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Nucleoside transport in human and sheep erythrocytes

EVIDENCE THAT NITROBENZYLTHIOINOSINE BINDS SPECIFICALLY TO FUNCTIONAL NUCLEOSIDE-TRANSPORT SITES

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Nitrobenzyl[^{35}S]thioinosine binding and nitro[^{3}H]benzylthioinosine binding to nucleoside-permeable and nucleoside-impermeable sheep erythrocyte membranes was investigated, and compared with that found for human erythrocytes. High-affinity nitrobenzylthioinosine-binding sites (apparent $K_D \simeq 1$ nM) were present on human and nucleoside-permeable but not nucleoside-impermeable sheep erythrocyte membranes (8400 and 18 sites/cell for human and sheep nucleoside-permeable cells respectively). Nitrobenzylthioinosine binding to nucleoside-permeable sheep erythrocytes was displaced by nitrobenzylthioguanosine and dipyridamole. Uridine, inosine and adenosine inhibited binding. The smaller number of nitrobenzylthioinosine sites on nucleoside-permeable cells compared with human erythrocytes corresponded to a considerably lower $V_{max.}$ for uridine influx in these cells (0.53×10^{-20} mol/cell per s at 25°C compared with 254×10^{-20} mol/cell per s). It is suggested that high-affinity nitrobenzylthioinosine binding represents a specific interaction with functional nucleoside-transport sites. The uridine-translocation capacity for each transport site at 25°C is 180 molecules/site per s for both nucleoside-permeable sheep cells and human erythrocytes (assuming a 1:1 interaction between nitrobenzylthioinosine and the nucleoside-transport system).

Nucleosides cross cell membranes by facilitated diffusion. Kinetic studies of nucleoside uptake by a variety of cell types, including human erythrocytes, have identified a broad-specificity transport mechanism for both purine and pyrimidine nucleosides (Berlin & Oliver, 1975; Cass & Paterson, 1972, 1973; Lum *et al.*, 1979; Wohlhueter *et al.*, 1979). Nucleoside uptake is inhibited by a diverse range of compounds including 6-thiopurines such as nitrobenzylthioinosine [6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine] (Paul *et al.*, 1975). Nitrobenzylthioinosine inhibits nucleoside transport at concentrations $< 0.1\ \mu$M, and loss of transport activity in human erythrocytes and HeLa cells is associated with high-affinity binding of inhibitor to the cell membrane (Pickard *et al.*, 1973; Cass *et al.*, 1974; Lauzon & Paterson, 1977).

This binding is displaced by nitrobenzylthioguanosine and dipyridamole, other potent inhibitors of nucleoside translocation. Some nucleosides have also been shown to inhibit nitrobenzylthioinosine binding, although deoxycytidine, a transported nucleoside, has no effect on inhibitor binding to human erythrocytes (Cass & Paterson, 1976; Paterson, 1979). For HeLa cells, the relationship between nitrobenzylthioinosine binding and transport inhibition is complex, with only partial loss of activity occurring, despite saturation of high-affinity nitrobenzylthioinosine-binding sites (Lauzon & Paterson, 1977; Paterson *et al.*, 1977*a*,*b*, 1980). Residual transport activity can be abolished, but only at high nitrobenzylthioinosine concentrations. In human erythrocytes, however, there is strict proportionality between the amount of nitrobenzylthioinosine bound to high-affinity sites and the degree of transport inhibition, suggesting that high-affinity binding may represent a specific interaction with the nucleoside transport system (Cass *et al.*, 1974). Nevertheless, it has not been possible to exclude the possibility that nitrobenzylthioinosine binds to high-affinity sites not associated with nucleoside translocation. For example, human erythrocytes have two, or possibly three, high-affinity cytochalasin B-binding sites, only one of which is relevant to cytochalasin B inhibition of glucose transport (Jung & Rampal, 1977; Lin & Snyder, 1977).

