Photoaffinity labelling of nucleoside-transport proteins in plasma membranes isolated from rat and guinea-pig liver

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Nitrobenzylthioinosine (NBMPR) was employed as a probe of the nucleoside transporters from rat and guinea-pig liver. Purified liver plasma membranes prepared on self-generating Percoll density gradients exhibited 16-fold (rat) and 10-fold (guinea pig) higher [^3H]NBMPR-binding activities than in crude liver homogenates (3.69 and 14.7 pmol/mg of protein for rat and guinea-pig liver membranes respectively, and 0.23 and 1.47 pmol/mg of protein for crude liver homogenates respectively). Binding to membranes from both species was saturable (apparent K_d 0.14 and 0.63 nM for rat and guinea-pig membranes respectively) and inhibited by uridine, adenosine, nitrobenzylthioguanosine (NBTGR) and dilazep. Uridine was an apparent competitive inhibitor of high-affinity NBMPR binding to rat membranes (apparent K_i 1.5 mM). There was a marked species difference with respect to dipyrindamole inhibition of NBMPR binding (50% inhibition at 0.2 and $>100\text{ }\mu\text{M}$ for guinea-pig and rat respectively). These results are consistent with a role of NBMPR-binding proteins in liver nucleoside transport. Exposure of rat and guinea pig membranes to high-intensity u.v. light in the presence of [^3H]NBMPR resulted in the selective radiolabelling of membrane proteins which migrated on sodium dodecyl sulphate/polyacrylamide gels with apparent M_r values in the same range as that of the human erythrocyte nucleoside transporter (45000–66000). Covalent labelling of these proteins was abolished when photolysis was performed in the presence of non-radioactive NBTGR as competing ligand.

Permeation of physiological and cytotoxic nucleosides across the plasma membrane of animal cells is mediated largely by nucleoside-specific transport mechanisms sensitive to inhibition by nanomolar concentrations of the 6-thiopurine ribonucleoside, NBMPR (Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1981; Young & Jarvis, 1983). In erythrocytes, NBMPR inhibition of nucleoside transport is competitive with respect to substrate concentration (Eilam & Cabantchik, 1977; Roger-Brown & Parks, 1980; Jarvis *et al.*, 1982a) and associated with high-affinity binding of inhibitor to the transporter (apparent K_d 10^{-10} – 10^{-9} M) (Cass *et al.*, 1974; Jarvis & Young, 1980a). Binding is inhibited by dipyrindamole, and competitively

blocked by transported nucleosides such as uridine (Cass & Paterson, 1976; Jarvis & Young, 1980a; Paterson *et al.*, 1980; Hammond *et al.*, 1981; Jarvis *et al.*, 1982a).

Specific high-affinity NBMPR-binding sites have also been shown to be present on other, more complex, cells. For example, mouse S49 lymphoma cells transport nucleosides and bind NBMPR (approx. 10^5 sites per cell), whereas AE₁ lymphoma cells (a nucleoside-transport-deficient clone derived from S49 cells) lack functional nucleoside transport systems and do not bind inhibitor (Cohen *et al.*, 1979; Cass *et al.*, 1981). More recently, the tissue distribution of high-affinity NBMPR-binding sites has been investigated in the rat (Marangos *et al.*, 1982). Binding sites were present in a variety of tissues, including liver, a tissue recognized to play a central role in whole-body purine and pyrimidine nucleoside metabolism (Murray, 1971; Lerner & Lowy, 1974; Pritchard *et al.*, 1975).

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

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Binding of NBMPR to the nucleoside transporter is normally reversible, but covalent labelling of erythrocyte 'ghosts' has been demonstrated to occur when site-bound NBMPR is exposed to high-intensity u.v. light (Young *et al.*, 1983). Using this approach, we have developed experimental conditions which permit the specific photoaffinity labelling of the human and pig erythrocyte nucleoside transporters, identified as band-4.5 polypeptides [nomenclature of Steck (1974)] with apparent M_r values on SDS/polyacrylamide gels of 45000–66000 (Jarvis & Young, 1981; Wu *et al.*, 1983a,b). The objective of the present series of experiments was to extend these NBMPR-photolysis studies to liver tissue as a first step towards a comparison of the molecular properties of nucleoside transporters in different cell types.

