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Species differences in nucleoside transport

A study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes

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A kinetic study of the inward transport of uridine in erythrocytes of rabbit, human, mouse, rat and guinea-pig demonstrated that the apparent K_m of this process was similar (about 0.2 mM) in these cell types, but $V_{max.}$ values differed markedly. In this array of cell types, $V_{max.}$ values were proportional to the number of transport-inhibitory, high-affinity binding sites present per cell of each type. Transport of uridine or adenosine was not detected in dog erythrocytes, nor was saturable, high-affinity binding of nitrobenzylthioinosine demonstrable. These findings demonstrate that species differences in nucleoside transport capacity are attributable to differences in the cell-surface content of functional nucleoside transport sites, rather than to differences in the kinetic properties of these sites.

Studies with human erythrocytes have shown the presence of a facilitated diffusion system that accepts both purine and pyrimidine nucleosides (Oliver & Paterson, 1971; Cass & Paterson, 1972, 1973; Pickard & Paterson, 1972; Cabantchik & Ginsburg, 1977). Erythrocytes of the guinea-pig and the adult pig also possess a similar membrane transport system for nucleosides (Roos & Pfleger, 1972; Jarvis *et al.*, 1980). In contrast, erythrocytes from most sheep are virtually impermeable to nucleosides, lacking a nucleoside transport system (nucleoside-impermeable phenotype). However, erythrocytes of some individual sheep (nucleoside-permeable phenotype) do possess a nucleoside transport system with properties similar to the nucleoside transport system of human erythrocytes (Young, 1978). The permeability difference between erythrocytes from sheep of these two phenotypes is genetically determined (Jarvis & Young, 1978). In the sparse literature on nucleoside transport by erythrocytes from other species (Duhm, 1974; Cass & Paterson, 1975; Kolassa & Pfleger, 1975), kinetic constants derived from initial rates of transport have not been reported.

Abbreviations used: NBMPR, 6[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine); NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (nitrobenzylthioguanosine).

The present paper describes the kinetics of uridine entry into erythrocytes from humans and several species of commonly used laboratory animals. Also evaluated in these cells was the binding of a potent inhibitor of nucleoside transport, NBMPR [see Paterson *et al.* (1981) and Jarvis & Young (1982*a*) for reviews]. The results here reported indicate that, although species variation is considerable in the relative abilities of erythrocytes both to transport nucleosides and to bind NBMPR, the capacity of each transport site for uridine entry is similar (approx. 150 molecules/site per s) in a number of species.

Materials and methods

Cells and membranes

Blood samples were collected into heparinized tubes and cells were used within 4 h of collection. Erythrocytes were washed three times with Dulbecco's (Dulbecco & Vogt, 1954) phosphate-buffered saline. The buffy coat was discarded. Cell counts and haematocrit estimations were performed by established methods (Archer, 1965). Haemoglobin-free erythrocyte membranes, prepared by the method of Dodge *et al.* (1963), were washed three times and resuspended in 5 mM-sodium phosphate (pH 7.2). All experiments were performed at 22°C.

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Nucleoside transport

Uridine influx was measured by mixing 0.2ml portions of cell suspension (haematocrit, 20%) with 0.2ml of phosphate-buffered saline containing the appropriate concentration of [U-^{14}C]uridine (0.5μCi/ml). At specified time intervals, cells were collected from incubation mixtures by one of two methods. In the first, used in measuring the slow uptake fluxes in dog and guinea-pig erythrocytes, 1ml portions of ice-cold medium were added to incubation mixtures (after uptake intervals of 0.5–30min), cells were pelleted and then rapidly washed four times with 1ml ice-cold portions of phosphate-buffered saline using an Eppendorf 5412 microcentrifuge (10s, 12000*g*). Cells were then processed for radioactivity determinations as described by Young (1978). This washing procedure removed extracellular labelled permeant, without significant loss of the latter from cells with slow nucleoside uptake rates (Young, 1978). For more rapid fluxes (erythrocytes of the human, rabbit, mouse and rat), after incubation intervals of 2–60s, 0.2ml samples of incubation mixture were added to 0.8ml of ice-cold 'stopper' medium (containing 10μM-NBTGR, a potent inhibitor of nucleoside transport) layered on top of 0.5ml of ice-cold di-n-butyl phthalate contained in 1.5ml microcentrifuge tubes. Tubes were immediately centrifuged (12000*g*, 10s) and the aqueous and di-n-butyl phthalate layers were removed by suction. Radioactivity associated with the cell pellet was determined (Young, 1978). Blank values ([^{14}C]uridine that became associated with cells during uptake intervals of zeros) were obtained by processing cell samples exposed simultaneously to [^{14}C]uridine and 10μM-NBTGR at 0°C. Where applicable, kinetic constants of influx (apparent K_m and $V_{max.}$) were determined by linear regression analysis of [S]/v-versus-[S] plots, where v is the initial uptake rate and [S] is the extracellular nucleoside concentration.

