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Binding of nitrobenzylthioinosine to high-affinity sites on the nucleoside-transport mechanism of HeLa cells

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Nitrobenzylthioinosine (NBMPR) binds reversibly, but with high affinity (K_d 0.1–1.2 nM), to inhibitory sites on nucleoside-transport elements of the plasma membrane in a variety of animal cells. The present study explored relationships in HeLa cells between NBMPR binding and inhibition of uridine transport. The K_m value for inward transport of uridine by HeLa cells in both suspension and monolayer culture was about 0.1 mM. The affinity of the transport-inhibitory sites for uridine (K_d 1.7 mM), inosine (K_d 0.4 mM) and other nucleoside permeants was low relative to that for NBMPR. The pyrimidine homologue of NBMPR, nitrobenzylthiouridine, also exhibited low affinity for the NBMPR-binding sites. Pretreatment of HeLa cells with *p*-chloromercuribenzene sulphonate (*p*-CMBS) or *N*-ethylmaleimide (NEM) decreased binding of NBMPR to its high-affinity sites and inhibited uridine transport, indicating the presence of thiol groups essential to both processes. NEM, a more penetrable reagent than *p*-CMBS, inhibited binding and transport at much lower concentrations than the latter compound. Pretreatment of cells with concentrations of *p*-CMBS that alone had no effect on either NBMPR binding or uridine transport increased the sensitivity of transport to NBMPR inhibition and changed the shape of the NBMPR concentration-effect curve, suggesting synergistic inhibition of uridine-transport activity by these two agents.

NBMPR and related S^6-derivatives of 6-thiopurine nucleosides (Paterson *et al.*, 1977*a*,*b*) are potent inhibitors of nucleoside transport in animal cells [for example, in erythrocytes (Paterson & Oliver, 1971; Cass & Paterson, 1972), and in various types of cultured cells (Paterson *et al.*, 1977*a*,*b*; Eilam & Cabantchik, 1977; Rozengurt *et al.*, 1977; Wohlhueter *et al.*, 1978)]. The interaction of NBMPR with transport elements of the plasma membrane was established in studies of NBMPR inhibition of the nucleoside-transport mechanism in human erythrocytes (Cass *et al.*, 1974; Cass & Paterson, 1976). These studies identified a set of membrane-binding sites (about 10^4 per erythocyte) that exhibited high affinity for NBMPR (K_d of about 1 nM). Fractional occupancy of these sites was proportional to inhibition of exchange diffusion of uridine (Cass *et al.*, 1974). A number of nucleoside permeants, including uridine and thymidine, decreased binding of NBMPR to the inhibitory sites on erythrocytes, and exhibited K_d values at these sites similar to their K_m values for transport. However, the apparent failure of deoxycytidine, a substrate for the nucleoside transporter of human erythrocytes (Cass & Paterson, 1972), to compete with NBMPR for binding to the inhibitory sites suggested that permeant and inhibitor may interact with the transporter at separate, but functionally related, sites (Cass & Paterson, 1976). Jarvis & Young (1978, 1980) have reported genetic evidence that high-affinity binding of NBMPR represents interaction with functional nucleoside-transport sites. In nucleoside-impermeable sheep erythrocytes, site-specific binding of NBMPR was not detected, whereas such binding (K_d about 0.5 nM) was demonstrable with erythrocytes from related sheep in which an allelomorphic gene controlling nucleoside transporter function was expressed. In contrast with our previous results (Cass & Paterson, 1976), Jarvis & Young (1980) found that deoxycytidine inhibits binding of NBMPR to nucleoside-permeable sheep erythrocytes and to human erythrocytes

Abbreviations used: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NEM, *N*-ethylmaleimide; *p*-CMBS, *p*-chloromercuribenzene sulphonate.

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and suggested that permeant and inhibitor molecules bind to the same site on the nucleoside transporter.

In HeLa cells, the relationship between binding of NBMPR and inhibition of transport appears to be more complex than in erythrocytes, despite similarities exhibited by the two cell types in structural requirements for transport inhibitor activity (Paul *et al.*, 1975; Paterson *et al.*, 1977*a*,*b*). HeLa cells possess high-affinity sites, about 10^5 per cell, at which the K_d for bound NBMPR is 0.1–1 nM (Lauzon & Paterson, 1977; Cass *et al.*, 1979). Occupancy of the high-affinity sites on HeLa cells by NBMPR inhibits transport, however, as saturation of these sites is approached (at about 5 nM), substantial transport activity remains (25–30%), and increases of 1000-fold in concentrations of NBMPR are required to eliminate the remaining transport activity (Lauzon & Paterson, 1977). Concentration–effect curves for inhibition of uridine and adenosine transport are biphasic, raising the possibility of interaction of NBMPR with two or more classes of transport-inhibitory sites (Paterson, 1979; Paterson *et al.*, 1980).

Kinetic studies of NBMPR inhibition of uridine transport in hamster fibroblasts have indicated that interactions between NBMPR and nucleoside-transport mechanisms are also complex in this cell type (Eilam & Bibi, 1977; Eilam & Cabantchik, 1977; Heichal *et al.*, 1978). Inhibition was partially competitive ($K_i = 3.7$ nM), and a substantial fraction of transport activity (20–30%) was insensitive to increasing concentrations of inhibitor. These data, together with results from studies of the effects of organomercurials on NBMPR inhibition of uridine transport, led Eilam & Cabantchik (1977) to propose that binding of NBMPR occurs at transporter sites different from substrate-binding sites. In a subsequent study (Heichal *et al.*, 1978), four distinct classes of sites on the uridine carrier of hamster cells were postulated: a substrate-binding site, an NBMPR-binding site and two types of thiol-containing modifier sites (one stimulatory, the other inhibitory, after binding of organomercurials).

The present study investigated the relationship between (*a*) the binding of NBMPR to high-affinity sites on HeLa cells, and (*b*) the resulting inhibition of uridine transport. The effect of NBMPR on activity of the uridine transporter of HeLa cells was assessed by using a rapid assay procedure that satisfied criteria (Heichal *et al.*, 1979; Koren *et al.*, 1979) for distinguishing transport from metabolism in the uptake process. In the present paper, the term 'transport' refers only to the mediated passage of nucleosides across the plasma membrane. 'Uptake' refers to the total cellular content of a nucleoside permeant and includes both the permeant and metabolites thereof.

