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The volume changes associated with the operation of the 'simple' transporter

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The effects of hydrostatic pressure (0.1–50 MPa) on uridine transport mediated by the 'simple' facilitated nucleoside transporter of guinea-pig and human erythrocytes have been studied in an attempt to identify the volume changes which occur during transport. Pressure inhibited the zero-*trans* (influx or efflux) mode of uridine transport in guinea-pig cells significantly more (about 2.2-×) than equilibrium exchange. The equilibrium binding of ^{3}H-nitrobenzylthioinosine, a potent specific inhibitor of nucleoside transport, to human red cells and ghosts, was not significantly altered by pressure suggesting that the permeation site was unperturbed. Thus pressure inhibited the transporter primarily by preventing the volume increase associated with the translocation step. Furthermore, the return of the 'empty' transporter was found to be rate-limiting because it required a larger increase in volume than when the transporter was loaded with substrate.

Introduction

The transport of small molecules across biological membranes by carrier-mediated processes is characterized by four fundamental steps, (a) binding of substrate to the transporter, (b) translocation of substrate by the transporter, (c) release of substrate at the other membrane surface, and (d) the return of the transporter to its original position. Step (b) which involves the movement of substrate across the membrane phase is thought to be associated with a relatively large conformational change of the transport protein, and is therefore believed to be the principal rate-limiting step for the overall reaction [1]. The transporter can either return loaded with substrate (the so-called equilibrium exchange (EE) mode) or 'empty' (the so-called zero-*trans* (ZT) mode). For many red cell carriers the rate of transport is markedly stimulated when the transporter is operating in equilibrium exchange compared to zero-*trans*, a phenomenon termed *trans*-stimulation (see Ref. 1).

The volume changes of the transporter which occur during the translocation step may be an indication of the conformational change the protein has to undergo to mediate transport [2,3]. For most transporters the volume changes if any, are unknown, and there have been few studies on the volume changes associated with substrate binding or partial reactions (see Ref. 4 for review). There has, however, been a considerable amount of work on the effects of pressure on isolated enzymes (reviewed in Refs. 2 and 3). In these experiments because of the absence of associated lipids, the pressure-sensitivity of the enzymes is due directly to changes to the volume of the protein itself, for example by the binding of substrate or conformational changes.

The only practicable way to study the nature of the volume changes which are associated with the operation of membrane transporters is to investigate the effects of pressure. In one of the few studies on pressure effects on membrane transport, it was shown that the translocation step of the anion exchanger (Band 3) of human red cells was markedly inhibited by pressure (ΔV^* + 150 ml/mol), whereas it was argued that substrate binding did not involve a significant change in volume [5]. Clearly, if the various steps of the transport process involve different changes in volume this will be reflected in their differential pressure-sensitivity. Thus, if a process involves a volume increase (i.e. a positive apparent activation volume ($+\Delta V^*$)) it will be inhib-

Abbreviations: NBMPR, nitrobenzylthioinosine (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); ΔV^*, apparent activation volume; ZT, zero-*trans*; EE, equilibrium exchange.

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ited by pressure and a process involving a volume decrease (i.e. a negative activation volume ($-\Delta V^*$)) will be accelerated by pressure [6].

In the present study we have used the well-characterized nucleoside transporter of human and guinea-pig erythrocytes which shows the properties of a 'simple' facilitated transporter [7]. This system is inhibited with high affinity by NBMPR (nitrobenzylthioinosine; see Ref. 8). Inhibition by NBMPR is associated with tight, but reversible binding to specific sites on the cell membrane [9,10]. Kinetically, NBMPR is a competitive inhibitor of zero-*trans* and equilibrium exchange uridine influx in guinea-pig and nucleoside-permeable sheep erythrocytes but a non-competitive inhibitor of zero-*trans* uridine efflux [8,11]. These results suggest that NBMPR binds selectively to the outward-facing conformation of the permeation site. An external location of the NBMPR binding site in pig red cells has also been suggested from a comparison of the rate of association of NBMPR binding to inside-out and right-side-out membrane vesicles [12].