NBMPR binding

NBMPR binding to erythrocytes and membrane suspensions was determined as previously described (Jarvis & Young, 1980; Hammond *et al.*, 1981). Briefly, cell suspensions (3.0×10^7 cells) and membrane suspensions (equivalent to 0.3×10^9 cells for dog and guinea-pig erythrocyte ghosts) were incubated with phosphate-buffered saline and 5mM-sodium phosphate respectively, containing [G-^{3}H]-NBMPR (0.05–10nM) in the presence and absence of NBTGR (10μM). After 20min, incubations were terminated by centrifugation at 12000*g* in an Eppendorf 5412 microcentrifuge (10s for intact cells and 15min for membranes). Supernatants were retained for radioactivity determinations. Pellets of cells or membranes were washed once or twice respectively with 1ml of the appropriate ice-cold medium. Radioactivity associated with cell pellets was determined by scintillation counting with appropriate quench correction as described by Jarvis & Young (1980). Kinetic constants for high-affinity NBMPR 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.

Chemicals

[G-^{3}H]NBMPR (sp. radioactivity 20Ci/mmol) and [2-^{3}H]adenosine (sp. radioactivity 16Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA, U.S.A., and, if necessary, just before use, were repurified to >98% radiochemical purity by high-pressure liquid chromatography using a C_{18} μ Bondapak column (Waters) eluted with methanol/water solutions. [U-^{14}C]Uridine (sp. radioactivity 0.53Ci/mmol; >98% radiochemically pure) was purchased from Amersham, Oakville, Ontario, Canada. Dipyridamole [2,2′,2″,2‴-(4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine-2, 6-diyldinitrilo)tetraethanol] was a gift from Boehringer Ingelheim (Canada), Burlington, Ontario, Canada. NBMPR and NBTGR were prepared (Paul *et al.*, 1975) from 6-thioinosine and 6-thioguanosine respectively, provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.

Results

To compare the nucleoside permeability characteristics of human, rabbit, mouse, guinea-pig, rat and dog erythrocytes, time courses of uridine uptake at 22°C by these cells from medium containing 1mM-uridine were measured (see Fig. 1). Dog and guinea-pig erythrocytes were virtually impermeable to uridine, but the uridine content of rat, mouse, human and rabbit erythrocytes increased with incubation time. In other time-course experiments (results not shown), it was demonstrated that initial rates of uridine uptake were measured by the cellular content of uridine after incubation intervals of 2–5s for erythrocytes from mice, humans and rabbits, and after intervals of 60s and 3min respectively for erythrocytes from dogs and guinea-pigs.

Fig. 2 compares the concentration dependence of uridine influx into rabbit, human, guinea-pig and dog erythrocytes at uridine concentrations from 0.025 to 5mM. Uridine fluxes for rabbit, human and guinea-pig erythrocytes were saturable with apparent K_m values of 0.17, 0.20 and 0.27mM respectively, and $V_{max.}$ estimates of 155, 100 and 0.31 mmol/litre of cells per h respectively. Uridine fluxes into mouse and rat erythrocytes were also saturable with apparent K_m values of 0.16 and 0.20mM respectively, and $V_{max.}$ values of 43.5 and 3.6mmol/litre of cells per h respectively. In contrast, uridine