Also assessed in the present study was the ability of uridine and other nucleoside permeants, as well as that of nitrobenzylthiouridine {4-[(4-nitrobenzyl)-thio]-1-β-D-ribofuranosylpyrimidin-2-one} to compete with [G-^{3}H]NBMPR for binding to the transport-inhibitory sites. Because thiol groups are involved in nucleoside transport in hamster cells (Eilam & Bibi, 1977; Eilam & Cabantchik, 1977; Heichal *et al.*, 1978), the effects of NEM and the organomercurial, *p*-CMBS, on uridine transport and NBMPR binding were examined to determine if thiol groups were also required for interaction of permeant or inhibitor with the nucleoside-transport mechanism of HeLa cells.

Materials and methods

Cells

HeLa S3 cells were employed as described previously (Paterson *et al.*, 1977*a*,*b*). Cultures were restarted at 6–8 week intervals from frozen stocks and were demonstrated to be free of mycoplasma (Dr. J. A. Robertson, Department of Medical Bacteriology, University of Alberta). Binding and transport studies were conduced with 24-h replicate monolayer cultures containing $(1–2) \times 10^6$ exponentially proliferating cells in 2-oz prescription bottles.

Transport of uridine by monolayer cultures

Uridine-transport rates were measured at 20°C by an initial-rate method using replicate monolayer cultures of HeLa cells, as described previously (Paterson *et al.*, 1977*a*). Time courses of uridine uptake were linear under the conditions used and extrapolated through the uptake value at time zero. Time-zero values of uridine uptake by the monolayer cultures were determined by a 'mock-stop' procedure in which cell sheets without exposure to permeant-containing medium were flooded with 60 ml of ice-cold 0.15 M-NaCl containing 0.35 ml of permeant-containing medium (the average volume of medium remaining in the culture bottles after the aspiration step). Thereafter, the monolayers were processed as other samples. Time-zero values were less than 5% of total cell-associated radioactivity.

Uptake rates were obtained by measurement of cell content of ^{3}H after replicate cultures were exposed for 10- or 50-s intervals to various concentrations of [5-^{3}H]uridine in transport medium. The latter consisted of $NaHCO_3$-free Eagle's minimal essential medium supplemented with 12 mM-NaCl and 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate] at pH 7.4. Uptake intervals were ended as previously specified (Paterson *et al.*, 1977*a*), except that after removal of the cold 0.15 M-NaCl stopping solution, cell sheets were dissolved in 1.5-ml portions of 0.5 M-KOH. The KOH lysates were mixed with 8 ml of a xy-

lene/Triton X-100 scintillant (Pande, 1976) and assayed for ^3H by liquid-scintillation counting. Cell-associated ^3H at time zero was determined as before (Paterson *et al.*, 1977*a*) and was subtracted from ^3H uptake during assay intervals. The differences between rates of uridine uptake in the absence and presence of 5 μM-NBMPR were considered to measure rates of uridine transport. Assays were performed in triplicate except where noted. The water space and the extracellular space in monolayer cultures were determined, respectively, by using ^3H_2O or [^{14}C]inulin in place of radioactive uridine in the uptake assay.

The effects of NBMPR, *p*-CMBS or NEM on uridine transport were determined after incubating replicate cultures at 20°C for 20min in transport medium containing graded concentrations of the appropriate additive. In some cases, cells treated with *p*-CMBS or NEM were washed, incubated for a further 15min at 20°C in transport medium containing dithioerythritol and then assayed for uridine uptake in the absence of additives.

Transport of uridine by suspension cultures

The kinetic constants of uridine transport were determined in suspension cultures of HeLa cells with an uptake assay that allows measurements during intervals as short as 2s. A detailed description of the uptake assay is given elsewhere (Harley *et al.*, 1981). Briefly, 100μl of transport medium containing [5-^{3}H]uridine was layered over 150μl of a silicone oil/paraffin oil mixture (specific gravity, 1.03g/ml) in 1.5-ml polypropylene microcentrifuge tubes. Intervals of nucleoside uptake were started by rapid addition of 100μl of transport medium containing about 2×10^6 cells. Uptake intervals were stopped by addition of 200μl of transport medium containing 20μM-NBMPR, followed immediately by centrifugation for 30s (Eppendorf microcentrifuge, model 5412). Time-zero uptake values were determined with incubation mixtures completed by the addition of cells to 100-μl portions of medium containing both [^{3}H]uridine and 10μM-NBMPR. Immediately after the completion of such mixtures, cells were pelleted. The water space in cell pellets and the extracellular space were determined, respectively, by using ^3H_2O or [^{14}C]sucrose in place of nucleoside permeant in the uptake assay.

After pelleting cells, the supernatant medium was removed from microcentrifuge tubes and 1.4ml of water was added to each as a rinse. After removal of oil and water by aspiration, cell pellets were solubilized in 0.2ml of 5% Triton X-100. Each microcentrifuge tube was then placed in a polyethylene scintillation vial and its contents were mixed with 8ml of the xylene/Triton X-100 scintillant for assay of cellular ^3H content by liquid-scintillation counting.

Binding of NBMPR

The binding of NBMPR to high-affinity sites on HeLa cells was measured as previously described (Paterson *et al.*, 1979). Replicate monolayer cultures were incubated at 20°C for 20min (an interval sufficient to allow equilibration of cell-associated and free NBMPR) in 4ml of transport medium containing graded concentrations of [G-^{3}H]NBMPR (1–7nM) in the presence or absence of an excess (5μM) of the competing ligand, nitrobenzylthioguanosine {2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine}. The latter eliminated site-specific binding of NBMPR and provided a measure of non-specific binding. To end binding intervals, medium was removed by suction and the monolayers were washed with ice-cold 0.15M-NaCl, and cell sheets were then assayed for ^3H content as specified above. Assays were performed in triplicate except where noted.