Guinea-pig erythrocytes were used for flux studies because of the relatively slow transport of uridine (a non-metabolized nucleoside) by these cells which allows the measurements at pressure to be performed over a reasonable time period [13]. In contrast, human red cells were used for ^{3}H-NBMPR binding studies because they possess about 500-× more transporters/red cell compared to guinea-pig cells [14]. Therefore human erythrocytes are more sensitive for studying the effects of pressure on the binding of NBMPR to the external inhibitor binding site which has been proposed to overlap with the permeation site [8,10,11,15]. Taking into account the differences in numbers of transporters/red cell, however, the kinetic properties of the nucleoside transporters in the erythrocytes of the two species are similar [7,13,14].

Materials and Methods

Materials. [^{14}C]Uridine (0.5 Ci/mmol) was purchased from Amersham International, and ^{3}H-NBMPR (23 Ci/mmol) from Movarek Biochemicals, CA. Uridine, NBMPR and all other reagents were obtained from the Sigma Chemical Co., Poole.

Blood. Fresh blood from guinea-pigs (250–400 g) or from normal human volunteers was taken by cardiac puncture under anaesthesia or venepuncture, respectively, into heparin (15 IU/ml blood). The blood was centrifuged (3000 × *g*, 5 min) and the plasma and buffy coat discarded. The blood was then washed five times by centrifugation and aspiration in 20 volumes of a standard saline (solution A) comprising (in mM); NaCl (140), KCl (5), glucose (5), Mops (15), EDTA (0.1; disodium salt), pH 7.4 at 37°C with NaOH. Haematocrits were measured using Drabkin's reagent [16,17].

Flux experiments. Initial flux rates of ^{14}C-labelled uridine were measured in washed guinea-pig red cells (haematocrit about 5%) under three conditions; (a) zero-*trans* influx, (b) equilibrium exchange (EE) influx and (c) zero-*trans* efflux essentially using methods previously described [14,18]. Briefly, for ZT influx measurements, washed red cells were suspended in the above ice-cold saline containing [^{14}C]uridine added from a stock solution (20 mM, with [^{14}C]uridine at about 5 μCi/ml) to give final concentrations covering the range 0.05–2.5 mM. A identical set of cell suspensions was also prepared containing a maximally effective dose of NBMPR (20 μM) from a stock solution (10 mM in DMSO). Taking care to remove all air bubbles, samples (usually triplicates of 1 ml each) of cell suspension were placed in disposable syringes containing a stainless steel mixing bar to agitate the cells when the pressure vessel was inverted. The syringes were then sealed with a plastic cap and placed in a water bath or water-filled pressure vessel (both at 37 ± 0.2°C; see Refs.19 and 20) and pressure applied hydrostatically. The experimental pressure was reached within 30 s and there was no significant temperature change during this period. The samples were mixed frequently, and at the end of the flux period (4–10 min) the vessel was decompressed, the syringes immediately placed on ice and the contents transferred to 1.5 ml Eppendorf microcentrifuge tubes for processing. The cells were quickly washed free of extracellular tracer by centrifugation (five times, 10000 × *g*, 10 s), aspiration and resuspension with the cold standard saline described above. The final cell pellet was lysed (0.1% v/v Triton X-100 in water), deproteinized (5% w/v trichloroacetic acid) and centrifuged (10000 × *g*, 5 min). The supernatant was then decanted into a vial containing Picofluor 40 as scintillant and radioactivity measured in a β-counter.

