. 1 shows that the rate of movement of a slowly diffusing species will be speeded when it interdiffuses into a rapidly diffusing species and *vice versa*; the rate of interdiffusion of the slower species rises asymptotically towards the diffusion rate of the faster species as the concentration of the latter rises[13].

Since galactose has a lower membrane affinity and therefore a higher persorption rate[1] than glucose, Eqn. 1 explains why, even in the absence of a significant unstirred layer effect (*cf.* ref. 1, Fig. 7a), [^{14}C]glucose efflux from red cells is increased as the external galactose concentration is increased.

Eqn. 1 also explains why [^{14}C]galactose efflux is retarded by external glucose, although the retardation of [^{14}C]galactose efflux seen in Fig. 4 may also be in part due to formation of a layer of glucose within the cell preventing access of [^{14}C]galactose to the membrane (*cf.* Fig. 9, ref. 1).

The results in this paper do not support the model for sugar transport recently described by LIEB AND STEIN[15]. Their model, which requires conformational changes in the membrane to facilitate sugar movements, predicts that the maximal trans acceleration of [^{14}C]glucose efflux by external glucose should equal twice the maximal net efflux rate. This prediction is not consistent with the results reported here.

Trans acceleration of both chloride and calcium flux has been previously observed in artificial membranes[16,17] and efflux of methylene blue from Sephadex G-10 (Pharmacia, Uppsala) is increased by both agitation of the bulk solution (R. J. NAFTALIN, unpublished results), and by addition of either sucrose or glucose to the bulk solution[18], these effects are not additive to the effects due to agitation. From

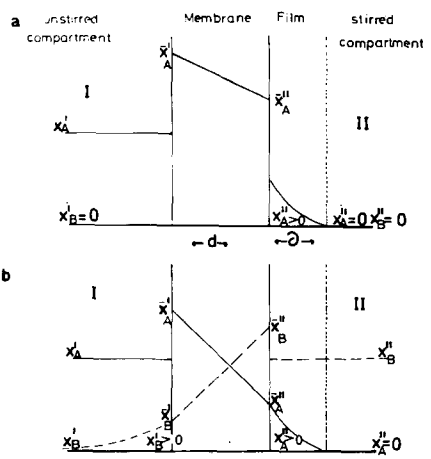

Diagram I. For explanation see DISCUSSION and APPENDIX.

these results it can be seen that there are similarities between solute flux across artificial membranes and sugar flux across red cell membranes.

In seeking a model for sugar transport across red cell membranes it therefore seems unnecessary to assume any membrane properties, other than an ability to reversibly bind sugars, at a restricted number of homogeneously distributed sites.

APPENDIX

In a sorbing membrane separating two compartments as in Diagram 1 the flux of A, J_A from Compartment I containing a solution of A into Compartment II containing a mixture of A and B may be represented as follows:

$$J_A = \frac{\bar{D}}{d}(\bar{x}'_A - \bar{x}''_A) \tag{2}$$

where $\bar{D}$ is the interdiffusion coefficient of A within the membrane, and d is the membrane thickness, $\bar{x}_i'$ and $\bar{x}_i''$ are the mole fractions of species i at the membrane surface in Compartments I and II, respectively.

If A is present in Compartment I in a sufficiently large concentration to saturate the membrane surface in I then

$$J_A = \frac{\bar{D}}{d}(1 - \bar{x}_A'') \tag{3}$$

If A and B are isotopes having equal affinity for the membrane (*e.g.* [^{14}C]glucose and glucose) it follows from the Langmuir equation[19] for monolayer adsorption of multiple species that

$$\bar{x}_A'' \simeq \frac{bx_A''}{1 + bx_A'' + bx_B''} \tag{4}$$

where x_i'' is the mole fraction of species i in Compartment II solution immediately adjacent to the membrane surface and b is the Langmuir constant for A and B. This ignores the small contribution to $\bar{x}_A''$ coming directly through the membrane from Compartment I.

If bx_A'' is large then in the absence of B in Compartment II $\bar{x}_A'' \simeq 1$ then on addition of B to Compartment II

$$\bar{x}_A'' \approx \frac{x_A''}{x_A'' + x_B''} \tag{5}$$

combining equations 3 and 5

$$J_A \approx \frac{\bar{D}}{d}\left(1 - \frac{x_A''}{x_A'' + x_B''}\right) \tag{6}$$

From Eqn. 6 it can be seen that J_A is a rectangular hyperbolic function of x_B''. This explains why [^{14}C]glucose efflux from red cells is a rectangular hyperbolic function of the concentration of unlabelled glucose in the bulk solution (Fig. 2).

From the law of mass action it may be deduced[20] that

$$\frac{\bar{x}_A''}{\bar{x}_B''} = \frac{x_A''}{x_B''} \tag{7}$$

Assuming x_B'' (bulk solution) $= x_B''$ (film layer); then when x_B'' (bulk solution) $= x_A''$ (film layer), then $\bar{x}_A'' = \bar{x}_B'' = 0.5$.

Hence, when the bulk concentration of unlabelled sugar is equal to the concentration of labelled sugar in the film layer, the flux of labelled sugar across the membrane is approximately half maximal. Thus the K_m (accelerated flux) *i.e.* the concentration of unlabelled sugar which gives half maximal acceleration of labelled sugar efflux, is an indicator of the concentration of labelled sugar in the film layer.

This explains why the K_m (accelerated flux) is reduced by agitation (see Table I).

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