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ERYTHROCYTE NUCLEOSIDE TRANSPORT: ASYMMETRICAL BINDING OF NITROBENZYLTHIOINOSINE TO NUCLEOSIDE PERMEATION SITES

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SUMMARY

1. Nitrobenzylthioinosine is a potent and specific inhibitor of nucleoside translocation in animal cells. Kinetic and inhibitor binding studies were undertaken to clarify how this inhibitor interacts with the nucleoside transporter from human and nucleoside-permeable type sheep erythrocytes.

2. [^{3}H]nitrobenzylthioinosine inhibition of zero-*trans* [U-^{14}C]uridine influx into nucleoside-permeable type sheep cells was consistent with simple competitive inhibition (apparent K_i 1 nmol/l). Analysis of results using total inhibitor levels instead of cell-free inhibitor concentrations did not affect the inhibition pattern, but increased the apparent K_i value by 5-fold.

3. In contrast, [^{3}H]nitrobenzylthioinosine was a non-competitive inhibitor of zero-*trans* [U-^{14}C]uridine efflux (apparent K_i 1·5 nmol/l). Dipyridamole, another potent inhibitor of nucleoside translocation, also inhibited zero-*trans* [U-^{14}C]uridine influx in a competitive manner (apparent K_i 20–40 nmol/l).

4. [^{3}H]nitrobenzylthioinosine bound to high-affinity sites on cell membranes from human and nucleoside-permeable type sheep cells (apparent K_D values $\simeq$ 1 nmol/l). Binding of inhibitor to these sites was competitively blocked by uridine, a well characterized substrate for the nucleoside transporter (apparent K_i 1·25 and 0·9 mmol/l, respectively). These apparent K_i values are close to the apparent K_m for uridine equilibrium exchange in human erythrocytes.

5. Similarly, deoxycytidine was found to be a competitive inhibitor of high-affinity [^{3}H]nitrobenzylthioinosine binding activity (apparent K_i 1·0 and 1·2 mmol/l for human and nucleoside-permeable type sheep cell membranes, respectively). This contrasts with a previous report that this nucleoside had no effect on inhibitor binding activity. Transport studies confirmed that deoxycytidine is a substrate for the erythrocyte nucleoside transporter. Apparent K_m and V_{max} values for [U-^{14}C]-deoxycytidine zero-*trans* influx into human and nucleoside-permeable type sheep cells were comparable to those obtained for [U-^{14}C]uridine.

6. It is suggested from these results that nitrobenzylthioinosine competes directly with nucleosides for the permeation site of the nucleoside transporter, but that inhibitor binds preferentially to the external membrane surface.

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INTRODUCTION

Transport of purine and pyrimidine nucleosides across the plasma membrane of animal cells occurs by a facilitated diffusion process which is independent of subsequent intracellular metabolism (Plagemann & Wohlhueter, 1980). Potent inhibitors of this translocation mechanism have been known for over 15 years, the best studied being nitrobenzylthioinosine (NBMPR; 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine) (Paterson & Simpson, 1965, 1966, 1967; Paterson & Oliver 1971; Paul, Chen & Paterson, 1975; Eilam & Cabantchik, 1977; Wohlhueter, Marz & Plagemann, 1978; Jarvis & Young, 1978*a*, 1980).

NBMPR inhibition of nucleoside transport is associated with reversible high-affinity binding of inhibitor to the cell membrane. In human erythrocytes, high-affinity NBMPR binding activity has an apparent dissociation constant (K_D) of $\sim$ 1 nM with a maximum binding capacity of approximately 10^4 sites per cell (Pickard, Brown, Paul & Paterson, 1973; Cass, Gaudette & Paterson, 1974). There is strict proportionality between the amount of NBMPR bound to these sites and the degree of transport inhibition. Experiments from this laboratory have shown that erythrocytes from some sheep (nucleoside-permeable type) exhibit both carrier mediated nucleoside transport and high-affinity NBMPR binding activity while cells from other sheep (nucleoside-impermeable type) lack a functional nucleoside transporter and do not bind inhibitor (Young, 1978; Jarvis & Young 1978*a*, 1980). This nucleoside transport polymorphism is under the simple genetic control of two autosomal alleles. There is therefore good evidence to suggest that high-affinity binding of NBMPR to the cell membrane represents a specific interaction with the nucleoside carrier. The molecular weight of the NBMPR binding component of the nucleoside transport mechanism from human erythrocyte membranes has been estimated to be 122,000 by radiation inactivation analysis (Jarvis, Young & Ellory, 1980*b*) and binding activity has been purified 13-fold following detergent extraction of isolated membranes (Jarvis & Young, 1981).

Nevertheless, the nature of the association between NBMPR and the nucleoside transporter is unclear. Thus, a number of nucleosides have been shown to inhibit NBMPR binding to human erythrocytes in an apparently competitive manner (Cass & Paterson, 1976). However, binding activity was unaffected by deoxycytidine, a nucleoside capable of stimulating uridine efflux from intact cells. This single observation has led to the widely held view that NBMPR and related inhibitors bind to the nucleoside carrier at a modifier site distinct from the nucleoside permeation site. Both competitive and non-competitive inhibition patterns have been reported for *S*-substituted 6-thiopurine inhibition of erythrocyte nucleoside transport (Eilam & Cabantchik, 1977; Turnheim, Plank & Kolassa, 1978) while studies using other more complex cells have found competitive, partially competitive and non-competitive inhibition of nucleoside uptake by NBMPR (Cass & Paterson, 1977; Eilam & Bibi, 1977; Eilam & Cabantchik, 1977; Paterson, Naik & Cass, 1977; Paterson, Babb, Paran & Cass, 1977; Wohlhueter *et al.* 1978). A common assumption in the kinetic analysis of transport inhibition by NBMPR has been that the concentration of free inhibitor in the medium is the same as the total (free + bound) inhibitor concentration of the cell suspension. However, radiolabelled-NBMPR binding studies have shown that substantial depletion occurs, particularly at lower

inhibitor levels and high cell densities (see e.g. Pickard *et al.* 1973; Cass *et al.* 1974). Thus the apparent K_i measured, and possibly the inhibition pattern observed, will depend on the experimental conditions employed. A further complication is that many of the published transport studies, particularly those using non-erythroid cells, make no clear distinction between transport and subsequent intracellular metabolism.