Materials and methods

Materials

[2-³H]AMP (sp. radioactivity 11.7 Ci/mmol; 98% radiochemically pure) was purchased from Amersham International, Amersham, Bucks., U.K. [G-³H]NBMPR (sp. radioactivity 17 Ci/mmol; >98% radiochemically pure) was obtained from Moravsek Biochemicals, Brea, CA, U.S.A. NBTGR was prepared (Paul *et al.*, 1975) from 6-thioguanosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A., and was generously given by Professor A. R. P. Paterson, University of Alberta Cancer Research Group. Dipyrindamole {2, 2', 2''-(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyl)dinitrilo}tetraethanol} (Persantin injection) was obtained from Boehringer Ingelheim, Bracknell, Berks., U.K., and dilazep {1,4-bis[3-(3,4,5-trimethoxybenzoyloxy)propyl]perhydro-1,4-diazepine} was a gift from Hoffmann-La Roche and Co., Basel, Switzerland. Percoll was from Pharmacia Fine Chemicals (Hounslow, Middx., U.K.). M_r standards for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other reagents were of analytical grade.

Liver plasma-membrane preparation

Male Sprague-Dawley rats (weighing 180–220 g) and Dunkin-Hartley guinea pigs (weighing 300–350 g) were anaesthetized by intraperitoneal injection of 5% (w/v) Nembutal (1 ml/kg body wt). Livers were perfused *in situ* through the hepatic portal vein with about 20 ml of ice-cold 0.3 M-sucrose to remove trapped erythrocytes. Livers were then dissected out, rinsed in 0.3 M-sucrose and cooled on ice. Tissue was minced with scissors and then homogenized in 5 vol. (v/w) of 0.25 M-sucrose containing 10 mM-Tris/HCl and 2 mM-EGTA

(pH 7.4 at 22°C) with ten strokes of a loose-fitting Teflon/glass homogenizer (0.15–0.23 mm clearance; Arthur H. Thomas Co., Philadelphia, PA, U.S.A.). All operations were performed at 0–4°C.

The homogenate was centrifuged at 1000 *g* for 30 s. The supernatant was collected and centrifuged for a further 30 min at 30000 *g*. The resulting pellet had two layers. The upper fluffy layer was resuspended gently in 1 vol. of homogenization buffer, leaving the bottom compact layer untouched. Then 0.75 ml of this resuspended fraction was mixed with 12 ml of 17.5% (v/v) Percoll containing 0.25 M-sucrose, 10 mM-Tris/HCl and 2 mM-EGTA (pH 7.4). The density of this solution was 1.05 g/ml. The whole mixture was then centrifuged in a fixed-angle rotor at 10000 *g* for 15 min to generate a self-forming gradient of Percoll (Belsham *et al.*, 1980).

The plasma-membrane fraction, which formed a band just below the surface of the gradient, was collected in a volume of about 1.5 ml. The membranes were washed twice by centrifugation (10000 *g* for 10 min) in a NaCl-based medium (0.15 M-NaCl/10 mM-Tris/HCl/1 mM-EGTA, pH 7.4). Finally, the washed plasma-membrane pellet was resuspended in NaCl medium and either used immediately or stored at –70°C for a maximum of 1 week. Human erythrocyte 'ghosts' were prepared from fresh heparinized blood as described previously (Dodge *et al.*, 1963).

Assay of enzyme activities

The activity of 5'-nucleotidase (EC 3.1.3.5) was assayed at 30°C as the release of [³H]adenosine from [2-³H]AMP as described by Belsham *et al.* (1980). Succinate dehydrogenase (EC 1.3.99.1) was also determined at 30°C as the succinate-dependent reduction of 2,6-dichlorophenol-indophenol (Bachmann *et al.*, 1966). NADH-cytochrome *c* reductase (EC 1.6.99.3) was assayed at room temperature as described by Dallner *et al.* (1966). A molar absorption coefficient of $29.5 \times 10^3 \text{ cm}^{-1} \cdot \text{M}^{-1}$ for cytochrome *c* at 550 nm was used in the calculation of enzyme activity. Protein was determined as described by Lowry *et al.* (1951).