In assessing the effects of various nucleosides on binding, monolayer cultures were incubated with graded concentrations of [G-^{3}H]NBMPR in transport medium at 20°C for 20min in the presence or absence of a test nucleoside, and an identical set of cultures was incubated with 5μM-nitrobenzylthioguanosine, as described above, to enable quantification of non-specifically-bound NBMPR.

In determining the effects of thiol reagents on the site-specific binding of NBMPR, cultures were first exposed to 4ml of transport medium containing graded concentrations of *p*-CMBS or NEM at 20°C or 37°C for 20min, a time interval sufficient to achieve maximal inhibitions under the conditions of the experiments reported here. Cultures were then (*a*) assayed for binding of NBMPR at 20°C in the absence of additives, or (*b*) washed, incubated for 15min at 20°C in transport medium containing dithioerythritol and assayed for binding of NBMPR in the absence of additives.

Materials

NBMPR and nitrobenzylthioguanosine were prepared (Paul *et al.*, 1975) from 6-thioinosine and 6-thioguanosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Nitrobenzylthiouridine was synthesized by the method of Sato & Kanaoka (1971). [G-^{3}H]NBMPR and [5-^{3}H]uridine were obtained from Moravek Biochemicals, Brea, CA, U.S.A. [*carboxy*-^{14}C]Inulin and [U-^{14}C]sucrose were purchased from ICN Pharmaceuticals Inc., Irvine, CA, U.S.A., and ^3H_2O was purchased from NEN Canada, Lachine, Que., Canada. NEM, *p*-CMBS and dithioerythritol were obtained from Sigma, St. Louis, MO, U.S.A. Cell-culture materials were purchased from GIBCO (Canada), Burlington, Ont., Canada.

Results and discussion

On entry into animal cells, the physiological nucleosides and many nucleoside analogues are metabolized, principally to phosphate esters. Such metabolic transformations remove influent nucleoside molecules from transport equilibria. Although time courses of nucleoside uptake may be complex because the uptake process involves a number of steps, the initial rate will be that of the first step, permeation. It has become apparent that rapid-sampling technologies are required to define the time course of nucleoside uptake under circumstances in which nucleoside entry rates exceed those of permeant transformation and trapping. Criteria for the use of uptake rates to measure transport rates have been discussed (Heichal *et al.*, 1979; Koren *et al.*, 1979), and the assay procedures used here for determination of uridine uptake rates in both monolayer and suspension cultures meet those criteria.

Initial rates of uridine uptake by suspended cells were determined by a rapid sampling method in which transport was stopped instantaneously by addition of NBMPR, followed by rapid centrifugal pelleting of cells under a layer of oil (Harley *et al.*, 1981). This method provided definitive time courses of uptake over a broad range of uridine concentrations and enabled characterization of kinetic parameters of uridine transport by HeLa cells. Results from two such experiments indicated that uridine transport was saturable with K_m and $V_{max.}$ values (±S.E.M.) respectively of $108 \pm 11\,\mu$M and 7.5 ± 0.5 pmol/μl of pellet water per s (data of Fig. 1), and $98 \pm 23\,\mu$M and 13 ± 2.2 pmol/μl of pellet water per s (results not shown). Plagemann *et al.*, (1978) obtained K_m and $V_{max.}$ values respectively of $84 \pm 29\,\mu$M and 7.5 ± 0.7 pmol/μl of cell water per s in studies of uridine transport at 25°C by ATP-depleted HeLa cells in which uptake was terminated by centrifugal pelleting of cells under a layer of oil.

Fig. 2 presents an example of time courses of uridine uptake by monolayer cultures. Such time courses were linear and extrapolated through values for cellular uptake of permeant at zero time. The latter were determined experimentally and apparently represent residual extracellular uridine in the monolayer cultures. Back extrapolation of time courses obtained for the NBMPR-insensitive uptake of uridine also passed through the zero-time uptake values.

In most of the work presented here, rates of uridine transport were estimated from uptake of uridine during the interval 0–10 s (determination of uptake during shorter intervals was not possible with the monolayer assay). Uptake rates have been expressed as pmol/10^6 cells per s rather than pmol/μl of pellet water per s because the extracellular and intracellular water spaces of monolayer cultures could not be determined with sufficient precision. In two experiments the total water content (±S.E.M.) was $2.9 \pm 0.5\,\mu$l and $3.6 \pm 0.6\,\mu$l, and the inulin space was about 55% and 75% of total water.

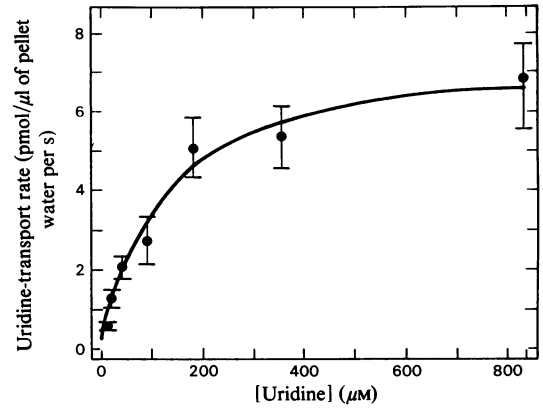

Fig. 1. *Kinetics of uridine transport by suspension cultures of HeLa cells*
Initial velocities of transport (and their standard errors) were estimated as described in the Materials and methods section from time courses (from 2 to 20 s) of uptake of [5-^{3}H]uridine at the concentrations indicated. Kinetic constants were obtained from weighted non-linear least-squares regression using the algorithm suggested by Cleland (1967) for analysis of the Michaelis–Menten equation. These values (±S.E.M.) were: K_m, $108 \pm 11\,\mu$M; $V_{max.}$, 7.5 ± 0.5 pmol/μl of pellet water per s.