In the present paper we present a detailed kinetic analysis of the interaction of NBMPR with the erythrocyte nucleoside transporter. Our study is divided into two parts. In the first, we present flux studies designed to test whether or not deoxycytidine is a substrate for the nucleoside transporter. In these experiments, deoxycytidine transport by human erythrocytes and nucleoside-permeable and nucleoside-impermeable sheep cells is compared with that of uridine, a well defined and non-metabolized substrate for the carrier. This section of the paper also describes the effects of NBMPR on uridine zero-*trans* influx and zero-*trans* efflux in nucleoside-permeable sheep erythrocytes. [^{3}H]NBMPR was used in these experiments to examine directly the influence of inhibitor depletion on transport inhibition. The second series of experiments investigate the ability of uridine and deoxycytidine to inhibit NBMPR binding to human and nucleoside-permeable sheep erythrocyte membranes. Our results suggest that NBMPR competes directly with nucleosides for the permeation site of the nucleoside transporter, but that inhibitor binds preferentially to the external membrane surface. The following paper (Jarvis & Young, 1982) provides evidence that the nucleoside transporter also exhibits chemical asymmetry.

METHODS

Whole blood from adult sheep was collected by jugular venepuncture into heparinized evacuated tubes. Animals were classified as to nucleoside-permeability type on the basis of nucleoside flux measurements (see Young, 1978). Blood from healthy human volunteers was withdrawn by syringe, also into heparin. Red cell counts, haematocrit and haemoglobin estimations were performed by established methods (Archer, 1965, 1977).

Materials

Uniformly labelled [^{14}C]uridine and [^{14}C]deoxycytidine were obtained from the Radiochemical Centre, Amersham, Bucks., and further purified on Poly-gram 0·25 mm silica gel thin-layer chromatography plates (Camlab, Cambridge) using butan-1-ol saturated with water as solvent. [G-^{3}H]NBMPR (6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine) (specific radioactivity 20 Ci/mmol) was purchased from Movarek Biochemicals, City of Industry, CA, U.S.A. Radiochemical purity was routinely checked by thin-layer chromatography with chloroform/methanol (17:3, v/v) as solvent and found to be > 98% pure. Non-radioactive NBMPR and nitrobenzylthioguanosine (2-amino-6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine, NBTGR) were generous gifts from Professor A. R. P. Patterson, Cancer Research Unit (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada. Other reagents were obtained as follows: uridine and 2′-deoxycytidine from Sigma (London) Chemical Co. Ltd., Kingston-Upon-Thames, Surrey; dipyridamole (2,2′,2″,2‴-(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyldinitrilo)tetraethanol: Persantin injection) from Boehringer Ingelheim Ltd., Bracknell, Berks. All other reagents were of analytical grade.

Cell and membrane preparation

Erythrocytes were washed three times with twenty volumes of a medium containing (mmol/l): NaCl 140, KCl 5, Tris-HCl 20 (pH 7·4 at 25 or 37 °C). $MgCl_2$ 2. EDTA 0·1 (disodium salt) and glucose 5. This solution is hereafter referred to as iso-osmotic NaCl medium. The buffy coat was discarded. Haemoglobin-free erythrocyte membranes were prepared by osmotic lysis as previously described and resuspended in sodium phosphate buffer 5 mmol/l (pH 7·2 at 25 °C) (Jarvis & Young, 1980).

Nucleoside transport studies

Initial rates of zero-*trans* nucleoside influx by human (25 °C) and sheep erythrocytes (25 and 37 °C) were determined by *n*-dibutyl phthalate and washing methods, respectively. Details of these techniques have been published previously (Pickard & Paterson, 1972; Ferreira & Lew, 1976; Young, 1978; Jarvis, Young, Ansay, Archibald, Harkness & Simmonds, 1980*a*). Incubation times (4 s and 2–3 min for human and sheep cells, respectively) were chosen such that maximum intracellular concentrations did not exceed 25% of extracellular levels in most instances. For inhibitor studies, NBMPR or dipyridamole were added to cells at the same time as the [^{14}C]permeant nucleoside. [^{3}H]NBMPR was used in experiments when it was necessary to measure the equilibrium free concentration of NBMPR in the medium at the end of the incubation period.

For zero-*trans* efflux experiments, nucleoside-permeable sheep erythrocytes were preloaded with various concentrations of isotopic uridine by mixing equal volumes (0·5 ml) of cells (50% haematocrit) and [^{14}C]uridine (0·5 μCi/ml) at 37 °C and allowing the nucleoside to equilibrate across the cell membrane. Extracellular isotope was removed by washing the cells with 5 × 1 ml portions of ice-cold iso-osmotic NaCl medium using an Eppendorf 3200 microcentrifuge (10 s, 15,000 *g*). The washed cell pellets were resuspended in 0·4 ml ice-cold medium. Portions (150 μl) of the resulting cell suspension were immediately added to 0·75 ml aliquots of prewarmed medium at 37 °C containing varying concentrations of NBMPR. As in the influx experiments, [^{3}H]NBMPR was used when it was necessary to measure cell-free NBMPR levels at the end of the incubation. The initial rate of uridine efflux was measured by following the appearance of ^{14}C-radioactivity in the medium. Incubations (2 min) were terminated by layering 0·8 ml of the incubation mixtures on 0·5 ml ice-cold *n*-dibutyl phthalate in microcentrifuge tubes (volume 1·5 ml). The tubes were immediately centrifuged at 15,000 *g* for 15 s. Portions (0·4 ml) of the upper layer of cell-free aqueous medium were transferred to 7 ml of Packard 299 scintillation fluid and counted for ^{14}C/^{3}H radioactivity in a Packard Tri-Carb scintillation counter with appropriate quench correction. Control experiments confirmed that no significant metabolism of [^{14}C]uridine occurred under these experimental conditions. None of the [^{3}H]NBMPR present in incubation mixtures was absorbed by the *n*-dibutyl phthalate present in the microcentrifuge tubes.