High-affinity NBMPR binding

NBMPR binding to liver plasma membranes was determined at room temperature as previously described (Jarvis & Young, 1980a). Briefly, membrane suspensions (0.4 ml; 0.5 mg of protein/ml) were equilibrated with various concentrations of [³H]NBMPR for 30 min in NaCl buffer in the absence or presence of NBTGR (20 μM). Incubations were terminated by rapid cooling on ice, followed by immediate centrifugation at 4°C for 6 min at 15000 *g*. Supernatants were retained for

radioactivity determination. Membrane pellets were washed once with 1 ml of ice-cold NaCl buffer and dissolved in 0.5 ml of 5% (w/v) Triton X-100. Portions of solubilized membrane (0.4 ml) were counted for ^3H by liquid-scintillation spectrometry, with appropriate quench correction.

To determine the maximum high-affinity binding (B_{max}) of NBMPR to crude liver homogenates, samples were first diluted to about 4 mg of protein/ml and then processed as described above, by using a saturating concentration of [^3H]NBMPR (25–50 nM). A small fraction (10–20%) of the high-affinity NBMPR-binding activity present in crude liver homogenates was not pelleted at 15000g. This binding was determined by passing 75 μl portions of the 15000g supernatants through 1 ml columns of Sephadex G-50 (medium grade) at 4°C to separate bound from free [^3H]NBMPR as described previously (Jarvis & Young, 1980b). Liver homogenate B_{max} values were calculated as the sums of pellet and supernatant binding activities, corrected for non-specific (NBTGR-insensitive) binding.

Photoaffinity labelling

Liver and erythrocyte membranes (approx. 2 mg of protein/ml in NaCl buffer) were equilibrated at room temperature for 30 min with a saturating concentration of [^3H]NBMPR (75 nM for erythrocyte 'ghosts', 50 nM for guinea-pig membranes and 25 nM for rat membranes) in the absence and presence of 20 μM -NBTGR as competing ligand. Membrane suspensions were then cooled to 4°C and supplemented with 50 mM-dithiothreitol (final concentration), added as a free-radical scavenger. Photolysis was performed in conventional 3 ml silica spectrophotometer cuvettes (10 mm light-path) with continuous stirring at 4°C. Samples were exposed to high-intensity u.v. light at a distance of 6.5 cm from a 450 W mercury arc lamp (Conrad-Hanovia, Newark, NJ, U.S.A.) for 45 s, the optimal exposure time for radiolabelling of erythrocyte 'ghosts' (Young *et al.*, 1983; Wu *et al.*, 1983b). Samples were then diluted 10-fold with NaCl buffer containing 10 μM -NBTGR and left at room temperature for 10 min before recovery of the membrane fraction by centrifugation at 35000g for 15 min. The membrane pellets were washed twice more with NBTGR-containing buffer and dissolved in gel sample buffer at room temperature.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in 1 mm-thick slab gels by the method of Thompson & Maddy (1982) with the Laemmli (1970) buffer system. Solubilized membranes in the gel sample buffer were not heated before application to the gel. Radioactivity in the various

regions of the gel was determined by slicing the gel into 2 mm fractions. The ^3H content of these slices was measured by liquid-scintillation counting in toluene containing 0.4% (w/v) Omnifluor and 3% (v/v) Protosol (New England Nuclear). Vials were left at 37°C for 20 h before radioactivity counting. Recoveries of applied radioactivity were in the range 75–80%.

Results

Preparation of liver plasma membranes

Plasma membranes were prepared from rat and guinea pig liver by the Percoll procedure described in the Materials and methods section. Some of the characteristics of the rat liver plasma-membrane fraction are summarized in Table 1(a). The specific activity of the plasma-membrane marker enzyme 5'-nucleotidase was increased about 17-fold relative to that in the crude liver homogenate, whereas there was a 2-fold decrease in the specific activity of succinate dehydrogenase (mitochondrial inner-membrane marker enzyme). The specific activity of NADH-cytochrome *c* reductase (endoplasmic-reticulum membrane marker enzyme) remained unchanged. Our plasma-membrane fraction contained about 15% of the total 5'-nucleotidase activity, and was cross-contaminated with mitochondria to the extent of 0.5% of the starting succinate dehydrogenase activity and with endoplasmic-reticulum material to the extent of 1% of the starting NADH-cytochrome *c* reductase activity.