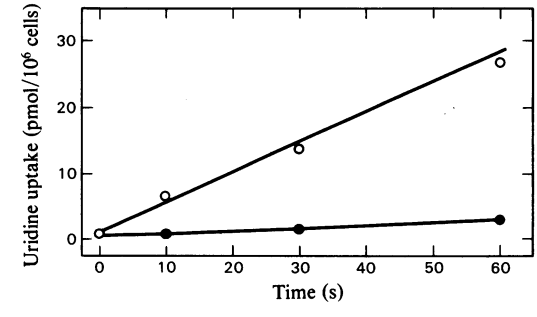

Fig. 2. *Inhibition of uridine transport by NBMPR*
Before assays for uridine uptake, replicate monolayer cultures of HeLa cells (1.8×10^6 cells/bottle) were incubated for 20 min in transport medium without or with NBMPR (5 μM). Time courses of total uptake (○) and NBMPR-insensitive uptake (●) from medium containing 4 μM-[5-^{3}H]uridine are shown.

The kinetic parameters for the inward transport of uridine by monolayer cultures were estimated from experiments in which uptake rates during intervals of 0–10s were measured at uridine concentrations ranging from 0.4 to 1660μM (results not shown). These values (±S.E.M.) were: K_m, 100 ± 21μM; $V_{max.}$, 11 ± 1.9pmol/10^6 cells per s. Although the monolayer assay for uridine uptake is less precise than the suspension assay at uridine concentrations near or above the K_m value, the close agreement between the K_m values obtained with both procedures indicated that the monolayer assay provided a valid measure of transport rates, particularly at concentrations well below the K_m value. The previously reported K_m value of 4μM (Paterson *et al.*, 1977*a*) was incorrect, apparently because the concentration range employed (1–30μM) was too narrow for characterization of a saturable process with a K_m value near 100μM.

The ability of NBMPR to inhibit uridine transport over a broad range of concentrations (0.04–400μM) is illustrated in Table 1. Because the binding of NBMPR at concentrations below saturation of the high-affinity sites was slow, HeLa cells were first pre-incubated with NBMPR under conditions that minimized depletion of free NBMPR during the binding reaction and then assayed for uridine-transport activity. At all concentrations of uridine tested, uptake was partially (50–60%) inhibited by concentrations of NBMPR from 1 to 10nM. However, uptake was insensitive to further increases in NBMPR concentration until the 1–10μM range was reached. It is noteworthy that the inhibitions of uridine uptake at low NBMPR concentrations correlated with the occupancy of the high-affinity binding sites for NBMPR, which, in suspended HeLa cells, are saturated at 5nM-NBMPR (Lauzon & Paterson, 1977). The low sensitivity to NBMPR of a substantial fraction of uridine permeation is not yet understood. Uridine entry by a second transport system of low NBMPR sensitivity is a possibility. It is also possible that the NBMPR-binding-site–transporter interactions are complex; for example, if nucleoside-transporter elements possess more than one NBMPR-binding site, the first NBMPR molecule bound may influence the binding (or effect) of successively bound molecules of NBMPR.

To determine if uridine and other nucleoside permeants compete with NBMPR for binding at the high-affinity sites, binding studies of the type illustrated in Fig. 3 were undertaken. Concentrations of 0.3–8.0mM decreased binding of NBMPR by 50%, indicating relatively weak interactions between these nucleoside permeants and the NBMPR-specific sites.

In the experiments of Fig. 4, which further characterized permeant inhibition of NBMPR binding, monolayer cultures of HeLa cells were equilibrated with graded, non-saturating concentrations of [G-^{3}H]NBMPR in the presence and absence of uridine (2, 4 or 8mM), or of inosine (0.5 or 2mM). Mass-law analysis of the relationship between cell-bound NBMPR and the medium content of NBMPR, as seen in the Scatchard plots of Fig. 4, indicated that both uridine and inosine competitively inhibited the high-affinity binding of NBMPR. Hanes–Woolf and reciprocal plots also demonstrated apparent competitive inhibition. The K_d

Table 1. *Concentration–effect relationships for NBMPR inhibition of uridine uptake by HeLa cells*

Replicate monolayer cultures of HeLa cells (1.2×10^6 cells/bottle) were used for assay of uptake of [5-^{3}H]uridine. Because cellular binding of NBMPR under the experimental conditions used decreased the medium content of NBMPR at low NBMPR concentrations, the following scheme was employed. Each culture was pre-incubated for 20min in 6ml of transport medium containing NBMPR at the concentrations indicated. NBMPR-containing transport medium from replicate cultures was then removed and pooled. Uridine was added to the latter to obtain solutions of the indicated uridine concentration, and uridine uptake from these solutions (4ml/culture) was measured during 10-s intervals. The NBMPR-insensitive component of uridine uptake has not been subtracted from uptake values reported here.

	Rates of uridine uptake from medium containing uridine (% of control rates)*				
[NBMPR] (μM) [Uridine] (μM) ...	0.04	0.4	4	40	400
0	100	100	100	100	100
0.001	39	45	46	78	73
0.01	31	33	35	35	54
0.1	21	27	25	26	28
1.0	7	15	25	25	22
10.0	0	0	6	6	11

* Control rates of uridine uptake (pmol/min per 10^6 cells) were: 0.04μM, 0.26; 0.4μM, 1.87; 4μM, 36.5; 40μM, 265; 400μM, 674.

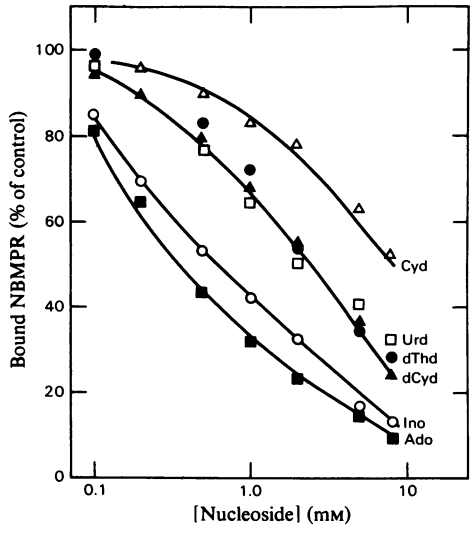

Fig. 3. *Effect of nucleoside permeants on high-affinity binding of NBMPR to HeLa cells*

Replicate monolayer cultures of HeLa cells were incubated for 20min with transport medium containing [G-^{3}H]NBMPR (1nM) and nucleosides at the concentrations indicated. In the six experiments shown, cultures contained (1–2) $\times 10^6$ cells/bottle, and the amounts of NBMPR bound in the absence of test nucleosides (controls) ranged from 0.31 to 0.42pmol/10^6 cells.