Studies from this laboratory have identified

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nucleoside-transport variation in sheep erythrocytes (Jarvis & Young, 1978*a*; Young, 1978). Cells from some animals (nucleoside-permeable type) rapidly transport nucleosides by a translocation mechanism similar to that found in human erythrocytes. This system is functionally absent from the erythrocytes of other sheep (nucleoside-impermeable type), with the transport difference between the two types being under simple genetic control. Nucleoside transport by nucleoside-permeable sheep cells is inhibited by nanomolar concentrations of nitrobenzylthioinosine (Jarvis & Young, 1978*a*). In the present paper we compare the binding of nitrobenzylthioinosine to erythrocyte membranes in the two types of sheep. Our results demonstrate that high-affinity nitrobenzylthioinosine-binding sites are present on nucleoside-permeable but not nucleoside-impermeable erythrocytes, providing strong independent evidence that nitrobenzylthioinosine interacts specifically with functional nucleoside-transport sites. A preliminary report of some of these results has been published (Jarvis & Young, 1978*b*).

Materials and methods

Chemicals

Uniformly ^{14}C-labelled nucleosides were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [U-^{14}C]Uridine was purified by t.l.c. as previously described (Jarvis & Young, 1978*a*). Nitrobenzyl[^{35}S]thioinosine (sp. radioactivity 760 Ci/mol), non-radioactive nitrobenzylthioinosine and nitrobenzylthioguanosine [2-amino-6-(4-nitrobenzyl)-thio-9-β-D-ribofuranosylpurine] were generous gifts from Profess A. R. P. Paterson, University of Alberta Cancer Research Unit, Edmonton,, Alberta, Canada. Nitro[G-^{3}H]benzylthioinosine (sp. radioactivity 4 Ci/mmol) was purchased from Movarek Biochemicals, City of Industry, CA 91745, U.S.A. Radiochemical purity of nitrobenzyl-[^{35}S]thioinosine and nitro[^{3}H]benzylthioinosine was checked by t.l.c. with chloroform/methanol (17:3, v/v) as solvent. Both were greater than 98% pure. Aldolase (grade I) from rabbit muscle, NAD^+ (grade II), D-fructose 1,6-bisphosphate and L-cysteine were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Dipyridamole [2,2',2'',2'''-(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyldinitrilo)tetraethanol; Persantin injection] was purchased from Boehringer Ingelheim, Bracknell, Berks., U.K. All other reagents were of analytical grade.

Cells and membranes

Whole blood was collected from sheep by jugular venepuncture into heparinized evacuated tubes. Animals were classified as to nucleoside permeability type by nucleoside flux measurements (Young, 1978). Blood from healthy human volunteers was withdrawn by syringe, also into heparin.

Erythrocytes were washed three times with 10 vol. of a medium containing 140 mM-NaCl, 5 mM-KCl, 20 mM-Tris/HCl (pH 7.2 at 37°C), 2 mM-$MgCl_2$, 0.1 mM-EDTA and 5 mM-glucose. The buffy coat was discarded. Cell counts, haematocrit and haemoglobin estimations were performed by established methods (Archer, 1965). Haemoglobin-free erythrocyte membranes were prepared by osmotic lysis by the method of Dodge *et al.* (1963), except that the final two washes were carried out in 5 mM-sodium phosphate (pH 7.2), which was also the medium used for subsequent binding assays. Measurement of glyceraldehyde 3-phosphate dehydrogenase activity in the presence and absence of Triton X-100 (Mawby & Findlay, 1978) confirmed that 'ghosts' from both species remained >85% unsealed under the experimental conditions used in the present study.