For equilibrium exchange influx experiments, aliquots of cells were pre-incubated (5 h, 37°C, 20% haematocrit) in the above saline including non-radioactive uridine at final concentrations ranging from 0.05–5 mM to allow equilibration with the intracellular pool. At the end of the loading period, the cells were rapidly washed (five times, 10000 × *g*, 10 s) in ice-cold uridine-free medium. The cells were then resuspended in cold saline containing [^{14}C]uridine at the same concentration as the intracellular unlabelled uridine in the presence or absence of NBMPR. The cell suspensions were then loaded into syringes and the flux performed as described above for ZT influx.

For ZT efflux experiments, aliquots of cells were pre-incubated as described for EE influx, except that [^{14}C]uridine (added from the stock solution see above) was present at concentrations to cover the range 0.05–5 mM. At the end of the loading period, the cells were washed in cold tracer-free saline (see above). The

erythrocytes were then suspended in fresh medium within syringes in the presence or absence of NBMPR. The incubation details were as described above, except that after the samples had been placed in Eppendorf tubes and centrifuged, aliquots of the supernatants were removed and placed into vials containing scintillant. The exact intracellular uridine concentration was determined by counting an aliquot of lysed cells.

No detectable haemolysis occurred following decompression of guinea pig or human red cells at the end of the flux period in any of these experiments. This stability of erythrocytes has previously been reported [4,19] making these cells suitable for membrane transport experiments at pressure.

Equilibrium ^{3}H-NBMPR binding studies. Washed human erythrocytes were suspended at about 2% haematocrit in ice-cold standard saline containing ^{3}H-NBMPR to give initial concentrations in the range 0.25–25nM [11,14]. For the estimation of non-specific binding, a parallel series of samples was prepared containing 20 μM non-radioactive NBMPR. The samples were then transferred to a water bath or pressure vessel as appropriate and incubated at 37°C for 30 min either at normal pressure or 50 MPa (0.1 MPa = 1 atmosphere). The pressure vessel was then decompressed, the samples quickly removed (total time taken was < 1 min) and placed on ice for 2 min. The cell suspensions were then rapidly decanted into cooled Eppendorf centrifuge tubes. The samples were centrifuged and an aliquot of the supernatant removed for the estimation of free (i.e. unbound) ^{3}H-NBMPR. The cells were washed (three times) to remove unbound tracer and the final cell pellet treated with 0.5 ml methanol, vortexed and centrifuged (10000 × g, 5 min) to remove the cellular debris. The supernatant was then counted as described above. Data were analyzed by linear regressions of Scatchard plots [21].

^{3}H-NBMPR binding at normal and high pressure was also studied by equilibrium dialysis using the following method. A concentrated suspension of human red cell ghosts (prepared by the method of Steck and Kant [22]) was added to the standard saline (A) and aliquotted into dialysis bags, which were then sealed and placed within large (50 ml) syringes (3–4 dialysis bags/syringe). Saline (A) with the concentration range of ^{3}H-NBMPR as described above was then drawn into each syringe and the syringes sealed with a cap. The syringes were then placed in a water-bath (at atmospheric pressure) or exposed to 50 MPa in the pressure vessel (both maintained at 37°C) for up to 24 h. Control experiments established that equilibration of ^{3}H-NBMPR across the dialysis membrane was complete in 12 h. At the end of the experiment, the syringes were quickly removed from the pressure vessel and water bath and a sample of the saline outside the dialysis bags (i.e. representing unbound ^{3}H-NBMPR) taken. The bags were then quickly removed from the syringes, blotted and also sampled (i.e. representing bound + unbound ^{3}H-NBMPR). The samples were then processed and analyzed as described above.

Analysis of data. The apparent kinetic constants (K_m and V_{max}) for the NBMPR-sensitive component of uridine flux were fitted to the Michaelis-Menten equation $V = V_{max} \cdot [S]/(K_m + [S])$ using a non-linear least squares regression analysis employing the HYPMIC programme [23]. The initial rate of NBMPR-sensitive uridine uptake, V, is given in units of mmol/l cells per h, [S] is the substrate (uridine) concentration (in mM) and K_m the Michaelis-Menten constant (in mM).