Results obtained with guinea-pig liver are shown in Table 1(b). The plasma-membrane fraction was contaminated with mitochondria and endoplasmic reticulum to a similar extent as the rat liver preparation. However, the yield of plasma membrane, in terms of the recovery of 5'-nucleotidase activity, was significantly higher. The relative specific activity of 5'-nucleotidase was also higher for guinea-pig membranes. Rat liver was found to have a consistently higher 5'-nucleotidase activity than guinea-pig liver.

NBMPR binding

The binding of [^3H]NBMPR to rat and guinea-pig liver plasma membranes is shown in Fig. 1, where the amounts of [^3H]NBMPR bound to membranes are plotted against the equilibrium free concentrations of ligand in the medium. Binding was saturable in both cases. Treatment of membranes with the structurally related nucleoside-transport inhibitor NBTGR abolished this saturable binding activity and revealed a residual NBTGR-insensitive non-saturable association (0.70 and 1.25 pmol/mg of protein at 10 nM-NBMPR for rat and guinea-pig membranes respectively). Scat-

Table 1. Enzyme and nitrobenzylthioinosine-binding activities in homogenates and plasma-membrane fractions from rat and guinea-pig liver

Enzyme specific activities (means $\pm$ S.E.M. for n experiments) are expressed in nmol of substrate produced/min per mg of protein and were determined as described in the text. [3 H]NBMPR binding to plasma-membrane fractions represent $B_{\max}$ values (pmol/mg of protein) determined by linear-regression analyses of Scatchard plots after subtraction of non-saturable (NBTGR-insensitive) binding components. Apparent K_d values for [3 H]NBMPR binding (means $\pm$ S.E.M.) were 0.14 ± 0.03 nM and 0.63 ± 0.11 nM for rat and guinea-pig membranes respectively. $B_{\max}$ values for NBTGR-sensitive binding to crude liver homogenates were determined at a single saturating concentration of ligand. In rat liver, 80% of the homogenate NBMPR-binding activity was associated with the 15000g pellet, compared with 90% in guinea-pig liver (see the text for experimental details). Recovery values refer to the percentage of homogenate activity recovered in the plasma-membrane fraction. Relative specific-activity values were calculated as the ratio plasma-membrane specific activity/homogenate specific activity.

		Homogenate specific activity	Plasma-membrane fraction			n
			Specific activity	Recovery (%)	Relative specific activity	
(a) Rat	NADH-cytochrome <i>c</i> reductase	126 ± 7	129 ± 8	0.98 ± 0.13	1.03 ± 0.10	4
	Succinate dehydrogenase	39 ± 2	22 ± 2	0.53 ± 0.08	0.57 ± 0.03	4
	5'-Nucleotidase	6.0 ± 0.4	98 ± 11	14.2 ± 0.4	16.6 ± 2.3	4
	[3 H]NBMPR binding	0.23 ± 0.03	3.69 ± 0.38	12.8 ± 1.1	14.9 ± 2.6	4
(b) Guinea pig	NADH-cytochrome <i>c</i> reductase	71 ± 7	53 ± 3	0.71 ± 0.04	0.77 ± 0.12	3
	Succinate dehydrogenase	55 ± 7	44 ± 12	0.78 ± 0.24	0.77 ± 0.14	3
	5'-Nucleotidase	0.38 ± 0.04	11.2 ± 0.4	28.0 ± 6.5	29.3 ± 3.5	3
	[3 H]NBMPR binding	1.47 ± 0.05	14.7 ± 2.5	11.1 ± 1.6	9.95 ± 0.7	4

Fig. 1. Concentration-dependence of nitrobenzylthioinosine binding to plasma membranes from rat and guinea-pig liver. Rat and guinea-pig membranes were incubated with [3 H]NBMPR (0.1–20 nM and 0.1–50 nM respectively) in the absence (●) and in the presence (○) of 20 μ M-NBTGR as competing ligand. Values are means of duplicate estimates. Apparent K_d and $B_{\max}$ values for NBTGR-sensitive binding were determined by Scatchard analysis (see the text for values).

chard analysis of NBTGR-sensitive binding gave apparent K_d values of 0.10 and 0.79 nM and $B_{\max}$ values of 3.9 and 14.3 pmol/mg of protein for the rat and guinea-pig membranes respectively.