Nitrobenzylthioinosine binding assays

NBMPR binding to membranes prepared from human and nucleotide-permeable sheep erythrocytes was determined at 25 °C as previously described (Jarvis & Young, 1980). Briefly, membrane suspensions (0·4 ml, 10 and 100% haematocrit equivalent for human and sheep ghosts, respectively) were first preincubated in the presence or absence of NBTGR (25 μmol/l). In the case of competition studies, the preincubation mixture also contained the test compound. After 25 min, 0·4 ml portions of prewarmed medium containing [^{3}H]NBMPR (1–60 nmol/l) were added. Incubations (20 min) were terminated by centrifugation at 15,000 *g* for 15 min. Supernatants were retained for radioactivity determinations. Membrane pellets were washed four times wth 1 ml aliquots of ice-cold sodium phosphate (5 mmol/l) and finally resuspended in 0·4 ml buffer. Supernatants and membrane suspensions were counted for ^{3}H-radioactivity as described above.

Octanol/water partition coefficients

Octanol (2 ml) was vigorously vortexed for 60 s at 25 °C with 2 ml of isosmotic NaCl medium containing radioactively labelled nucleoside (0·1 mmol/l, 0·25 μCi/μmol). Following centrifugation (5 min, 1000 *g*), the radioactivity present in 1 ml portions of each of the phases was measured.

RESULTS

Kinetics of nucleoside uptake by human and sheep erythrocytes

Fig. 1 compares the concentration dependence of uridine and deoxycytidine uptake by human, nucleoside-permeable and nucleoside-impermeable sheep erythrocytes over the concentration range 0·025–5 mmol/l at 25 °C. In agreement with previous studies at 37 °C (Young, 1978; Jarvis & Young, 1978*b*), uptake by nucleoside-permeable erythrocytes was saturable and conformed to simple Michaelis–Menten

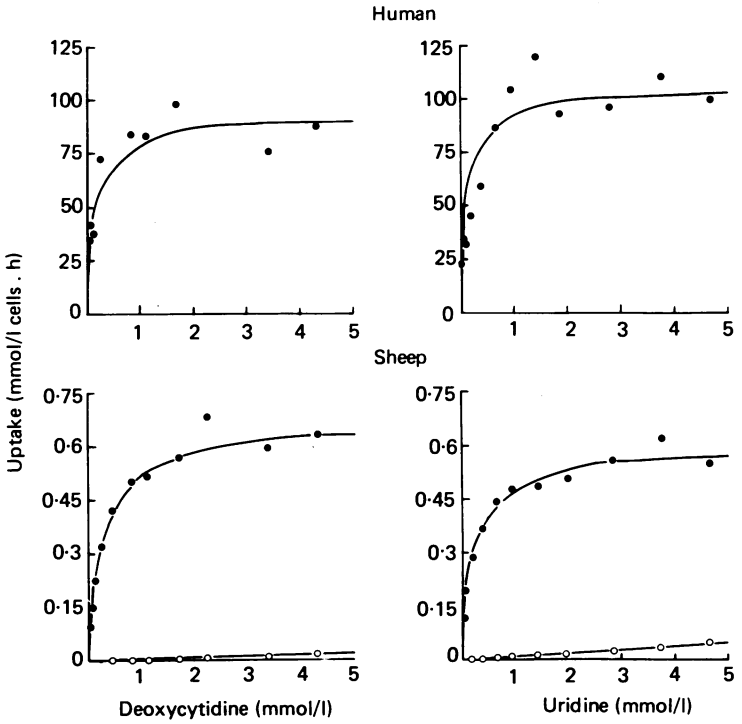

Fig. 1. Concentration dependence of uridine and deoxycytidine uptake by nucleoside-permeable and nucleoside-impermeable sheep erythrocytes and human erythrocytes. The [^{14}C]uridine and [^{14}C]deoxycytidine uptake curves for nucleoside-permeable sheep erythrocytes (●) are fitted as v (mmol/l cells.h) = 0·60 s/(0·26 + s) and v = 0·68 s/(0·27 + s), respectively, where s is the extracellular nucleoside concentration (mmol/l). For human cells the uptake curves were fitted as v = 106 s/(0·14 + s) and v = 87 s/(0·11 + s) for uridine and deoxycytidine respectively. The incubation temperature was 25 °C. Nucleoside-impermeable sheep erythrocytes (○).

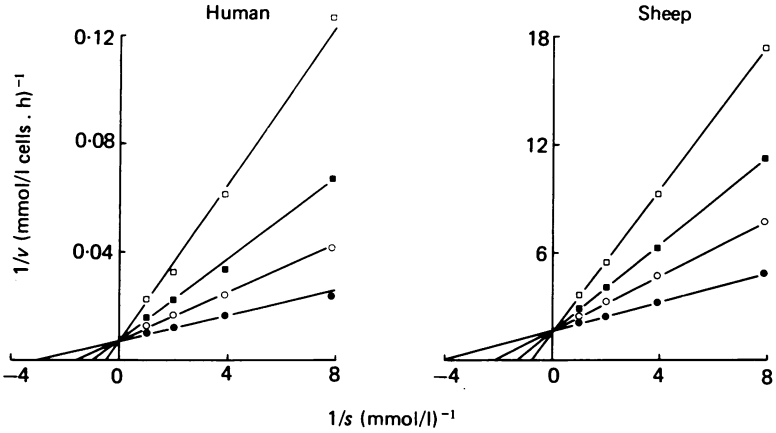

Fig. 2. Effect of deoxycytidine on uridine uptake by human and nucleoside-permeable sheep erythrocytes. The reciprocals of [^{14}C]uridine uptake rates in the presence of varying concentrations of deoxcytidine (25 °C) (0 (●), 0·25 (○), 0·5 (■) and 1·0 (□) mmol/l) are plotted against the reciprocals of the extracellular uridine concentrations. Apparent K_i values were 0·14 and 0·28 mmol/l for human and nucleoside-permeable sheep erythrocytes, respectively.

kinetics giving apparent K_m values of 0·26 and 0·27 mmol/l with V_{max} estimates of 0·60 and 0·68 mmol/l cells . h for uridine and deoxycytidine, respectively. In contrast, nucleoside-impermeable cells gave a slow linear uptake over the same concentration range (0·01 and 0·003 mmol/l cells . h (nucleoside concentration 1 mmol/l) for uridine and deoxycytidine, respectively).