Nitrobenzylthioinosine binding

Erythrocyte and membrane suspensions (0.4 ml, 10% haematocrit for erythrocytes and 20 and 70% haematocrit equivalent for human and sheep 'ghosts' respectively) were preincubated in iso-osmotic NaCl medium or phosphate buffer respectively, at 37°C in the presence and absence of nitrobenzylthioguanosine (50 μM). After 25 min, 0.4 ml portions of prewarmed medium containing nitrobenzyl-[^{35}S]thioinosine or nitro[^{3}H]benzylthioinosine (1–100 nM) were added. Incubations (15 min) were terminated by centrifugation at 15000 *g* in an Eppendorf 3200 microcentrifuge (15 s for intact cells and 15 min for membranes). Supernatants were retained for radioactivity determinations, and the cell and membrane pellets were washed four times with 1 ml portions of appropriate ice-cold medium. Protein measurements by a modified Lowry procedure (Markwell *et al.*, 1978) established that <10% of the 'ghost' protein was lost during washing. Supernatants, and membrane pellets resuspended in buffer (0.4 ml), were counted for radioactivity in a Packard Tri-Carb scintillation counter with quench correction (Young, 1978). Radioactivity present in cell pellets was extracted with methanol (1 ml). Precipitated protein was removed by centrifugation (2 min, 15000 *g*) before counting for radioactivity. Methanol extraction recovered 95% of the bound radioactivity (as judged from supernatant counts) and gave identical results to samples processed in the presence of NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL 60005, U.S.A) with benzoyl peroxide as decolourizing agent. Other methods of extraction were less efficient [Triton X-100 (0.5%, w/v) and trichloroacetic acid (5%, w/v) gave 25% recovery; $HClO_4$ (6%, w/v) gave 40% recovery and butanol gave 90%

recovery]. In experiments to determine the time course of nitrobenzylthioinosine binding to membranes, incubations were diluted with a 10-fold excess of ice-cold buffer and rapidly centrifuged. Otherwise samples were treated as described above. Further experiments established that the high-affinity nitrobenzylthioinosine binding constants were similar at 37 and 25°C. Kinetic constants for high-affinity nitrobenzylthioinosine binding to intact cells and membranes were determined by linear regression analysis of Mass Law (Scatchard) plots after subtraction of the non-saturable binding component.

It was necessary to establish that no high-affinity binding activity was lost during the multiple-washing procedure. For human erythrocytes, differences between supernatant radioactivity counts in the presence and absence of nitrobenzylthioguanosine could be used to estimate high-affinity binding. These values agreed closely with estimates obtained from cell and membrane pellets after washing, demonstrating that no significant loss of binding activity had occurred. Other control binding experiments employing either a single buffer wash (as opposed to the normal four) or no wash, but with [^{3}H]inulin as a space marker, confirmed that the method gave an accurate estimate of equilibrium binding activity in intact human erythrocytes and isolated membranes (see also Pickard *et al.*, 1973). Sheep cells, in contrast with human erythrocytes, have few high-affinity nitrobenzylthioinosine-binding sites and it proved impossible to estimate their number accurately without first removing unbound radioactivity. Nevertheless, control experiments of the type described above established that both nucleoside-permeable and -impermeable erythrocytes had <100 sites/cell.

Nucleoside uptake

Initial uptake rates for nucleoside transport by human and sheep erythrocytes (zero-*trans* influx) were determined by dibutyl phthalate and washing methods respectively (Pickard & Paterson, 1972; Young, 1978; Jarvis *et al.*, 1980). Incubation times (4s for human cells and 3min for sheep) were chosen such that maximum intracellular concentrations did not exceed 20% of extracellular values.

Results

The binding of nitrobenzyl[^{35}S]thioinosine to intact human and sheep erythrocytes is shown in Fig. 1, where cell-associated binding is plotted against the equilibrium free concentration of inhibitor in the medium. The data for human cells demonstrate two components of binding: a saturable association responsible for the binding of 23×10^{-21} mol/cell with an apparent K_D of 2.1 nM,

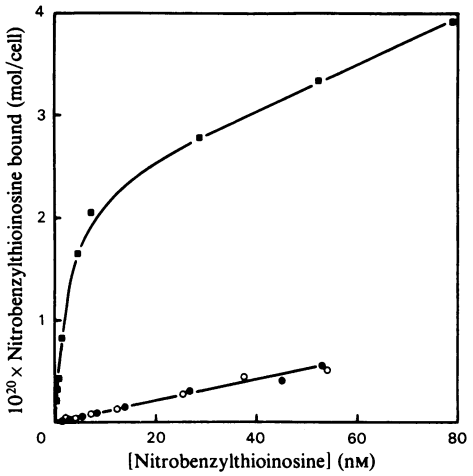

Fig. 1. *Binding of nitrobenzyl[^{35}S]thioinosine to nucleoside-permeable and -impermeable sheep and human erythrocytes*