Table 2. *Inhibition of high-affinity binding of NBMPR to HeLa cells by nitrobenzylthiouridine*

Monolayer cultures of HeLa cells (1.2×10^6 cells/bottle) were incubated for 20min at 20°C in transport medium containing 1nM-[G-^{3}H]NBMPR in the absence or presence of nitrobenzylthiouridine at the concentrations indicated.

[Nitrobenzylthiouridine] (μM)	Bound NBMPR (pmol/10^6 cells)	Binding (% of control value)
0	0.74	100
4.5	0.63	85
9	0.48	65
13.5	0.43	58
18	0.39	52
27	0.34	46
36	0.31	41
45	0.27	36

values (means from Fig. 4 data) for uridine and inosine respectively were 1.7 and 0.4mM. Thus physiological nucleosides interacted with the transport-inhibitory sites, but with relatively low affinity.

We reported previously that the uridine homologue of NBMPR was a less effective inhibitor of uridine transport than NBMPR, nitrobenzylthioguanosine and certain other purine nucleoside homologues of NBMPR (Paterson *et al.*, 1977*a*). In those experiments, permeant and inhibitor (at several different concentrations) were presented simultaneously to cells, and concentrations of inhibitor that decreased rates of nucleoside uptake to 50% of rates in the absence of inhibitor were calculated (IC_{50} values). Values of 0.06, 0.1 and 40μM were obtained respectively for inhibition by NBMPR, nitrobenzylthioguanosine and nitrobenzylthiouridine of uridine uptake (4μM) by monolayer cultures of HeLa cells (Paterson *et al.*, 1977*a*). The ability of graded concentrations of nitrobenzylthiouridine to inhibit binding of NBMPR to its high-affinity sites was also tested (Table 2). Nitrobenzylthiouridine decreased NBMPR binding with an IC_{50} value of about 20μM, a value similar to its IC_{50} for inhibition of uridine transport. The low affinity of the transport-inhibitory sites for nitrobenzylthiouridine, relative to that for NBMPR, suggested that these sites are 'purine-specific'.

Recent studies with hamster fibroblasts have demonstrated involvement of thiol groups in nucleoside-transporter activity and in inhibition of that activity by NBMPR (Eilam & Bibi, 1977; Eilam & Cabantchik, 1977; Heichal *et al.*, 1978). To detect thiol groups required for nucleoside transport in HeLa cells, the effects of *p*-CMBS and NEM on uridine transport and high-affinity binding of NBMPR were assessed. Because NEM evidently enters cells more readily than *p*-CMBS (Rothstein, 1970), differences in the sensitivity of either process to the two reagents may indicate whether reactive thiol groups are superficial or on the interior aspect of the plasma membrane.

The limited ability of the poorly penetrable *p*-CMBS to inhibit transport and binding is shown in Table 3. The concentration–effect relationships for *p*-CMBS inactivation of the two processes were similar (Expts. 1 and 2), with transport exhibiting somewhat greater sensitivity than binding. *p*-CMBS inhibition of transport and binding was readily reversed by treatment of cells with the reducing agent dithioerythritol, indicating that the inhibition was due to interaction of *p*-CMBS with thiol groups required for both transport and binding. The inhibitory effect of *p*-CMBS on NBMPR binding was enhanced by conducting the *p*-CMBS treatment at 37°C.

We have reported (Paterson *et al.*, 1980) that NBMPR inhibition of uridine transport in HeLa cells is characterized by a biphasic concentration–effect curve, suggesting (among several possibilities) that NBMPR may interact with more than one class of transport-inhibitory sites. In assessing the combined

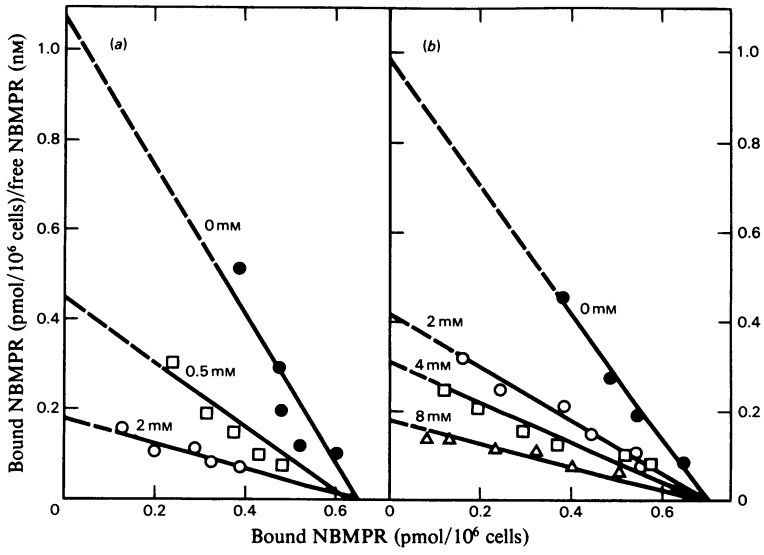

Fig. 4. *Inhibition of high-affinity binding of NBMPR by uridine (b) and inosine (a)*
Replicate monolayer cultures of HeLa cells were incubated for 20 min with [G-^{3}H]NBMPR in the presence and absence of competing nucleosides with or without nitrobenzylthioguanosine (5 μM). Displacement of [G-^{3}H]NBMPR by the latter provided a measure of non-specific binding to the high-affinity sites. Results reported are for specific binding of NBMPR. (*a*) Scatchard plots for inosine inhibition of NBMPR binding. Cultures (1.7×10^6 cells/bottle) were incubated with 1–7 nM-[G-^{3}H]NBMPR in the absence (●) and presence of 0.5 mM (□) and 2 mM-inosine (○). The K_d value for NBMPR in the absence of inosine was 0.6 nM. Individual K_d values for inosine at 0.5 and 2 mM respectively were 0.3 and 0.4 mM. (*b*) Scatchard plots for uridine inhibition of NBMPR binding. Cultures (1.5×10^6 cells/bottle) were incubated with 0.5–7 nM-[G-^{3}H]NBMPR in the absence (●) or in the presence of 2 mM-(○), 4 mM- (□) and 8 mM-uridine (△). The K_d value for NBMPR in the absence of uridine was 0.7 nM. Individual K_d values for uridine at 2, 4 and 8 mM respectively were 1.5, 1.8 and 1.8 mM.