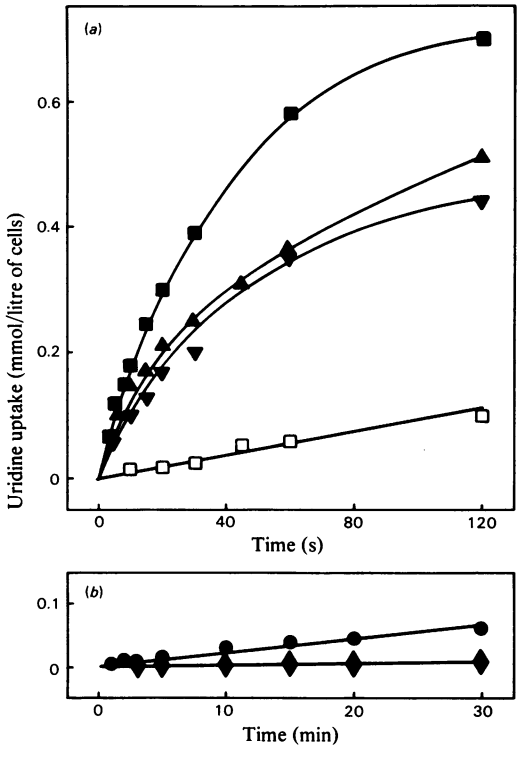

Fig. 1. *Time courses for the uptake of uridine by rabbit, human, mouse, rat, guinea-pig and dog erythrocytes*
The uridine concentration was 1 mM (22°C). Symbols: ■, rabbit; ▲, human; ▼, mouse; □, rat; ●, guinea-pig; ♦, dog erythrocytes. Note the difference in scale and units on the abscissa between the upper panel and the lower panel.

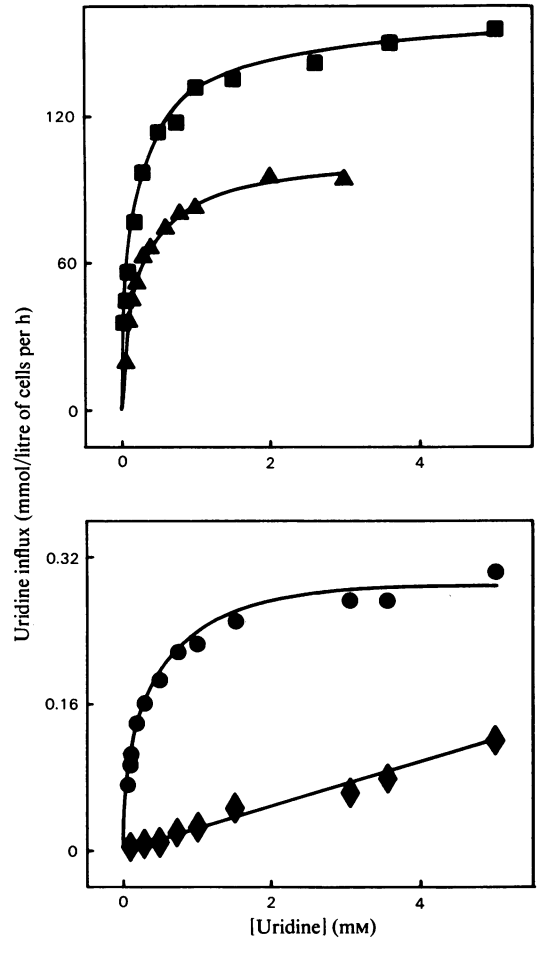

Fig. 2. *Concentration dependence of uridine entry by rabbit, human, guinea-pig and dog erythrocytes*
The curves describing the influx of uridine in rabbit (■), human (▲), dog (♦) and guinea-pig (●) erythrocytes were calculated from the equation:

$$v \text{ (mmol/litre of cells per h)} = V_{max.}[S]/(K_m + [S])$$

where v is the velocity of influx at an extracellular uridine concentration of [S]. The kinetic constants K_m and $V_{max.}$ were calculated for each experiment from a least-squares analysis of an [S]/v-versus-[S] plot of the data. The influx of uridine in dog erythrocytes was directly proportional to uridine concentration and was calculated as $v = 0.024 \cdot [S]$. Note the difference in scale on the ordinate between the upper and lower panel.