The flux data were further analysed using the 'resistance' parameters for the 'simple' transporter as defined by Lieb and Stein, [24]. The resistance parameter, R, represents the average time it takes the transporter to complete a single transport cycle under the appropriate experimental conditions. Thus, the R parameters are equal to the reciprocals of the maximum velocities for transport and are defined as follows; (i) $R_{12} = 1/V_{max}$ for ZT influx; (ii) $R_{21} = 1/V_{max}$ for ZT efflux; (iii) $R_{ee} = 1/V_{max}$ for EE which represents the resistance parameter for the loaded carrier; and (iv) R_{oo} is the resistance parameter for the empty carrier. R_{oo} is not directly measurable but is defined by the relationship; $R_{12} + R_{21} = R_{oo} + R_{ee}$.

Results

When plotted semi-logarithimically, pressure up to 50 MPa linearly inhibited both NBMPR-sensitive (i.e. nucleoside transporter-mediated; ZT influx mode) and NBMPR-insensitive components of uridine influx (Fig. 1). To exclude effects of pressure on the K_m of the transporter, a fully saturating uridine concentration (5

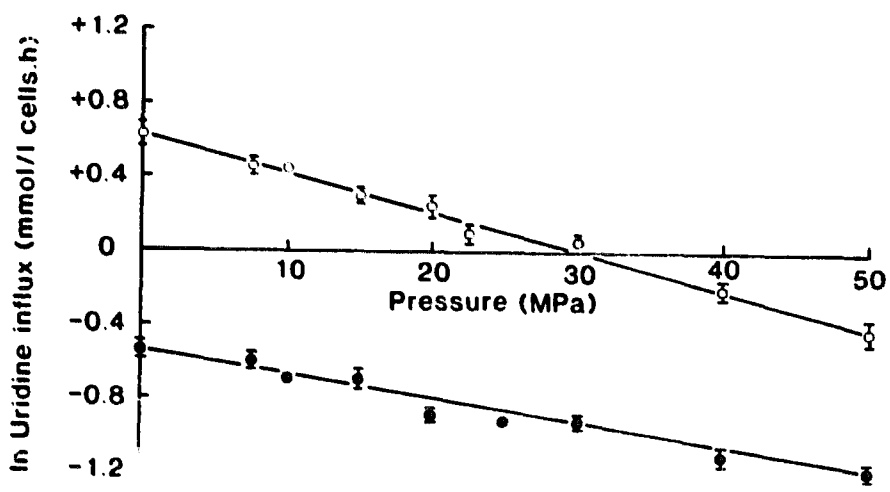

Fig. 1. The pressure-dependence of the nucleoside transporter, and passive uridine influx in guinea-pig red cells. Uridine influx was studied under zero-*trans* (ZT) conditions at a saturating concentration (5 mM) as follows; NBMPR-sensitive (transporter-mediated; open circles) and NBMPR-insensitive ('passive'; closed circles). The slopes of the data for ZT uridine influx calculated by least-squares regression analysis were 0.00208 ± 0.00017 MPa^{-1} and 0.00133 ± 0.00012 MPa^{-1}, respectively, with correlation coefficients of 0.995 and 0.973, respectively. Results are means ± S.E. for at least three experiments at each pressure.