Table 3. *The effect of p-CMBS on uridine transport and high-affinity binding of NBMPR*
Replicate monolayer cultures of HeLa cells were pretreated in duplicate for 20 min at 20°C (Expts. 1 and 2) or at 37°C (Expt. 3) in transport medium containing *p*-CMBS at the concentrations indicated. Cultures were then (*a*) assayed at 20°C for transport or binding in the presence of *p*-CMBS, or (*b*) washed with transport medium, incubated in transport medium containing 1 mM-dithioerythritol (15 min at 20°C) and then assayed for transport or binding in the absence of dithioerythritol or *p*-CMBS. In Expt. 1, uridine-transport rates were calculated from uptake of radioactivity after exposure of cells (1.6×10^6/bottle) for 10 s to 4 μM-[5-^{3}H]uridine. In Expt. 2, binding was determined after exposure (1.2×10^6 cells/bottle) for 20 min to 1 nM-[G-^{3}H]NBMPR and in Expt. 3, after exposure (1.4×10^6 cells/bottle) to 1 nM-[G-^{3}H]NBMPR. Not shown are results from the portions of these experiments in which cultures were treated with *p*-CMBS together with dithioerythritol; the values obtained for transport rates and bound NBMPR were similar to those presented below for untreated control cultures, indicating complete reversal of *p*-CMBS effects by treatment with dithioerythritol.

	Uridine transport at 20°C (Expt. 1)		Site-bound NBMPR at 20°C (Expt. 2)		Site-bound NBMPR at 37°C (Expt. 3)	
[*p*-CMBS] (mM)	Rate (pmol/min per 10^6 cells)	(% of control vlaue)	Bound (pmol/ 10^6 cells)	(% of control value)	Bound (pmol/ 10^6 cells)	(% of control value)
0	30	100	0.57	100	0.49	100
0.1	29	96	0.54	95	0.44	90
0.2	27	90	0.53	93	0.42	85
0.3	17	86				
0.4			0.52	91	0.36	73
0.5	24	80				
0.6			0.49	86	0.24	49
0.8			0.45	79	0.17	34
1.0	16	53	0.45	79	0.11	22

effects of *p*-CMBS and NBMPR on uridine transport, it became apparent that *p*-CMBS pretreatment altered the response of HeLa cells to NBMPR (Fig. 5). Pretreatment of cells with 100 μM-*p*-CMBS, which alone was without significant effect on transport (see inset, Fig. 5), increased the sensitivity of the transport mechanism to NBMPR and changed the shape of the complex concentration–effect curve. These pretreatment conditions were also without effect on high-affinity binding of NBMPR (Table 3, Expt. 2), suggesting that the altered sensitivity to NBMPR inhibition resulted from *p*-CMBS interaction with thiol groups that are not required for site-specific, high-affinity binding of NBMPR.

The effects of pretreating cells at 4°C and 20°C with the more penetrable thiol reagent, NEM, on uridine transport and NBMPR binding are illustrated in Fig. 6. The two processes exhibited similar sensitivities to NEM inhibition, with IC_{50} values of 20–50 μM-NEM, suggesting that thiol groups may be involved in both processes. Uridine transport (Fig. 6*a*) was virtually eliminated by pretreatment at 4°C or 20°C with 160 μM-NEM. NBMPR binding (Fig. 6*b*) exhibited dose–response curves similar to those observed for transport, except that complete inhibition was not achieved at the higher concentrations of NEM, and binding was somewhat more sensitive to NEM at 4°C than at 20°C. The dose–response curve for inactivation of binding by exposure to NEM at 37°C in an experiment similar to that of Fig. 6(*b*) was almost identical with the dose–response curve obtained at 20°C, indicating relatively little dependence on temperature.

Experiments (not presented) similar to those of Fig. 6 were conducted with dithioerythritol to show that NEM effects were due to reaction with thiol groups. When dithioerythritol (1 mM) was present together with NEM during pretreatment of cells, binding of NBMPR was unaffected, indicating complete protection by addition of the reducing agent.

The experiment of Table 4 represents an attempt to determine whether the presence of uridine would protect against NEM inactivation of thiol groups

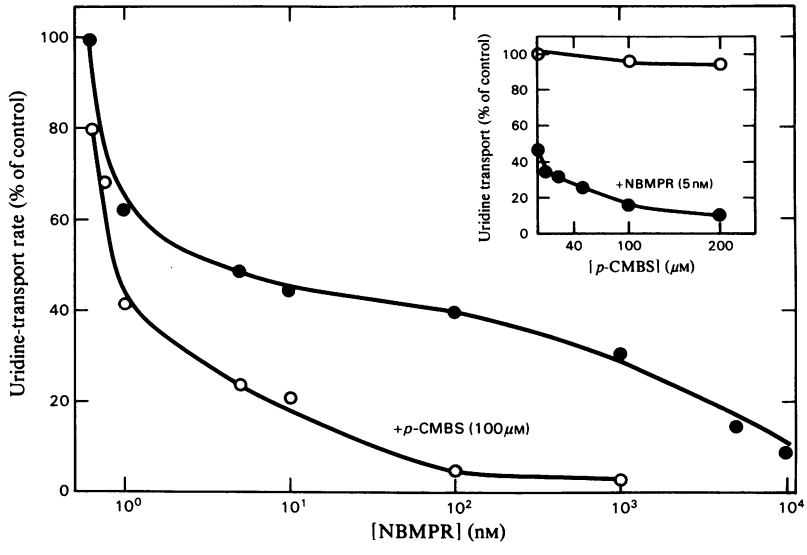

Fig. 5. *Synergism between p-CMBS and NBMPR in the inhibition of uridine transport*