The uridine permeability of erythrocytes from five animals of each type was compared at 25 and 37 °C using a saturating uridine concentration (5 mmol/l). Nucleoside-permeable cells showed a 4-fold difference in uridine uptake rate between the two temperatures (2·32 ± 0·09 mmol/l cells . h at 37 °C compared to 0·62 ± 0·07 mmol/l cells . h at 25 °C). Uridine uptake by nucleoside-impermeable erythrocytes was less temperature sensitive (0·19 ± 0·02 and 0·095 ± 0·013 mmol/l cells . h at 37 and 25 °C, respectively). These transport rates were used to estimate the apparent activation energies for uridine influx in the two cell types, giving values of 20·1 and 10·5 kcal/mol for nucleoside-permeable and nucleoside-impermeable erythrocytes, respectively. A value of 20 kcal/mol for the saturable transport system present in nucleoside-permeable cells corresponds closely with previously published estimates for nucleoside uptake mechanisms in other systems (Duhm, 1974; Cabantchik & Ginsburg, 1977; Kolassa, Plant & Turnheim, 1978; Wohlhueter, Marz & Plagemann, 1979). The V_{max} for deoxycytidine entry into nucleoside-permeable sheep cells showed a similar 4-fold difference between 25 and 37 °C. Apparent K_m values for the two nucleosides were less temperature sensitive (0·26, 0·47 mmol/l for uridine and 0·27, 0·48 mmol/l for deoxycytidine at 25 and 37 °C, respectively).

Uptake of both nucleosides by human erythrocytes was also saturable and conformed to simple Michaelis–Menten kinetics, giving apparent K_m values of 0·14 and 0·11 mmol/l for uridine and deoxycytidine, respectively (25 °C). The estimated V_{max} values (per litre of cells) were approximately 150-fold higher for human erythrocytes than for nucleoside-permeable sheep cells (106 and 87 mmol/l cells . h for uridine and deoxycytidine uptake in human cells at 25 °C, respectively).

Fig. 2 shows the effect of 0·25–1·0 mmol/l deoxycytidine on uridine uptake (0·125–1·0 mmol/l) at 25 °C by human and nucleoside-permeable sheep erythrocytes. The results demonstrate that deoxycytidine is a simple competitive inhibitor of uridine uptake by both cell types (apparent K_i 0·14 and 0·28 mmol/l for human and nucleoside-permeable sheep erythrocytes, respectively). Similarly, uridine was a competitive inhibitor of deoxycytidine uptake by the two cell types (apparent K_i 0·14 and 0·20 mmol/l, respectively). Similar findings have been reported for adenosine and uridine as inhibitors of inosine uptake by nucleoside-permeable sheep erythrocytes (Young, 1978). Deoxycytidine had no significant effect on uridine uptake by nucleoside-impermeable sheep cells and vice versa.

The magnitude of the non-saturable uptake of nucleosides by nucleoside-impermeable sheep cells differs considerably for different permeants (1·70, 0·19, 0·033 and 0·036 mmol/l cells . h for adenosine, uridine, deoxycytidine and inosine at 37 °C (5 mmol/l), respectively (data from one animal)) (see also Fig. 1 and Young, 1978). To test whether these influx rates correlate with the lipid solubilities of the individual nucleosides, we measured their oil:water partition coefficients (pH 7·4, 25 °C and 0·1 mmol/l). Octanol was used in preference to the more commonly employed solvents (heptane and chloroform) because of the greater solubility of nucleosides in octanol

(Ross & Pfleger, 1972). Partition coefficients were 0·10, 0·037, 0·014 and 0·0087 for adenosine, uridine, deoxycytidine and inosine, respectively. Under similar conditions the octanol:water partition coefficient of 100 nmol/l [^{3}H]NBMPR was 29·6.

NBMPR inhibition of nucleoside influx and efflux

The first series of experiments were carried out at 25 °C. Deoxycytidine uptake by human cells was readily inhibited by NBMPR (Fig. 3). The concentration required to produce 50% inhibition of deoxycytidine (1 mmol/l) uptake was 75 nmol/l

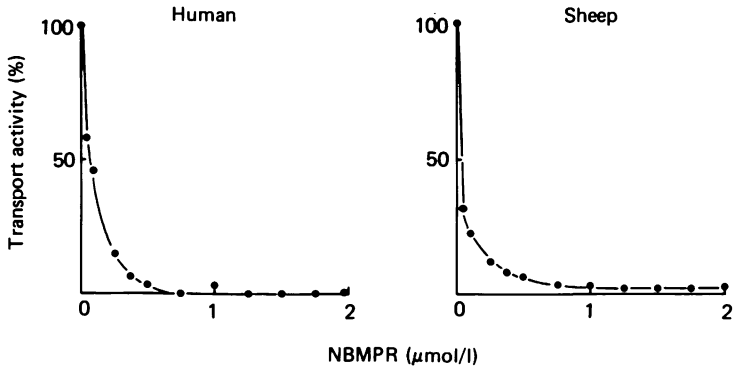

Fig. 3. Effect of nitrobenzylthioinosine on deoxycytidine uptake by human and nucleoside-permeable sheep erythrocytes. [^{14}C]Deoxycytidine (extracellular concentration 1 mmol/l; 25 °C) and NBMPR were added to cells simultaneously. Results are plotted as a percentage of control uptake values measured in the absence of inhibitor.

(haematocrit 11%). In a parallel experiment, deoxycytidine uptake by nucleoside-permeable sheep erythrocytes (measured at the same haematocrit) was inhibited 50% by 25 nmol/l NBMPR, a 3-fold lower inhibitor concentration. In agreement with previous studies, NBMPR had no effect on nucleoside uptake by nucleoside-impermeable sheep cells (Jarvis & Young 1978*b*). A small fraction of deoxycytidine uptake by nucleoside-permeable cells is also not inhibited by NBMPR (see e.g. Fig. 3). This component of uptake is separate from the carrier mediated influx via the nucleoside transporter and is equivalent to the NBMPR-insensitive uptake of nucleosides by nucleoside-impermeable cells. Subtraction of this flux component (estimated in the presence of NBMPR 10 μmol/l) from the uptake data in Figs. 1 and 2 had no significant effect on the calculated kinetic constants.