Mean apparent K_d and $B_{\max}$ values for high-affinity NBMPR binding to liver plasma membranes from a number of animals of each species are summarized in Table 1. The mean apparent K_d

and $B_{\max}$ values for guinea-pig membranes are both about 4-fold higher than those for rat membranes. When the $B_{\max}$ values of the purified plasma membranes are compared with those of crude liver homogenates, 15- and 10-fold increases in specific activity are apparent for rat and guinea-pig membranes respectively (Table 1). Recoveries of NBMPR-binding activity in the plasma-mem-

Fig. 2. Nitrobenzylthioinosine binding to rat and guinea-pig liver plasma membranes: inhibition by adenosine, uridine, dilazep and dipyridamole

NBTGR-sensitive [^3H]NBMPR binding (2.5 nM) to rat (closed symbols) and guinea pig (open symbols) liver membranes was measured in the presence of uridine (■, □), adenosine (▲, △), dilazep (●, ○) and dipyridamole (◆, ◇) as described in the text, except that the membrane protein content of incubations was adjusted to 0.36 and 0.07 mg/ml for rat and guinea pig respectively. Under these conditions, the equilibrium concentration of unbound [^3H]NBMPR in the absence of added inhibitor was the same (1.7 nM) for the two species. Results are expressed as a percentage of control binding activities. Values are means of duplicate estimates.

brane fraction were 13 and 11% respectively. As detailed in the legend to Table 1, 80% of the NBTGR-sensitive binding activity present in rat liver homogenates was pelleted by centrifugation at 15000g for 6 min. The value for guinea-pig liver was 90%. Previously, Marangos *et al.* (1982) have reported an apparent K_d value of 0.20 nM and a B_{max} of 0.16 pmol/mg of protein for NBMPR binding to a crude membrane fraction prepared by homogenization of rat liver tissue in Tris buffer, followed by high-speed centrifugation at 30000g for 30 min.

Inhibition studies

The effects of two physiological nucleosides, adenosine and uridine, on NBTGR-sensitive [^3H]NBMPR binding to rat and guinea pig liver membranes are shown in Fig. 2. The concentrations of nucleosides required to induce 50% inhibition (IC_{50}) of high-affinity NBMPR binding (2.5 nM) were similar in the two species: 20 mM and 2 mM for uridine and adenosine respectively. Higher concentrations of nucleosides induced essentially complete inhibition of NBMPR-binding activity. Fig. 3 shows the effects of various concentrations (0–25 mM) of uridine on high-affinity binding of [^3H]NBMPR to rat liver plasma membranes, plotted as the reciprocal of specific bound [^3H]NBMPR versus the reciprocal of the equilibrium free concentration of ^3H -labelled ligand. Uridine was an apparent competitive inhibitor of NBMPR binding, with an apparent K_i value of 1.5 mM. The linearity of the replot of apparent K_d versus uridine concentration (inset to Fig. 3) provides further evidence of simple competitive inhibition of binding activity.

Fig. 3. Effect of uridine on [^3H]nitrobenzylthioinosine binding to rat liver plasma membranes

The reciprocals of high-affinity (NBTGR-sensitive) [^3H]NBMPR bound are plotted against the respective reciprocals of the free equilibrium concentrations of [^3H]NBMPR in the medium. Concentrations of uridine were: ●, 0 mM; ○, 2.5 mM; ■, 12.5 mM; □, 25 mM. The apparent K_i value was 1.5 mM

The properties of [^3H]NBMPR binding to rat and guinea-pig liver membranes were investigated further by studying the effects of dilazep and dipyridamole on high-affinity binding activity in the two species. Fig. 2 shows that dilazep was a

Fig. 4. Photoaffinity labelling of rat and guinea-pig liver plasma membranes and human erythrocyte 'ghosts' with $[^3\text{H}]$ nitrobenzylthioinosine under equilibrium binding conditions