Replicate monolayer cultures of HeLa cells were incubated for 20 min at 20°C with transport medium containing NBMPR with (○) or without (●) *p*-CMBS (100 μM) at the concentrations indicated. After this treatment, a portion of the medium from each incubation mixture was combined with [5-^{3}H]uridine (final concentration 4 μM) alone or together with NBMPR (final concentration 5 μM). Using these uridine-containing solutions, uridine uptake was assayed (uptake interval of 10 s) by the procedure described in the Materials and methods section. Transport rates (total uptake less NBMPR-insensitive uptake) are expressed as percentages of control rates. The concentration–effect relationship for NBMPR inhibition of uridine transport in the presence and absence of 100 μM-*p*-CMBS is shown. The inset illustrates the concentration–effect relationship of *p*-CMBS inhibition of uridine transport in the presence (●) and absence (○) of 5 nM-NBMPR. The results are averages from several separate experiments; control transport rates ranged from 14 to 32 pmol/min per 10^6 cells, and replicate cultures contained (1.0–1.9) × 10^6 cells/bottle.

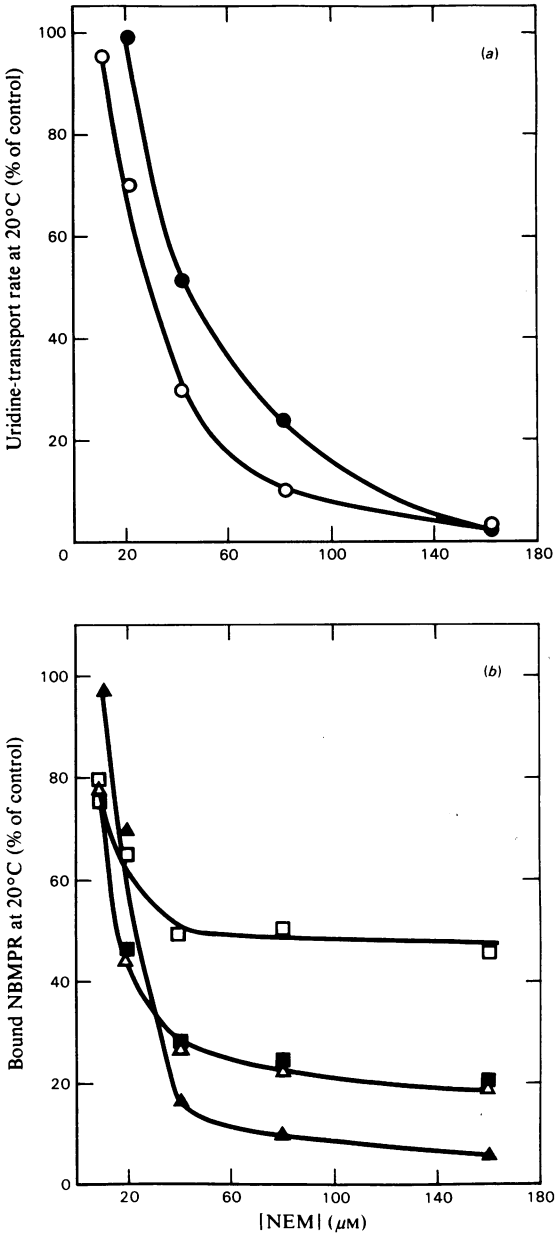

Fig. 6. *The effects of NEM on uridine transport and high-affinity binding of NBMPR*

Replicate monolayer cultures of HeLa cells were pretreated for 20min at 4°C (●, ▲ and ■) or 20°C (○, △ and □) in transport medium containing NEM at the concentrations indicated. Cultures were then washed with transport medium and assayed in the absence of NEM at 20°C for transport of 4μM-uridine (*a*) or binding of [G-^{3}H]NBMPR (*b*), at 1nM (△ and ▲) or at 5nM (□ and ■) as described in Table 3. In the several experiments presented, cultures contained from (1.3–1.9) x 10^6 cells/bottle. Control rates for uridine transport were 4 and 27pmol/min per 10^6 cells after NEM treatment at 4°C and 20°C respectively. Control values for NBMPR binding at 1nM were 0.30 and 0.57pmol/10^6 cells at 4°C and 20°C respectively. When the NBMPR concentration was 5nM, the corresponding values were 0.56 and 0.87pmol/10^6 cells.

Table 4. *Failure of uridine to protect against NEM-induced inhibition of high-affinity binding of NBMPR*

Monolayer cultures of HeLa cells (1.2 x 10^6/bottle) were pretreated for 20min at 20°C in transport medium containing graded concentrations of NEM in the presence and absence of 100μM-uridine before assay of binding of 5nM-[G-^{3}H]NBMPR. Results are expressed as percentages of control binding for cells pretreated in the absence of either NEM or uridine (0.71pmol/10^6 cells).

	NBMPR binding (% of control value)	
[NEM] (μM)	Without uridine	With uridine
20	88	97
80	64	60
120	56	49
160	49	45

required for binding of NBMPR. Uridine did not alter NEM inhibition of NBMPR binding, a result consistent with the idea that different NEM-sensitive thiol groups are involved in transport and binding.

The experiments of Table 5 were undertaken to determine the extent to which *p*-CMBS or NEM inhibition of binding of NBMPR was influenced by the presence of NBMPR during the inactivation reaction. At each concentration of *p*-CMBS or NEM tested, the inhibitions by the latter of binding were markedly dependent on NBMPR concentration, suggesting that binding of NBMPR protected sensitive thiol groups from reaction with *p*-CMBS or NEM. Although it was not possible from such experiments to distinguish between true substrate protection of thiol groups associated with NBMPR binding sites and indirect protection resulting from NBMPR-induced conformational changes in the NBMPR binding protein(s), these results suggested the presence of thiol groups in the vicinity of the NBMPR-binding sites.