To investigate the kinetics of NBMPR inhibition of nucleoside transport, the effect of varying concentrations of NBMPR (initial concentration 5–100 nmol/l) on zero-*trans* uridine uptake (0·26–10 mmol/l) by nucleoside-permeable sheep erythrocytes was investigated at 37 °C. Sheep cells were chosen for these experiments because of their lower maximum velocity of nucleoside transport. [^{3}H]Inhibitor and [^{14}C]uridine were added to cells simultaneously. The NBMPR-insensitive component of uridine uptake, estimated in the presence of excess non-radioactive NBMPR, was subtracted from the total uptake data before kinetic analysis. Fig. 4 shows Dixon plots (Dixon, 1953) ($1/v$ *versus* I) of such an experiment, where reciprocals of the initial rates of uridine influx are plotted against either the free [^{3}H]NBMPR concentration (A) or

the total inhibitor concentration (*B*). Both plots are consistent with simple competitive inhibition. However, the apparent K_i values differ by a factor of 5 depending on whether inhibitor depletion is considered (apparent K_i 1 and 5 nmol/l for free and total inhibitor concentrations, respectively). Similarly, competitive inhibition plots were obtained when the data were analysed by the Cornish-Bowden method (*s/v versus I*) (Fig. 5) (Cornish-Bowden, 1974). Deviations from linearity were

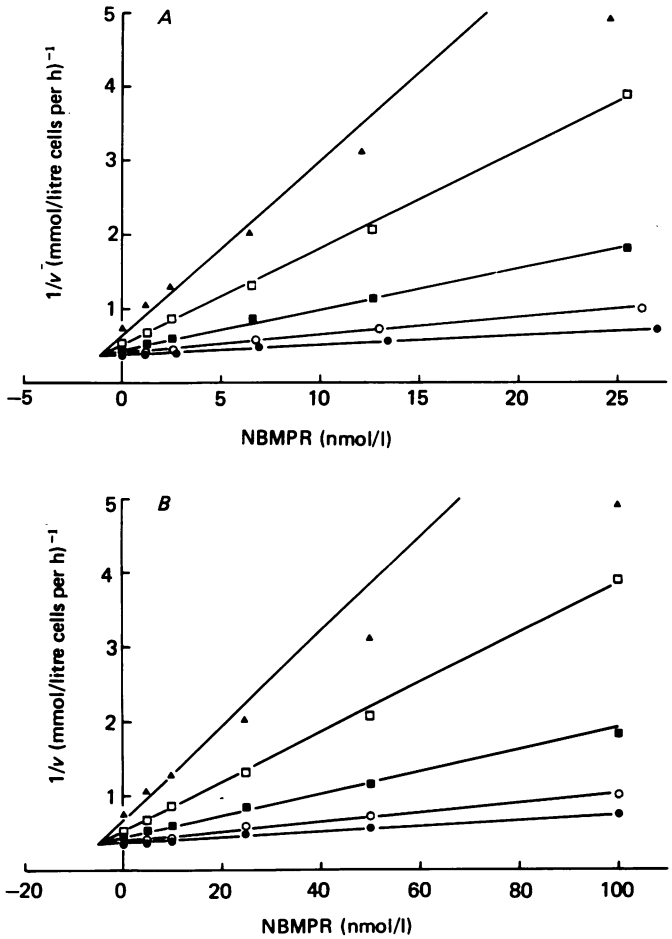

Fig. 4. Effect of [^{3}H]nitrobenzylthioinosine on uridine uptake by nucleoside-permeable sheep erythrocytes. The reciprocals of [^{14}C]uridine uptake rates at 37 °C (corrected for the NBMPR-insensitive component) are plotted against either the final equilibrium [^{3}H]NBMPR concentrations (*A*, apparent K_i 1 nmol/l) or the initial [^{3}H]NBMPR levels (*B*, apparent K_i 5 nmol/l). Extracellular uridine concentrations (mmol/l) were 10 (●), 3·1 (○), 1·06 (■), 0·51 (□), and 0·26 (▲). Other experimental details are given in the text.

observed at the high inhibitor and lower substrate concentrations. These data points are the most inaccurate with transport rates only 2-fold above blank values. Under these conditions, additional errors arise from the separation of ^{3}H and ^{14}C-radioactivity. Other experiments established that NBMPR also acted as a competitive inhibitor of uridine influx when [^{3}H]NBMPR was preincubated with

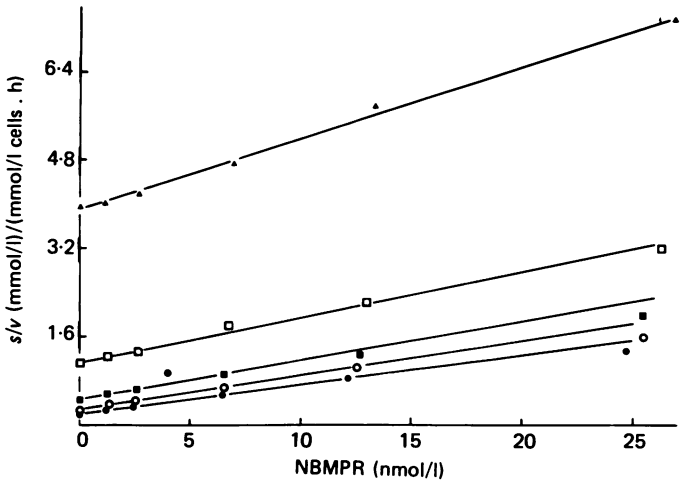

Fig. 5. Cornish-Bowden plot of [^{3}H]nitrobenzylthioinosine inhibition of uridine uptake by nucleoside-permeable sheep erythrocytes. The data from Fig. 4*A* are replotted as s/v *versus* I where s and I are the extracellular [^{14}C]uridine (mmol/l) and [^{3}H]NBMPR concentrations (nmol/l), respectively, and v is the initial rate of uridine influx (mmol/l cells . h). Uridine concentrations were 0·26 (●), 0·51 (○), 1·06 (■), 3·1 (□) and 10 (▲) mmol/l.