Cells were incubated with nitrobenzyl[^{35}S]thioinosine at initial inhibitor concentrations of 1–100 nM. Cell-associated radioactivity was measured as described in the Materials and methods section. The amount of nitrobenzylthioinosine bound per cell is plotted against the equilibrium concentration of free nitrobenzylthioinosine. Symbols: ■, human erythrocytes; ●, nucleoside-permeable sheep erythrocytes; ○, nucleoside-impermeable sheep erythrocytes.

and a non-saturable component responsible for the binding of 22×10^{-23} mol/cell at 1 nM. Cells from four separate subjects gave a mean apparent K_D ($\pm$ S.E.M.) of 1.27 ± 0.67 nM with a maximum binding of $(1595 \pm 255) \times 10^{-23}$ mol/cell for the saturable component. The saturable association was abolished by pretreating cells with nitrobenzylthioguanosine (25 μM). These results are consistent with previously published data for human cells (Cass *et al.*, 1974). In contrast with human erythrocytes, however, both nucleoside-permeable and -impermeable sheep cells showed no detectable high-affinity binding ($< 20 \times 10^{-23}$ mol/cell), and the non-saturable association was 2-fold lower than that present in human erythrocytes. Non-saturable nitrobenzylthioinosine binding in both species was significantly inhibited by nitrobenzylthioguanosine (approx. 20%), suggesting that nitrobenzylthioinosine binding to these sites may in fact be saturable at higher inhibitor concentrations.

The studies of Pickard *et al.* (1973) and Cass *et al.* (1974) demonstrated that the non-saturable binding of nitrobenzylthioinosine to human erythrocytes was considerably decreased when haemo-

globin-free cell membranes were used instead of intact cells, presumably due to the removal of intracellular low-affinity binding proteins. Fig. 2 shows the concentration dependence of nitrobenzyl-[^{35}S]thioinosine binding to membranes prepared from nucleoside-permeable and nucleoside-impermeable sheep erythrocytes. For both cell types, the non-saturable association previously seen in intact cells was decreased 50-fold. The presence of a previously masked high-affinity binding component was now revealed in nucleoside-permeable but not nucleoside-impermeable membranes (3.2×10^{-23} mol/cell; apparent K_D 0.54 nM). Erythrocytes from three nucleoside-permeable-type animals gave a mean high-affinity binding of $(2.85 \pm 0.30) \times 10^{-23}$ mol/cell with an apparent K_D of 0.71 ± 0.09 nM. This high-affinity association was abolished by nitrobenzylthioguanosine and in contrast with intact cells, the gradient of the non-saturable binding component was the same in the presence and absence of nitrobenzylthioguanosine (Fig. 3). Under identical conditions, human cells had a saturable binding component of $(1390 \pm 145) \times 10^{-23}$ ($n = 4$) mol/cell with an apparent K_D of 0.50 ± 0.13 ($n = 4$) nM, parameters similar to those found with intact erythrocytes.

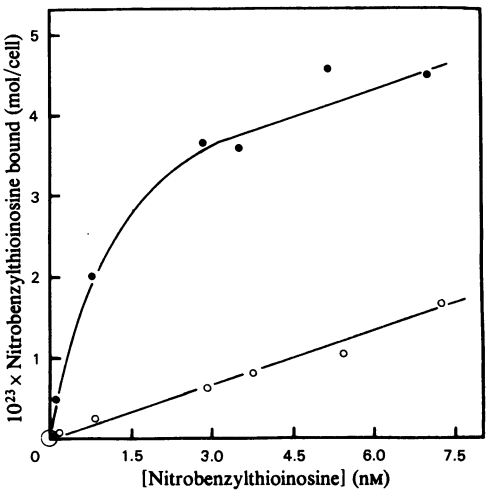

Fig. 2. *Binding of nitrobenzyl[^{35}S]thioinosine to membranes from nucleoside-permeable and -impermeable sheep erythrocytes*

Nitrobenzyl[^{35}S]thioinosine bound to nucleoside-permeable cells (●) is fitted as:

$$[3.2\,I/(0.54 + I) + 0.21.I] \times 10^{-23}\ \text{mol/cell}$$

where I is the equilibrium free concentration of nitrobenzylthioinosine (nM). Binding to nucleoside-impermeable membranes (○) was linear (0.19×10^{-23} mol/cell at 1 nM). See the text for experimental details.