uptake by dog erythrocytes was slow and rates were proportional to uridine concentration (0.12 mmol/litre of cells per h at 5 mM) over the Fig. 2 concentration range. Saturability of adenosine uptake rates in dog erythrocytes was not detected in similar experiments (results not shown). Erythrocyte samples from three to six animals of these species were employed to determine the following mean apparent $V_{max.}$ values for NBMPR-sensitive entry of uridine: rabbit, 155 ± 9 (3); human, 102 ± 12 (6); mouse, 52.9 ± 5.9 (4); rat, 3.5 ± 0.6 (6); and guinea-pig, 0.22 ± 0.044 (3) mmol/litre of cells per h [means ± S.E.M. (n)]. These data are further considered in Table 1. Neither NBMPR (10 μM) nor dipyridamole (10 μM) inhibited the uptake of uridine (1 mM) or adenosine (0.025–15 mM) by dog erythrocytes.

The determination of site-specific binding of [^{3}H]-NBMPR to intact rabbit and guinea-pig erythrocytes *in vitro* is illustrated in Fig. 3, in which cell-associated NBMPR is plotted against the equilibrium concentration of the inhibitor in the medium. The NBMPR associated with rabbit cells was resolved into two components: (*a*) an association of NBMPR with specific sites which, when saturated, bound 11×10^{-21} mol of NBMPR per cell with an apparent K_D of 1.1 nM, and (*b*) a non-saturable association responsible for the binding of 0.35×10^{-21} mol/cell

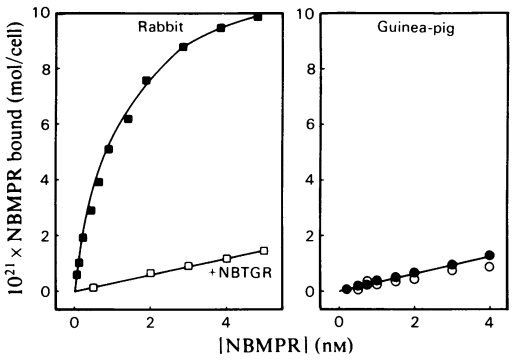

Fig. 3. *Binding of NBMPR to rabbit and guinea-pig erythrocytes*

Cells were incubated in [G-^{3}H]NBMPR (0.05–10 nM) in the presence (non-specific binding; □ and ○) and absence (total binding; ■ and ●) of a second transport inhibitor, NBTGR (10 μM), as described in the Materials and methods section. The amount of NBMPR bound is plotted as a function of the equilibrium free NBMPR concentration. The kinetic constants K_D (1.1 nM) and $B_{max.}$ (11.1×10^{-21} mol/cell) for site-specific binding of NBMPR to rabbit erythrocytes were calculated by a least-squares analysis of a Scatchard plot (bound/free versus bound) of the data (corrected for non-specific binding). Site-specific binding of NBMPR to intact erythrocytes of the guinea-pig was not demonstrable.

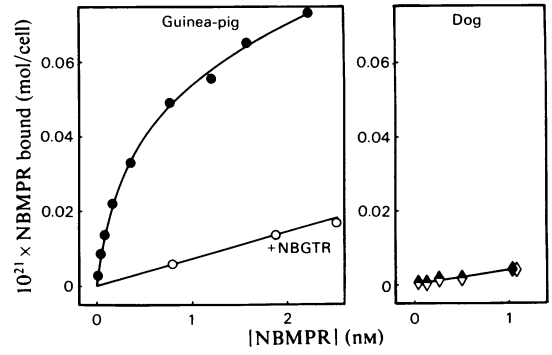

Fig. 4. *Binding of NBMPR to membranes from guinea-pig and dog erythrocytes*

Membrane suspensions were incubated in [G-^{3}H]NBMPR (0.05–10 nM) in the presence (non-specific binding; ○ and ◊) and absence (total binding; ● and ♦) of a second transport inhibitor, NBTGR (10 μM), as described in the Materials and methods section. The kinetic constants K_D (0.35 nM) and $B_{max.}$ (0.06×10^{-21} mol/cell) for site-specific binding of NBMPR in guinea-pig membranes were calculated as indicated in the legend to Fig. 3. Site-specific binding of NBMPR to dog erythrocyte membranes was not demonstrable.