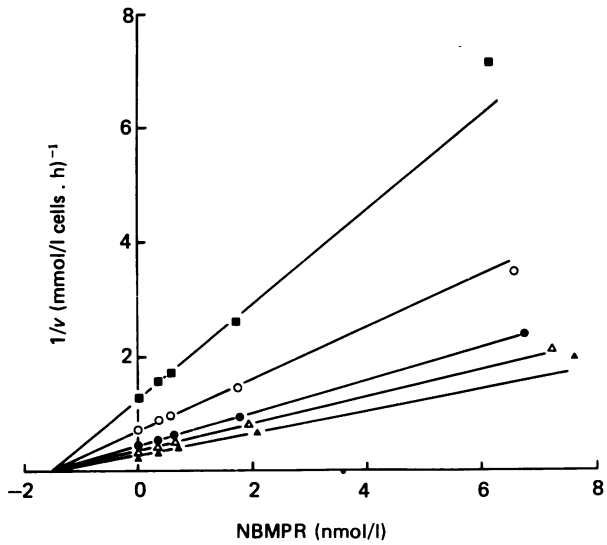

Fig. 6. Effect of [^{3}H]nitrobenzylthioinosine on uridine efflux from nucleoside-permeable sheep erythrocytes. The reciprocals of [^{14}]uridine efflux rates at 37 °C (corrected for the NBMPR insensitive component) are plotted against the final free [^{3}H]NBMPR concentrations in the medium. Initial intracellular uridine concentrations (mmol/l cell water) were 1·80 (▲), 0·55 (△), 0·40 (●), 0·19 (○) and 0·10 (■). Apparent K_i 1·5 nmol/l. See text for other experimental details.

nucleoside-permeable sheep cells for 30 min at 37 °C before addition of [^{14}C]uridine (data not shown).

In other experiments, NBMPR was also found to inhibit zero-*trans* uridine efflux (intracellular concentration 0·94 mmol/l cell water) from pre-loaded nucleoside-permeable sheep cells. However, the concentration of NBMPR required to inhibit efflux by 50% was only 2·5 nmol/l at a haematocrit of 20%, a value 10-fold lower

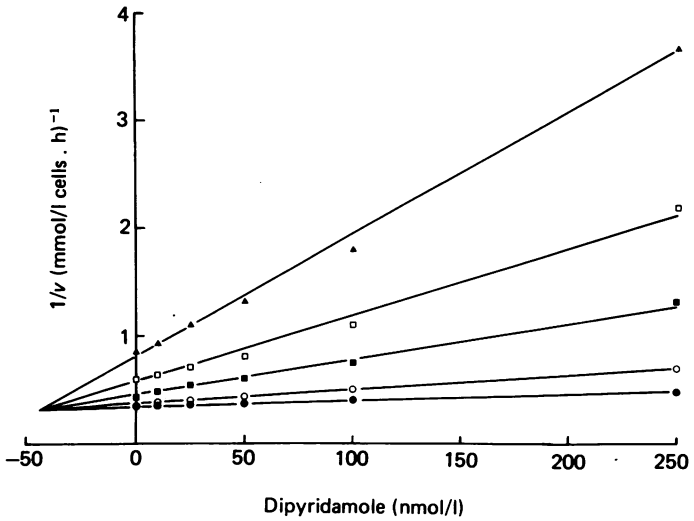

Fig. 7. Effect of dipyridamole on uridine uptake by nucleoside-permeable sheep erythrocytes. The reciprocals of initial [^{14}C]uridine uptake rates at 37 °C (corrected for the NBMPR-insensitive component) are plotted against initial inhibitor concentrations. Extracellular uridine concentrations (mmol/l) were 10 (●), 3·24 (○), 1·06 (■), 0·52 (□) and 0·26 (▲). Apparent K_i 44 nmol/l.

than required to produce comparable inhibition of nucleoside influx under similar conditions (see e.g. Fig. 3). [^{3}H]NBMPR inhibition of [^{14}C]uridine efflux was therefore investigated in detail using cells pre-loaded with varying concentrations of isotopic uridine. As in the influx experiments, NBMPR-insensitive uridine efflux (estimated in the presence of non-radioactive NBMPR 10 μmol/l) was subtracted from the total efflux data before kinetic analysis. Fig. 6 shows the results of one such experiment (37 °C) where the data is plotted as $1/v$ *versus* I. In contrast with the effect of NBMPR on uridine influx, the lines intersect on the I axis, consistent with non-competitive inhibition of zero-*trans* uridine efflux (apparent $K_i \simeq 1{\cdot}5$ nmol/l).

Data presented in Fig. 6 allow calculation of the kinetic constants for NBMPR-sensitive uridine efflux. The apparent K_m value was 0·5 mmol/l with a V_{max} of 5·0 mmol/l cell water . h, constants similar to those of uridine influx. This contrasts with a previous report of marked differences in the kinetic constants for uridine influx and efflux in human cells from outdated blood (Cabantchik & Ginsburg, 1977).

Dipyridamole inhibition of nucleoside influx

Dipyridamole is another potent inhibitor of nucleoside translocation in a variety of cell types, including erythrocytes (see e.g. Young, 1978). Fig. 7 shows the effect

of dipyridamole (initial concentration 10–250 nmol/l) on uridine uptake (0·26–10 mmol/l; 37 °C) by nucleoside-permeable sheep cells where the results are plotted as $1/v$ versus I (total dipyridamole concentration). Inhibition was apparently competitive, with an apparent K_i value of 44 nmol/l. A second independent experiment gave an apparent K_i estimate of 22 nmol/l. Analysis of the data by $1/v$ *versus* $1/s$ and s/v *versus* I plots confirmed that dipyridamole inhibited zero-*trans* uridine influx in an apparently competitive manner.