Membranes were equilibrated with $[^3\text{H}]\text{NBMPR}$ (75 nM for human erythrocyte 'ghosts'; 50 nM and 25 nM for guinea-pig and rat liver membranes respectively), with or without 20 μM -NBTGR as competing ligand, and exposed to u.v. light for 45 s in the presence of 50 mM-dithiothreitol, a free-radical scavenger added to minimize non-specific labelling. Samples (0.3 and 0.75 mg of protein for human erythrocyte and liver membranes respectively) were dissolved in gel sample buffer and subjected to SDS/polyacrylamide-gel electrophoresis as described in the text. (a), Comparison of rat liver

very effective inhibitor of high-affinity NBMPR binding to liver plasma membranes. NBMPR binding activity was at least 1000-fold more sensitive to dilazep than to adenosine, Dilazep IC_{50} values (2.5 nM-NBMPR) were 1.2 μM for rat membranes and less than 0.1 μM for guinea-pig membranes. Interestingly, guinea-pig liver membranes were considerably more sensitive to dipyridamole inhibition than were rat membranes. The IC_{50} value for inhibition of NBMPR binding to guinea-pig membranes was 0.2 μM , and binding was inhibited completely when the concentration of dipyridamole was increased to 20 μM . In contrast, the IC_{50} value for rat membranes was $>100 \mu\text{M}$.

Photoaffinity-labelling experiments

In our previous studies with erythrocyte membranes, we established that exposure of membranes to high-intensity u.v. light in the presence of $[^3\text{H}]\text{NBMPR}$ resulted in substantial covalent radiolabelling of the nucleoside transporter, a band-4.5 polypeptide with an apparent M_r on SDS/polyacrylamide gels of 45 000–66 000 (Young *et al.*, 1983; Wu *et al.*, 1983a,b). In the present series of experiments, we investigated photolysis of $[^3\text{H}]\text{NBMPR}$ bound to rat and guinea-pig liver membrane preparations, with human erythrocyte 'ghosts' as a control (Fig. 4). In agreement with our previous results, the electrophoretograms of human erythrocyte membranes exhibited a major broad peak in the band-4.5 region of the gel. Minor high- and low- M_r peaks were also apparent. Covalent labelling of membrane protein was abolished when photolysis was performed in the presence of NBTGR (results not shown). The electrophoretograms of rat and guinea-pig liver membranes also exhibited symmetrical peaks of radioactivity in the band-4.5 region of the gels, with small amounts of labelling in higher- and lower- M_r ranges. The guinea-pig membrane peak coincided with that of the human erythrocyte transporter, whereas the rat membrane peak migrated with a slightly higher apparent M_r . In both cases, radiolabelling was abolished by NBTGR. The total radioactivity detected in the band-4.5 peaks, corrected for NBTGR-insensitive labelling, was about 4-fold higher for guinea-pig membranes than for rat membranes, a result consistent with their difference in B_{max} values. We calculate that

and human erythrocyte membranes; (b), comparison of guinea-pig liver and human erythrocyte membranes. ^3H profiles and positions of M_r standards are from the same slab gels. Symbols: $\bullet$, rat or guinea-pig liver membranes; $\square$, human erythrocyte 'ghosts'; $\circ$, photolysis of rat or guinea-pig liver membranes in the presence of NBTGR.

approx. 10% of the high-affinity binding sites present in the two liver membrane preparations were covalently labelled under the present experimental conditions. All samples exhibited non-specific labelling in the lipid region of the gel.

Discussion

Purified plasma membranes from rat and guinea-pig liver were prepared for the present study on self-generating Percoll density gradients by a modification of the method of Belsham *et al.* (1980) for the isolation of adipocyte plasma membranes. The use of Percoll self-generating gradients for the preparation of liver membranes is simpler and more rapid than conventional methods with sucrose density gradients and gives a plasma-membrane fraction of comparable purity and yield (see, e.g., Jones *et al.*, 1973; Van Amelsvoort *et al.*, 1978; Hubbard *et al.*, 1983). Liver plasma membranes prepared by the present method exhibited a 10–15-fold higher NBMPR-binding activity than in crude liver homogenates, and the recovery of high-affinity NBMPR binding activity in the plasma-membrane fraction was broadly similar to that obtained with the plasma-membrane marker 5'-nucleotidase. These results are consistent with a role of NBMPR-binding proteins in cellular nucleoside transport. $B_{\max}$ values for high-affinity NBMPR binding to rat and guinea-pig liver membranes were approx. 4 and 15 pmol/mg of protein respectively, compared with approx. 1 and 0.1 pmol/mg of protein for rat and guinea-pig erythrocyte membranes respectively (Jarvis *et al.*, 1982b). Rat and guinea-pig liver membranes therefore seem to have a higher density of NBMPR-binding sites than do erythrocyte membranes from the two species.