at 1 nM. The latter component of binding was revealed when the saturable association was abolished by prior treatment of cells with 10 μM-NBTGR. When site-specific association of NBMPR with erythrocytes from a number of individual animals was determined, the following mean saturation values [$B_{max.}$, 10^{-21} × mol of NBMPR bound per cell, means ± S.E.M. (*n*)] were obtained: rabbit, 15.5 ± 0.5 (3); human, 18.3 ± 1.1 (9); mouse; 5.5 ± 0.8 (4); and rat, 0.5 ± 0.05 (6). The corresponding mean K_D values [nM, means ± S.E.M. (*n*)] for site-bound NBMPR were: rabbit, 1.1 ± 0.4 (3); human, 0.31 ± 0.29 (9); mouse, 0.14 ± 0.02 (4); and rat, 0.28 ± 0.11 (6). These data are further considered in Table 1. The values here reported for human cells are consistent with previous published data (Cass *et al.*, 1974; Jarvis & Young, 1980; Hammond *et al.*, 1981). In contrast, both guinea-pig and dog erythrocytes showed no detectable high-affinity binding.

Previous studies (Pickard *et al.*, 1973; Cass *et al.*, 1974; Jarvis & Young, 1980) demonstrated that the non-saturable NBMPR binding component of human and sheep erythrocytes was considerably decreased when haemoglobin-free cell membranes were used instead of intact cells. Fig. 4 shows the concentration-dependence of NBMPR binding to unsealed erythrocyte membranes (ghosts) prepared from guinea-pig and dog erythrocytes. Non-saturable binding by the membrane preparations was decreased 50-fold relative to that by intact cells of both types (Fig. 3), thereby revealing the presence of a high-affinity NBMPR-binding site in guinea-pig erythrocyte membranes ($B_{max.}$ $10^{-21} \times 0.06$ mol/cell; apparent K_D, 0.35 nM). Such sites were absent from dog erythrocyte membrane. NBTGR abolished the high-affinity component of NBMPR binding to guinea-pig membranes (Fig. 4), but had no effect on the non-saturable binding component. Erythrocyte membranes from three guinea-pigs bound NBMPR in the amount of $10^{-21} \times (0.045 \pm 0.007)$ mol/cell with an apparent K_D of 0.22 ± 0.11 nM.

Discussion

The results presented here demonstrate that a saturable mechanism is responsible for the inward transport of uridine by human, rabbit, mouse, rat and guinea-pig erythrocytes. The apparent affinity (K_m) of uridine for the nucleoside transporter was similar in these instances (approx. 0.2 mM for uridine entry at 22°C), although observed $V_{max.}$ values for uridine influx differed by as much as 370-fold between erythrocytes of the five species. These findings confirm and extend the previous observations of Roos & Pfleger (1972), Duhm

(1974), Cass & Peterson (1975) and Jarvis & Young (1982*a*,*b*). In contrast with previous studies (Kubler & Bretschneider, 1964; Kubler *et al.*, 1970), saturable uridine or adenosine transport was not detected in dog erythrocytes, and high-affinity binding of NBMPR was absent from these cells. This finding supports a previous conclusion that high-affinity NBMPR binding to erythrocyte membranes is a specific association of NBMPR with functional nucleoside-transport sites (Jarvis & Young, 1980). A possible reason for the discrepancy between the present results and those of Kubler and co-workers is that, in the present study, NBMPR made possible a clear distinction between transporter-mediated entry of permeant and entry by diffusion.