In another series of experiments, differences between the binding of saturating concentrations of nitro[^{3}H]benzylthioinosine (initial concentration 30 nM) in the presence and absence of nitrobenzylthioguanosine (25 μM) were used to quantify further the magnitude of the saturable binding component in a number of nucleoside-permeable- and -impermeable-type sheep. Membranes from five nucleoside-permeable-type animals bound $(2.92 \pm 0.28) \times 10^{-23}$ mol/cell and a control experiment confirmed that there was strict proportionality between the numbers of sites detected and the cell count of the incubation. No significant saturable binding ($< 0.2 \times 10^{-23}$ mol/cell) was detected on

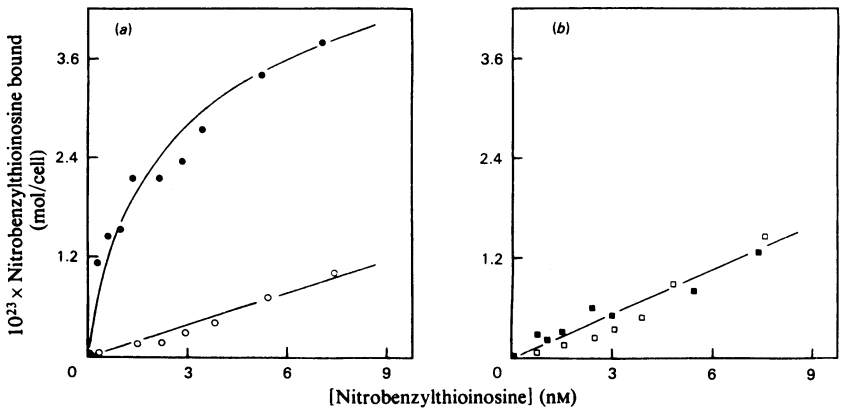

Fig. 3. *Effect of nitrobenzylthioguanosine on nitrobenzyl[^{35}S]thioinosine binding to nucleoside-permeable and -impermeable sheep erythrocyte membranes*

Membranes from nucleoside-permeable (*a*) and nucleoside-impermeable (*b*) sheep erythrocytes were preincubated with (open symbols) or without (filled symbols) excess nitrobenzylthioguanosine for 25 min at 37°C before addition of nitrobenzyl[^{35}S]thioinosine. Experimental details are given in the text.

Table 1. *Correlation between nitro[^{3}H]benzylthioinosine binding activity and $V_{max.}$ for zero-trans uridine influx in human and nucleoside-permeable and -impermeable sheep erythrocytes*

$V_{max.}$ for nitrobenzylthioinosine-sensitive uridine uptake was determined by incubating cells with 5 mM-uridine in the presence and absence of 2 μM-nitrobenzylthioinosine. Nitro[^{3}H]benzylthioinosine binding was measured at saturating nitrobenzylthioinosine concentrations and corrected for non-specific nitrobenzylthioguanosine-insensitive binding as described in the Materials and methods section. Uridine uptake by human cells at 37°C was too rapid to be measured accurately. Abbreviations used: N.D., not detected. The numbers of observations are indicated in parentheses.