As predicted from studies of nucleoside transporters in other cell types, high-affinity NBMPR binding to liver plasma membranes was inhibited by adenosine, uridine, dilazep and dipyridamole. Adenosine was a more effective inhibitor of NBMPR binding than was uridine, a result consistent with the known relative affinities of these two nucleosides for the transporter in other systems, adenosine usually having the lower apparent K_m (Young, 1978; Lum *et al.*, 1979; Jarvis *et al.*, 1980, 1983; Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1981; Harley *et al.*, 1982). We were also able to demonstrate that uridine inhibition of NBMPR binding to rat liver membranes was competitive. The measured apparent K_i value of 1.5 mM is similar to that reported previously for uridine inhibition of NBMPR binding to human and nucleoside-permeable sheep erythrocyte membranes (1.25 and 0.9 mM respec-

tively) (Jarvis *et al.*, 1982a) and is in the same range as the half-saturation constant for NBMPR-sensitive cytidine uptake by isolated perfused mouse liver (Kolassa & Paterson, 1982).

As shown in Fig. 2, NBMPR binding to rat and guinea-pig liver membranes was equally susceptible to nucleoside inhibition. In contrast, there was a large species difference in dipyridamole sensitivity (IC_{50} values of 0.2 and $>100 \mu\text{M}$ for guinea-pig and rat membranes respectively), correlating with previous studies, which have shown that nucleoside uptake by rat erythrocytes and other rat tissues is relatively insensitive to dipyridamole inhibition (Hopkins & Goldie, 1971; Kolassa *et al.*, 1971; Kolassa & Pfeleger, 1975; Williams *et al.*, 1984). We have also found a similar species difference with respect to dipyridamole inhibition of NBMPR binding to rat and guinea-pig lung membranes (Shi *et al.*, 1984). Interestingly, NBMPR binding to rat liver membranes was also less susceptible to dilazep inhibition than was NBMPR binding to guinea-pig liver membranes.

These studies establish that purified plasma membranes from rat and guinea-pig liver exhibit substantial high-affinity NBMPR-binding activity and that this binding has the properties expected of NBMPR binding to nucleoside transporter proteins. Using human erythrocyte 'ghosts', we have developed photolysis conditions which permit the selective photoaffinity labelling of the erythrocyte nucleoside transporter by [^3H]NBMPR (Jarvis *et al.*, 1983; Wu *et al.*, 1983a,b; Young *et al.*, 1983). In the present series of experiments with rat and guinea-pig liver membranes, exposure of site-bound [^3H]NBMPR to high-intensity u.v. light under equilibrium binding conditions also resulted in substantial radiolabelling of plasma-membrane protein. For both species, most of the radiolabelled protein migrated on SDS/polyacrylamide gels as symmetrical peaks with apparent M_r values in the same range as for the human erythrocyte nucleoside transporter (45 000–66 000). Significant species differences were, however, apparent. The rat liver radioactive peak had a slightly higher apparent M_r . No labelling of membrane protein occurred when photolysis was performed in the presence of NBTGR as competing ligand. It is likely that the minor high- and low- M_r peaks correspond to aggregates of the transporter and degradation products respectively.

We therefore conclude from these studies that NBMPR-sensitive nucleoside transporters are present in plasma membranes isolated from rat and guinea-pig liver and that the transporters have broadly similar apparent M_r values to that of the nucleoside carrier present in human erythrocytes. We expect that photoaffinity-labelling studies with [^3H]NBMPR will find widespread application in

studies of the molecular properties of the nucleoside transporters in these and other systems.

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