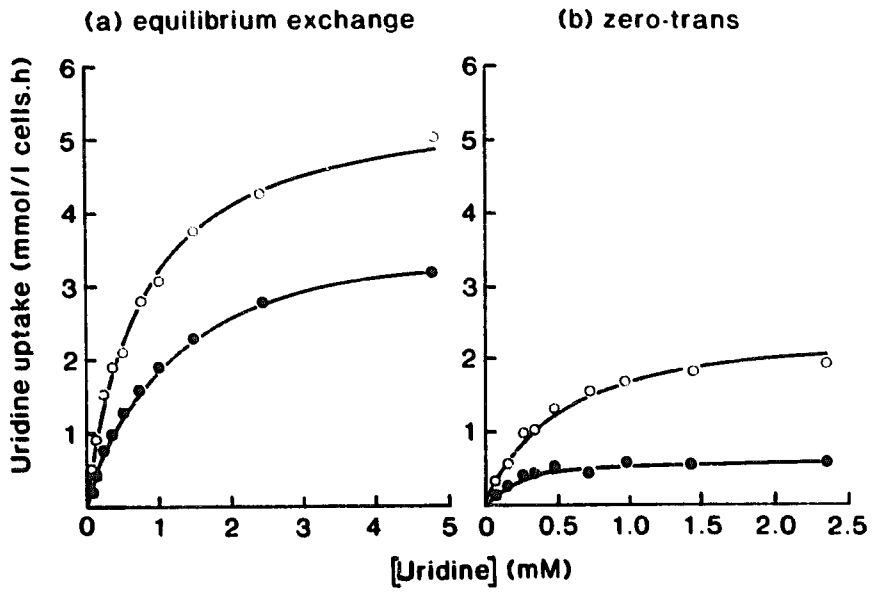

Fig. 2. Inhibition of the nucleoside transporter in guinea-pig red cells by pressure. NBMPR-sensitive uridine influx was studied at normal (open symbols) or high pressure (50 MPa; closed symbols) with the transporter operating either in (a) EE or (b) ZT modes as described in Methods. Data shown are means of duplicate determinations from representative experiments; pooled data are given in Table I. The curves are fitted to the Michaelis-Menten equation (see Methods) using the following values:

		K_m (mM)	V_{max} (mmol/l cells per h)
EE influx	0.1 MPa	0.71	5.6
	50 MPa	0.94	3.8
ZT influx	0.1 MPa	0.34	2.4
	50 MPa	0.14	0.65

mM) was used which is more than an order of magnitude higher than the K_m (0.35 mM; Fig. 2). Since there was a linear relationship between the logarithm of the flux and pressure this indicates that the apparent activation volume (ΔV^*) does not change with pressure [6,19] and therefore any pressure tested within this range should give the same value for the ΔV^*. In all subsequent experiments 50 MPa was used because of the substantial inhibition it produced. Values for the ΔV^* (calculated using a standard equation, see Ref. 19) were 53 ± 7 and 34 ± 5 ml/mol (means $\pm$ S.E., $n = 6$) for transporter-mediated and passive uridine fluxes, respectively.

Hydrostatic pressure markedly influenced the kinetic characteristics of the nucleoside transporter. Fig. 2 shows NBMPR-sensitive uridine influx mediated by the transporter operating in either EE or ZT modes was well-described by a standard Michaelis-Menten type equation (see Methods). Data for ZT efflux experiments performed at normal and high pressure could also be fitted to this equation (data not shown). Note the phenomenon of *trans*-stimulation such that at both normal and high pressure the V_{max} and K_m for uridine flux in the EE mode was markedly elevated compared to the flux in ZT mode (Fig. 2 and Table I; see also Refs. 7 and 25).

In both ZT and EE modes, the V_{max} was inhibited by pressure whereas the K_m was only significantly reduced (by about 50%) when the transporter was in the ZT (either influx or efflux) mode (Table I). In the EE mode the K_m was not significantly altered by pressure. Pressure inhibited the V_{max} of the transporter more when it was operating in ZT (both influx and efflux) modes compared to the EE mode. Thus at pressure (50 MPa), $35 \pm 4\%$ and $36 \pm 3\%$ of the flux remained in ZT influx and ZT efflux modes, respectively, whereas for EE influx $63 \pm 7\%$ of the flux at normal pressure was present. Corresponding values for

TABLE I

The effects of hydrostatic pressure on (a) [^{14}C]uridine flux mediated by the guinea-pig erythrocyte nucleoside transporter operating in three different modes, and (b) the calculated resistance parameters for the operation of the transporter

Definitions of the resistance parameters are given in Methods. Units for K_m, V_{max} and the resistance parameter R are mM, mmol/l cells per h and h/mmol per l cells, respectively. Data are given as means $\pm$ S.E. for n independent experiments; a single asterisk indicates a significant difference ($P < 0.05$) between normal and high pressure.