	Uridine $V_{max.}$				
	25°C		37°C		
	(mmol/litre of cells per h)	(mol/cell per s)	(mmol/litre of cells per h)	(mol/cell per s)	10^{23} × Nitro[^{3}H]-benzylthiainosine bound (mol/cell)
Nucleoside-permeable sheep erythrocytes	0.62 ± 0.06 (5)	$(0.53 \pm 0.05) \times 10^{-20}$ (5)	3.18 ± 0.18 (5)	$(2.73 \pm 0.16) \times 10^{-20}$ (5)	2.92 ± 0.28 (5)
Nucleoside-impermeable sheep erythrocytes	N.D.	N.D.	N.D.	N.D.	N.D.
Human erythrocytes	99 ± 6 (3)	$(254 \pm 15) \times 10^{-20}$ (3)			1390 ± 130 (3)

membranes prepared from five different nucleoside-impermeable-type animals. Under the same experimental conditions human cell membranes bound $(1390 \pm 130) \times 10^{-23}$ ($n = 3$) mol/cell, a value identical with that found from complete concentration-dependence curves. Table 1 correlates these values with $V_{max.}$ estimates for zero-*trans* uridine uptake. In agreement with previous studies (Young, 1978; Jarvis & Young, 1978*a*), the $V_{max.}$ for saturable nitrobenzylthioinosine-sensitive influx was considerably greater in human erythrocytes than nucleoside-permeable sheep cells. The ratio (uridine $V_{max.}$ per cell/nitrobenzylthioinosine bound per cell) was similar for human and nucleoside-permeable sheep erythrocytes.

Fig. 4 shows the time course of nitro[^{3}H]benzylthioinosine binding to nucleoside-permeable sheep erythrocyte membranes (initial concentration 30 nM). Binding to both non-saturable and saturable sites was complete within a few minutes at 37°C and addition of nitrobenzylthioguanosine or dipyridamole, another inhibitor of nucleoside transport, resulted in complete and rapid displacement of nitro[^{3}H]benzylthioinosine bound to high-affinity but not non-saturable sites. Bound nitro[^{3}H]benzylthioinosine was also lost during incubation at 37°C, even in the absence of displacing agent, but this loss of binding activity was only partial (approx. 50%) and presumably reflected the re-equilibration of free and bound nitrobenzylthioinosine (new free nitrobenzylthioinosine concentration was 0.21 nM). In contrast, nitro[^{3}H]benzylthioinosine bound to cell membranes kept at 4°C was stable for at least 5 h (< 10% loss). Results presented in Table 2 show that nucleoside substrates for the transport system (inosine, adenosine and uridine) effectively inhibited saturable but not non-saturable binding of nitro-

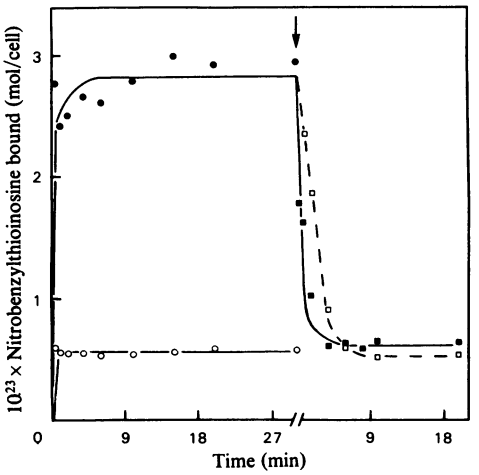

Fig. 4. *Nitro[^{3}H]benzylthioinosine binding to nucleoside-permeable sheep erythrocyte membranes: time course of binding and displacement by nitrobenzylthioguanosine and dipyridamole*

Membrane suspensions were incubated with nitro-[^{3}H]benzylthioinosine (initial concentration 30 nM) (●) at 37°C and assayed for bound nitro[^{3}H]-benzylthioinosine at predetermined time intervals as described in the text. Nitrobenzylthioguanosine (25 μM) (■) or dipyridamole (50 μM) (□) were added at the time indicated by the arrow after removal of free extracellular nitro[^{3}H]benzylthioinosine by washing. The time course of nitro[^{3}H]benzylthioinosine binding in the presence of 25 μM-nitrobenzylthioguanosine is also shown (○).

[^{3}H]benzylthioinosine to nucleoside-permeable membranes. The ability of these nucleosides to inhibit nitro[^{3}H]benzylthioinosine binding correlated with their respective apparent K_m values for influx.