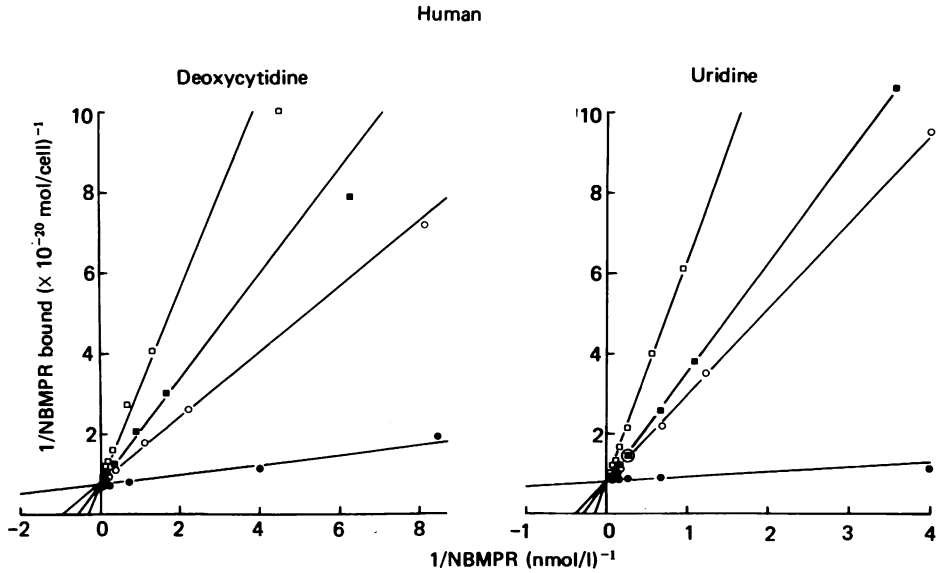

Fig. 8. Effects of uridine and deoxycytidine on [^{3}H]nitrobenzylthioinosine binding to human erythrocyte membranes. The reciprocals of [^{3}H]NBMPR bound to high-affinity sites in the presence of varying concentrations of uridine ($K_i \sim 1{\cdot}25$ mmol/l) and deoxycytidine ($K_i \sim 1{\cdot}0$ mmol/l) at 25 °C are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]NBMPR. Symbols, concentration of nucleoside (mmol/l): ●, 0; ○, 5; ■, 15 and □, 30. Other experimental details are given in the text.

NBMPR binding studies

In a previous study we have shown that nucleoside substrates for the transport system (uridine, inosine and adenosine) effectively inhibit saturable but not non-saturable binding of [^{3}H]NBMPR to nucleoside-permeable sheep erythrocyte membranes (Jarvis & Young, 1980). In the present study, the ability of two nucleosides, uridine and deoxycytidine, to inhibit high-affinity NBMPR binding to human and nucleoside-permeable sheep erythrocyte membranes were investigated in detail. Isolated membranes were chosen in preference to intact cells for these experiments because the non-saturable component of NBMPR binding is very much reduced in membranes compared with cells (Pickard *et al.* 1973; Cass *et al.* 1974; Jarvis & Young, 1980; Jarvis *et al.* 1980*b*). All experiments were performed at 25 °C.

Fig. 8 shows the effect of varying concentrations (0–15 mmol/l) of uridine and deoxycytidine on high-affinity binding of [^{3}H]NBMPR (initial concentration 0·75–25 nmol/l) to nucleoside-permeable sheep erythrocyte membranes, plotted as the

reciprocal of specific bound [^{3}H]NBMPR *versus* the reciprocal of the equilibrium free concentration of inhibitor. NBMPR binding was reduced in the presence of both nucleosides in an apparently competitive manner. Plots of apparent K_D values *versus* nucleoside concentration were linear for both uridine and deoxycytidine, further evidence of simple competitive inhibition of binding activity (see Segel, 1975). The apparent inhibition constants were 0·9 and 1·2 mmol/l for uridine and deoxycytidine, respectively. Similar values were obtained when the data was plotted as NBMPR bound/NBMPR free *versus* NBMPR bound (Scatchard plot; Scatchard, 1949).

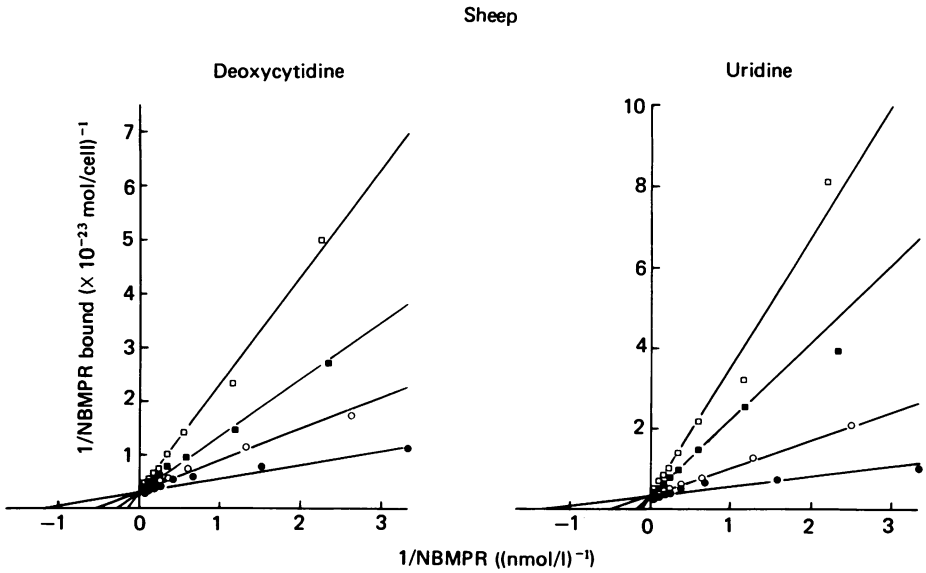

Fig. 9. Effects of uridine and deoxycytidine on [^{3}H]nitrobenzylthioinosine binding to nucleoside-permeable sheep erythrocyte membranes. The reciprocals of [^{3}H]NBMPR bound to high-affinity sites in the presence of varying concentrations of uridine ($K_i \sim 0{\cdot}9$ mmol/l) and deoxycytidine ($K_i \sim 1{\cdot}2$ mmol/l) at 25 °C are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]NBMPR. Symbols, concentration of nucleoside (mmol/l): ●, 0; ○, 2; ■, 7·5 and □, 15.

The effects of varying concentrations (0–30 mmol/l) of uridine and deoxycytidine on specific binding of NBMPR (initial concentration 0·5–20 nmol/l) by human 'ghosts' are shown in Fig. 9. Both nucleosides were competitive inhibitors of high-affinity NBMPR binding, giving similar apparent inhibition constants to those obtained with nucleoside-permeable sheep erythrocyte membranes (1·25 and 1·0 mmol/l for uridine and deoxycytidine, respectively).