(a) Uridine flux	Pressure (MPa)						
Flux measured	0.1				50		
	(n)	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
ZT influx	(6)	0.29 ± 0.03	1.88 ± 0.25	6.48 ± 0.18	0.17 ± 0.03 *	0.66 ± 0.05 *	3.88 ± 0.05 *
ZT efflux	(3)	0.27 ± 0.04	1.76 ± 0.16	6.52 ± 0.66	0.16 ± 0.04 *	0.63 ± 0.05 *	3.94 ± 0.45 *
EE influx	(3)	0.85 ± 0.16	5.96 ± 0.35	7.01 ± 1.35	1.04 ± 0.14	3.73 ± 0.08	3.59 ± 0.50 *

(b) Resistance parameters	Pressure (MPa)	
	0.1	50
R_{12}	0.53 ± 0.08	1.52 ± 0.12 *
R_{21}	0.57 ± 0.05	1.59 ± 0.12 *
R_{ee}	0.17 ± 0.01	0.27 ± 0.01 *
R_{oo}	0.93 ± 0.12	2.84 ± 0.23 *
R_{oo}/R_{ee}	5.47 ± 1.09	10.50 ± 1.30 *

the ΔV^* were significantly different ($P < 0.001$); ZT influx 53 ± 4, ZT efflux 52 ± 4 and EE influx 24 ± 3 ml/mol (data are means ± S.E., $n > 3$). Table I also shows that the nucleoside transporter exhibits directional symmetry at both normal and high pressure, i.e. ZT influx was equal to ZT efflux both at normal and high pressures. This symmetry has also been observed at reduced temperature [13,26].

There might be concern that pressure could change red cell volume and thereby alter intracellular uridine concentrations which might influence the flux calculations. There is, however, only a slight decrease (about 4%) in red cell volume at pressure (40 MPa) which has been explained by the activation of the KCl transporter resulting in cell shrinkage [19]. We consider that this volume change is too small to alter intracellular uridine concentrations or to influence flux measurements.

At normal pressure the V_{max}/K_m ratios measured in the different flux modes were not significantly different with a mean (±S.E.) value of 6.6 ± 0.4 ($n = 11$). At pressure, there was also agreement between the ratios measured under the different flux conditions, however the average value was decreased to 3.9 ± 0.2 (Table I). This was due to effects of pressure on both V_{max} and K_m. These data also indicate that within experimental error, at both normal or high pressure the V_{max}/K_m ratio for ZT influx was not significantly different from the V_{max}/K_m ratio for EE. The R_{oo}/R_{ee} ratio increased by more than a factor of two at 50 MPa indicating that the mobility of the unloaded transporter was more pressure-sensitive than the loaded transporter (Table I).

In human erythrocytes, there was no significant effect ($P < 0.05$) of hydrostatic pressure on either the number of specific ^{3}H-NBMPR binding sites or their affinity (Table II). Non-specific binding was also unaffected by the application of pressure (data not shown). This method does not actually measure ^{3}H-NBMPR binding at pressure, and there was concern that during the short time at atmospheric pressure before and after pressure was applied there might be changes to binding. To check this, the effect of pressure on ^{3}H-NBMPR binding was studied using the equilibrium dialysis technique on erythrocyte ghosts (see Methods). At normal pressure values for the K_d and B_{max} were 3.6 ± 0.4 nM and 26.3 ± 4.0 pmol/mg, respectively; at 50 MPa these values were not significantly ($P > 0.05$) altered (3.8 ± 0.6 nM and 23.9 ± 4.0 pmol/mg, respectively).