Table 2. *Effect of nucleosides on nitro[^{3}H]benzylthioinosine binding to nucleoside-permeable sheep erythrocyte membranes*
Membranes were preincubated with nucleoside ± nitrobenzylthioguanosine (25 μM) for 20 min before addition of nitro[^{3}H]benzylthioinosine (final equilibrium concentration 1.6 nM). Control membranes bound 1.88×10^{-23} mol/cell of which 1.75×10^{-23} mol/cell was displaced by nitrobenzylthioguanosine. Values are means of duplicate estimates. Other experimental details are given in the text.

	Nitro[^{3}H]benzylthioinosine bound (% of control value)		Apparent K_m for zero-*trans* influx at 37°C (mM)*
	Saturable	Non-saturable	
Adenosine (10 mM)	11	94	0.13
Inosine (16 mM)	24	96	0.26
Uridine (16 mM)	38	113	0.47

* Data from Young (1978).

Discussion

Previous studies have shown that nitrobenzylthioinosine inhibition of nucleoside transport in HeLa cells and human erythrocytes is associated with high-affinity binding (apparent $K_D \approx 1$ nM) of inhibitor to the cell membrane (Cass *et al.*, 1974; Cass & Paterson, 1977; Lauzon & Paterson, 1977). It is generally assumed that this binding represents a specific interaction with the nucleoside-transport mechanism. Thus nitrobenzylthioinosine binding and inhibition studies have been used to estimate the numbers of nucleoside-transport sites present in cell membranes from various sources and during cell division and growth (Cass *et al.*, 1974, 1979; Eilam & Cabantchik, 1977; Lauzon & Paterson, 1977; Wohlhueter *et al.*, 1978). One study has employed nitrobenzylthioinosine in an attempt to identify nucleoside-transport components during membrane extraction (Pickard & Paterson, 1976). The aim of the present study was to provide an independent test of the hypothesis that nitrobenzylthioinosine interacts specifically with functional nucleoside-transport sites by comparing the inhibitor binding characteristics of human, nucleoside-permeable sheep and nucleoside-impermable sheep erythrocytes.

In the present experiments we have been able to show that nucleoside-permeable sheep erythrocytes possess high-affinity nitrobenzylthioinosine-binding sites with similar properties to those found in human cells. In particular, the apparent K_D for binding is similar in the two species, and nitrobenzylthioinosine binding to nucleoside-permeable cells is inhibited by nitrobenzylthioguanosine, dipyridamole and both purine and pyrimidine nucleosides. In contrast with nucleoside-permeable cells, this high-affinity binding component is absent from nucleoside-impermeable sheep erythrocytes. We have previously shown that these cells lack a saturable nucleoside-uptake mechanism (Jarvis & Young, 1978*a*; Young, 1978). Nucleoside-permeable cells bound 2.92×10^{-23} mol of nitrobenzylthioinosine/cell compared with 1390×10^{-23} mol/cell for human erythrocytes. These values correspond to 18 and 8400 nitrobenzylthioinosine sites/cell for nucleoside-permeable sheep and human erythrocytes respectively. This 470-fold difference in binding capacity parallels the difference in $V_{max.}$ for zero-*trans* uridine influx in the two cell types (Table 1). Our data therefore provide direct evidence that high-affinity nitrobenzylthioinosine binding to erythrocyte membranes is a specific association with functional nucleoside-transport sites. The calculated translocation capacity for each transport site is the same in human and nucleoside-permeable sheep erythrocytes and equal to 180 molecules/site per s at 25°C, assuming that one transport site binds a single molecule of nitrobenzylthioinosine. It remains to be established whether nitrobenzylthioinosine binds to the actual nucleoside permeation site of the nucleoside transport mechanism or to a modifier site as suggested by the inability of deoxycytidine to inhibit binding and the complex transport inhibition patterns observed with other cell types (Cass & Paterson, 1976; Eilam & Cabantchik, 1977; Wohlhueter *et al.*, 1978; Paterson, 1979).

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