This demonstration of substantial differences in nucleoside transport rates among erythrocytes from commonly used laboratory animals bears importantly on attempts to understand the influence of erythrocytes on nucleoside concentrations in the circulation. Thus, in biological systems where the erythrocyte transport system can influence plasma concentrations of adenosine and other nucleosides, significant species differences in the potency and rates of removal of these agents should be expected. Similarly, the apparent potency of the adenosine receptor antagonist, theophylline, is also influenced by the removal of adenosine from the vicinity of its receptors by the nucleoside transport mechanism (Clanachan & Muller, 1980).

The influence of nucleoside transport inhibitors on adenosine concentration in the extracellular space is related to the quantitative importance of the nucleoside transport system as a means of nucleoside removal. If, as has been suggested (Roos & Pfleger, 1972), erythrocytes inactivate adenosine released into the coronary circulation, then the effects of the vasoactive inhibitors of nucleoside transport (such as dipyridamole or dilazep) should be expected to be significantly greater in humans than in dogs, guinea-pigs or rats. The rabbit would appear to possess an erythrocytic nucleoside transport system that is more comparable with that of man than would be those of the dog, guinea-pig or rat. In the dog, commonly used in coronary circulation studies, adenosine is not transported into erythrocytes, but may be inactivated by other cellular elements of blood.

Species differences in nucleoside transport have also been noted in other cells. In cardiac muscle, dipyridamole potentiates the negative inotropic action of adenosine in guinea-pigs, but not in rats (Hopkins & Goldie, 1971). This may be due to a lower rate of nucleoside transport-dependent accumulation of adenosine in rat cardiac tissue.

The higher nucleoside transport capacity ($B_{max.}$) of rabbit, human, mouse, rat and guinea-pig erythrocytes relative to dog erythrocytes is related to the cell-surface density of high-affinity NBMPR-binding sites (see Table 1). If each high-affinity NBMPR-binding site is assumed to represent a single nucleoside transport site, then the translocation capacity for each transport site is similar in erythrocytes of the rabbit, human, mouse, rat and guinea-pig (approx. 150 molecules/site per s at 22°C; Table 1). These values are similar to those reported previously for sheep erythrocytes, sheep reticulocytes, foetal erythrocytes from newborn lambs, pig erythrocytes and human erythrocytes (Jarvis & Young, 1982*a*,*b*). These findings demonstrate that species differences

Table 1. *Comparison of $V_{max.}$ for zero-trans uridine influx with $B_{max.}$ for the binding of NBMPR in erythrocytes from various species*

$V_{max.}$ values for uridine influx and maximum high-affinity NBMPR-binding activities for erythrocytes are determined as described in the text. The numbers of observations are indicated in parentheses. Results are means ± S.E.M.

	Uridine $V_{max.}$		NBMPR $B_{max.}$		
Species	(mmol/litre of cells per h)	(10^{19} × mol/cell per s)	(10^{21} × mol/cell	(molecules/cell)	Translocation capacity (molecules/sites per s)
Human (6)	102 ± 12	26 ± 3	18.3 ± 1.1	11,040 ± 640	142 ± 28
Rabbit (3)	155 ± 9	29 ± 2	15.5 ± 0.5	9,290 ± 280	190 ± 22
Mouse (4)	52.9 ± 5.9	6.8 ± 0.8	5.5 ± 0.8	3,310 ± 480	123 ± 48
Rat (6)	3.5 ± 0.6	0.7 ± 0.1	0.5 ± 0.05	300 ± 30	140 ± 29
Guinea-pig (3)	0.22 ± 0.04	0.07 ± 0.01	0.045 ± 0.007	27 ± 4	155 ± 12
Dog (4)	Not detectable		Not detectable		—
Pig*	57.5 ± 9.5	10.9 ± 1.8	8.5 ± 0.7	5,120 ± 420	128
Sheep* (5)					
Nucleoside-permeable type	0.62 ± 0.06	0.053 ± 0.005	0.029 ± 0.003	18 ± 2	181 ± 43
Nucleoside-impermeable type	Not detectable		Not detectable		—

* Taken from Jarvis & Young (1982*b*).

in the nucleoside transport capacity of erythrocytes are due to differences in the cellular numbers of functional nucleoside transport sites, rather than to differences in the kinetic properties of the sites.

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