TABLE II

Lack of effect of hydrostatic pressure on ^{3}H-NBMPR binding to human red cells

Specific binding, non-specific binding and calculation of binding constants were determined by equilibrium binding experiments as described in Methods; data are means ± S.E. from six independent experiments.

	Pressure (MPa)	
	0.1	50
K_d (nM)	0.173 ± 0.004	0.188 ± 0.013
B_{max} (pmol/ml cells)	110 ± 17	111 ± 15

Discussion

These results show that the nucleoside transporter of guinea-pig erythrocytes is inhibited by high pressure indicating that a volume change occurs during the transport process. The lack of effect of pressure on ^{3}H-NBMPR binding to human red cells supports the notion that the inhibition of the transporter by pressure is not due to impairment of the external permeation site or to a decrease in the number of functional carriers. The translocation step therefore appears to account primarily for the change in volume. (It should be noted, however, that NBMPR has a considerably higher affinity than uridine for the nucleoside transporter [8] and thus subtle changes by pressure to the site which interacts with uridine may not be detected). Of particular interest is the finding that there is a significantly larger (about 2-fold) volume increase associated with the return of the 'empty' compared to the loaded transporter (i.e zero-*trans* compared to equilibrium exchange mode).

Pressure might inhibit the transporter either by a direct action on the transport protein itself, indirectly via an ordering of the lipid environment or possibly by disrupting the lipid/protein interface. The data presented here and consideration of previous studies do not allow us to determine which of these sites is primarily responsible, however, there are some pertinent comments which can be made.

In artificial and biological membranes lipid acyl chain mobility tends to be ordered by elevated pressure (and lowered temperature) [27,28] and this could constrain the conformational change(s) of the transport protein. Indeed, lipid ordering tends to be more sensitive to the application of hydrostatic pressure than conformational changes of proteins [2,3] although there are a few examples of apparent activation volumes of conformational changes of isolated proteins which are similar to those measured here (about +50 ml/mol [2]). In general however, much higher pressures (> 100 MPa) than those used in this study are necessary to disrupt protein structure. Thus, the relatively high sensitivity of the nucleoside transporter to inhibition by pressure tends to favour a lipid site. It seems unlikely, however, that pressure inhibits the transporter by altering the phase state (i.e. from 'disordered' to 'ordered') of membrane lipids because there was no evidence of a 'break-point' (i.e. non-linearity) which would implicate a phase transition of lipids controlling transporter op-

eration (see Fig. 1). Furthermore, the differential effect of pressure on ZT and EE modes (ZT/EE = 2.2; Table I) was also observed when experiments were repeated at 21°C (ZT/EE = 2.3; Hall, A.C., unpublished data). Thus, the similarity of the pressure effect at low temperature indicates that the phase state of the lipids are not crucial for transporter operation.

The finding that hydrostatic pressure significantly increased the ratio of the resistance parameters R_{oo}/R_{ee} indicating that the 'mobility' of the empty transporter was impaired by pressure to a greater extent than that of the loaded transporter is particularly noteworthy. Similar differential changes in the 'mobility' of the 'empty' compared to the loaded transporter are also observed with uridine fluxes measured in fresh cells exposed to reduced temperature and also those studied in outdated human erythrocytes compared to fresh cells [7,13,26,29]. It has been suggested that these latter manoeuvres primarily influence the lipid environment and thereby perturb transporter operation, and it is possible that hydrostatic pressure also has the same site of action. At high pressure the directional symmetry of the nucleoside transporter was maintained. Furthermore, the similarity of the V_{max}/K_m ratio for both ZT and EE modes is consistent with the nucleoside transporter continuing to operate as a 'simple' transporter at high pressure [24].