DISCUSSION

The experiments described in this paper were designed to clarify the mechanism by which the high-affinity ligand NBMPR interacts with the erythrocyte nucleoside transporter. Two complementary approaches were employed. In the first we investigated the kinetics of erythrocyte nucleoside transport and its inhibition by NBMPR. The second approach was to characterize the interaction of uridine and deoxycytidine

with high-affinity NBMPR binding to isolated erythrocyte membranes. The detailed strategy behind these experiments is outlined in the Introduction.

In agreement with previous studies, human and nucleoside-permeable sheep erythrocytes were shown to possess a saturable uptake mechanism for uridine (see e.g. Cabantchik & Ginsburg, 1977; Young, 1978; Jarvis & Young, 1980). Transport by this route in sheep cells was highly temperature dependent, with the V_{max} for zero-*trans* influx about twice as sensitive to temperature (25–37 °C range) as the apparent K_m. Uptake by this mechanism in both cell types was completely inhibited by micromolar concentrations of NBMPR and the structurally unrelated nucleoside transport inhibitor, dipyridamole. Essentially identical results were obtained for deoxycytidine entry into these cells. In addition, deoxycytidine was an effective competitive inhibitor of uridine entry and vice versa, with the apparent K_i values of the two nucleosides the same as their respective apparent K_m values for influx. These observations strongly suggest that uridine and deoxycytidine are transported by the same mechanism. Further support for this conclusion comes from the finding that entry of both deoxycytidine and uridine into nucleoside-impermeable sheep erythrocytes was very slow and linear with concentration. Previous studies have shown that these cells lack a functional nucleoside transporter (see e.g. Young, 1978). There is therefore good evidence to support the view that deoxycytidine enters human and nucleoside-permeable sheep cells by the same nucleoside carrier as uridine, confirming the limited data of Cass & Paterson (1972). The residual non-saturable entry of nucleosides into nucleoside-impermeable sheep erythrocytes has a lower temperature sensitivity than the saturable uptake mechanism and previous studies have shown that it is not inhibited by NBMPR, dipyridamole or nucleosides (Young, 1978; Jarvis & Young, 1978*b*). The magnitude of this flux differs for different nucleosides and corresponds to their lipid solubility, suggesting that it represents simple diffusion through the lipid bilayer.

NBMPR was found to be a potent competitive inhibitor of carrier-mediated uridine entry into nucleoside-permeable sheep erythrocytes. Analysis of the inhibition data using equilibrium free NBMPR concentrations rather than total NBMPR levels did not affect the pattern of inhibition, but dramatically reduced the estimated apparent K_i value from 5 to 1 nmol/l. The latter value is close to the apparent K_D value for NBMPR binding to high-affinity sites on membranes prepared from these cells. Uridine equilibrium exchange efflux in human erythrocytes is also inhibited by NBMPR in an apparently competitive manner (apparent $K_i \simeq 0{\cdot}6$ nmol/l) (Eilam & Cabantchik, 1977). These results are consistent with the conclusion that NBMPR competes directly with nucleosides for the permeation site of the transport system. In contrast with an earlier study (Cass & Paterson, 1976), deoxycytidine, a nucleoside substrate for the transport system, inhibited high-affinity NBMPR binding to membranes prepared from human erythrocytes. Similarly, deoxycytidine blocked NBMPR binding to nucleoside-permeable sheep erythrocyte membranes. Furthermore, inhibition by deoxycytidine in both cases was apparently competitive. The reason for the discrepancy between the present results and those of Cass & Paterson (1976) is unclear. Similar results were also obtained with uridine. The apparent K_i value for uridine inhibition of NBMPR binding to human erythrocyte membranes was 1·25 mmol/l at 25 °C, a value higher than the apparent K_m for

uridine influx in intact cells (approximately 0·14 mmol/l at 25 °C, see Fig. 1) but close to the apparent K_m of equilibrium exchange (1·3 mmol/l) (Cabantchik & Ginsburg, 1977; Shohami & Koren, 1979). The close similarity of the apparent K_i value for uridine inhibition of high-affinity NBMPR binding to the apparent K_m value for uridine equilibrium exchange is further evidence that NBMPR competes directly with nucleosides for the permeation site of the transport system.

NBMPR binding studies with intact erythrocytes suggest that the inhibitor penetrates cell membranes rapidly, a conclusion supported by the present finding that NBMPR has a high octanol: water partition coefficient. Thus NBMPR has access to both membrane surfaces. The observation that NBMPR is a competitive inhibitor of uridine influx, even after prolonged preincubation of cells with inhibitor, therefore implies that the NBMPR inhibition site is located largely on the outer surface of the cell membrane. A formal test of this hypothesis is to perform zero-*trans* uridine efflux studies where it is predicted that the inhibition pattern would be non-competitive (see Deves & Krupka, 1978*a*). The results presented in Fig. 6 show that this is indeed the case. The suggestion that NBMPR binds largely to an outer membrane site is opposite to the situation for cytochalasin B, an inhibitor of glucose transport in human erythrocytes which has been postulated to bind only to permeation sites on the inner surface of the cell membrane (Basketter & Widdas, 1978; Deves & Krupka, 1978*b*).

Although the structures of dipyridamole and nucleosides have little in common, the competitive nature of dipyridamole inhibition of uridine influx by nucleoside-permeable sheep erythrocytes suggests that dipyridamole also binds within the permeation site of the nucleoside transport mechanism. The finding that dipyridamole displaced high-affinity NBMPR binding to nucleoside-permeable sheep erythrocyte membranes is consistent with this proposal (Jarvis & Young, 1980). In HeLa cells, such displacement is competitive (Paterson, 1979; Paterson, Lau, Dahlig & Cass, 1980) with an apparent K_i value very similar to the apparent K_i for dipyridamole inhibition of uridine influx calculated from the present experiments. As was the case for NBMPR, such experiments probably over-estimate the magnitude of dipyridamole inhibitor constants. No radioactive dipyridamole was available to evaluate the effects of inhibitor depletion.

In conclusion the data presented here are consistent with the notion that NBMPR and dipyridamole compete with nucleosides for the permeation site of the nucleoside transport system and that the NBMPR binding site is located largely on the outer surface of the cell membrane. Further evidence of nucleoside transporter asymmetry is presented in the following paper.

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