The greater volume change associated with the return of the 'empty' compared to the loaded transporter (Table I) may be because the binding pocket is hydrated when substrate is not present and this increases the volume of the protein. This explanation however appears unlikely because the volume change is considerably larger than that expected from hydration alone which should be a few ml/mol [2,3,6]. A more likely explanation is that the 'empty' transporter (i.e. in ZT mode) can be considered a 'looser' structure and requires a larger volume change for its 'return' across the membrane. When substrate is bound however, the transporter becomes more 'condensed' resulting in a smaller conformational change which will presumably necessitate a smaller change in volume. Thus, it is possible that the phenomenon of *trans*-stimulation could be explained by proposing that because the volume change for transporter operation in the EE mode is less than that in the ZT mode, transporter turnover will be more rapid.

The interpretation of the effects of pressure on the K_m of the transporter operating in different modes is complex because this constant includes all 8 rate constants each of which may show differential pressure-sensitivity. Despite this, the data show that in the EE mode pressure had no significant effect on the K_m in contrast to a 50% reduction when ZT uridine influx or efflux were studied (Table I). The increased affinity of the transporter when operating in the ZT mode by pressure is probably due to the general slowing of the transporter so that the binding sites are available for longer and therefore for a given substrate concentration more sites will be filled. In addition, the reduced K_m by pressure in this mode supports the idea that substrate binds to a more compact conformation of the transporter (see above). Although the K_m is reduced by pressure this does not mean that the transporter will operate faster because as noted above, translocation of the empty carrier across the membrane is the rate-limiting step.

When the transporter was operating in the EE mode however, pressure had no significant effect on the K_m (Table I). Since transporter turnover in this mode will be faster, the time during which it is unloaded will be considerably less than when the transporter is in the ZT mode. In addition as we have noted above, the transporter occupies a larger (about 2-times) volume in the unloaded compared to the loaded form and therefore increasing pressure would favour the latter state of the transporter. Hence it is possible that although the transporter may be slowed by pressure, an effect on the K_m is not observed because this is compensated by the increased translocation rate and by pressure favouring the EE mode of operation.

In the presence of a maximally effective dose of NBMPR, uridine fluxes show the characteristics of transport mediated by 'simple' diffusion presumably across the lipid bilayer [10,11,14,30]. The pressure-sensitivity of this pathway (ΔV^* of about +34 ml/mol) is similar to that reported for passive diffusion of other molecules across lipid and red cell membranes (D-glucose across liposomes, +37 ml/mol [31,32]; lysine, glycine, alanine, across red cells about +30 ml/mol [4], and Hall, A.C. unpublished data). The simplest explanation is that passive permeation is across the lipid bilayer perhaps within the voids created by the motion of the acyl chains of the phospholipids, the so-called Trauble 'kink' hypothesis [33]. Hydrostatic pressure (and reduced temperature) would therefore inhibit passive permeation by reducing the motion of lipid hydrocarbon chains and decreasing the void volume and their rate of transfer across the membrane. However in biological membranes estimates of the volume available are very difficult [32,33] and it is possible that the rate-determining step is at the water/bilayer interface rather than in the bilayer interior. It is worth noting that the diffusional cation permeability of erythrocytes particularly to small monovalent cations (K, Rb, Na, Cs), is markedly elevated by hydrostatic pressure [4,19,34]. This is in contrast to the inhibition observed to the basal Na and K permeability of liposomes subjected to pressure [31,32]. A role for integral membrane proteins mediating diffusional cation permeation has been suggested [4,34].

In conclusion, the relative simplicitiy of the

NBMPR-sensitive nucleoside transporter has made it possible to separate out distinct reaction steps and to investigate their pressure-sensitivity. It is clearly difficult if not impossible, to predict the volume changes which occur with transporter operation without performing pressure experiments similar to those described here. The volume changes are fundamental to the operation of membrane transporters, and therefore it seems worthwhile to extend these experiments to other membrane carriers in an attempt to determine whether or not their partial reactions show similar sensitivity to pressure.

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