The effects of pretreating cells with either *p*-CMBS or NEM on the binding of NBMPR to the high-affinity sites were complex, as is evident from the Scatchard plots presented in Fig. 7. Inactivation of thiol groups after exposure to relatively low concentrations of NEM (20 or 30μM) and to much

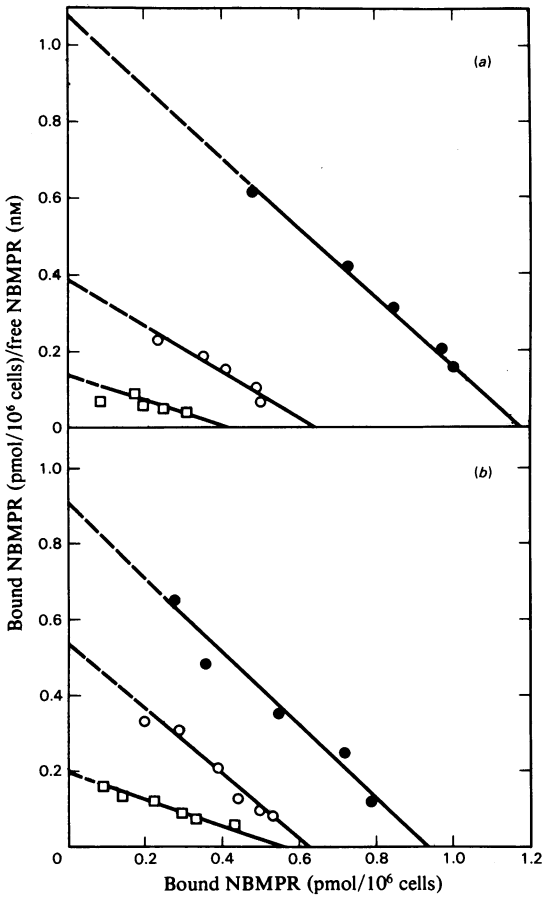

Fig. 7. *The effects of pretreatment with p-CMBS and NEM on site-specific binding of NBMPR to HeLa cells*
(*a*) Replicate monolayer cultures (2×10^6 cells/bottle) were pretreated for 20min at 37°C with transport medium without additives (●), or with transport medium containing *p*-CMBS at 0.5mM (○) or 2mM (□). Site-specific binding of [G-^{3}H]NBMPR was then determined at 37°C as described for Fig. 4 in the presence of the pretreatment medium. (*b*) Replicate monolayer cultures (1.9×10^6 cells/bottle) were pretreated for 20min at 20°C with transport medium without additives (●), or with transport medium containing NEM at 20μM (○) or 30μM (□). Cultures were then washed with additive-free transport medium and site-specific binding of [G-^{3}H]NBMPR was determined at 20°C as described in the legend to Fig. 4 in the presence of the pretreatment medium.

higher concentrations of *p*-CMBS (0.5 or 2mM) decreased the number of NBMPR-binding sites and altered in a complex way the ability of NBMPR to bind to these sites, presumably through structural alterations of protein(s) involved in the binding reaction. The insensitivity of binding to *p*-CMBS, relative to that of the more penetrable NEM, suggested that the thiol groups required for binding of NBMPR are located near the cytoplasmic side of the membrane.

In summary, we have described results of experiments that explored the relationship between NBMPR binding and inhibition of uridine transport in monolayer cultures of HeLa cells. The basis for the biphasic concentration–effect curves for inhibition of uridine transport by NBMPR obtained in this work (Table 1) and previously (Paterson *et al.*, 1977*a*) is not known. Inhibition of uptake of uridine over a broad range of concentrations (0.4–400μM; see Table 1) was achieved by pretreatment of cells with concentrations of NBMPR that were expected to only partially saturate the high-affinity sites, suggesting that these sites are transport-inhibitory. We do not understand why near total inhibition of uridine transport could only be obtained at concentrations of NBMPR three to four orders of magnitude greater than those that saturated the high-affinity sites. Although uridine entry by a second transport system of low NBMPR sensitivity is a possibility, the characteristics of nucleoside-impermeable sheep erythrocytes (Jarvis & Young, 1980) and of the transport-defective mouse lymphoma line, AE_1 (Cass *et al.*, 1981) suggest that a single NBMPR-sensitive transporter mediates uptake of uridine, and probably that of other nucleoside permeants as well. The results from the current study are inconclusive as to whether transport inhibition results from binding of NBMPR to permeant binding sites or to a separate set of regulatory sites, as proposed by Eilam & Cabantchik (1977). Uridine and other nucleoside permeants competed with NBMPR for binding, indicating affinity of permeants for the 'NBMPR-specific' sites, and the K_d value (1.7mM) obtained from competitive studies for dissociation of uridine from these sites was an order of magnitude greater than the K_m values (about 100μM) for inward transport of uridine.

Pretreatment of HeLa cells with low concentrations of the penetrable thiol-specific reagent NEM and with high concentrations of the less penetrable *p*-CMBS decreased the number of binding sites and inhibited uridine transport. Thus both processes required the presence of free thiol groups at or near the interior face of the plasma membrane. The sensitivity of the binding process to both thiol-specific reagents was decreased by the presence of NBMPR during the inactivation reaction, suggesting that the critical thiol groups are located in the vicinity of the NBMPR-binding sites.

Table 5. *Effects of NEM and p-CMBS with and without pretreatment on NBMPR binding to HeLa cells*
Replicate monolayer cultures of HeLa cells[(1–2) × 10^6 cells/bottle] were incubated for 20 min with [G-^{3}H]NBMPR together with NEM at 20°C or with *p*-CMBS at 37°C (simultaneous exposure). In parallel experiments, cells were incubated with NEM at 20°C or with *p*-CMBS at 37°C for 15 min (pretreatment), washed with 4 ml of additive-free transport medium and then incubated for 20 min with [G-^{3}H]NBMPR at the concentrations indicated.

	NBMPR binding (% of control value)									
	Simultaneous exposure to NEM at these concentrations (μM)			Pretreatment with NEM at these concentrations (μM)			Simultaneous exposure to *p*-CMBS at these concentrations (mM)		Pretreatment with *p*-CMBS at these concentrations (mM)	
[NBMPR] (nM)	20	40	80	20	40	80	0.5	1	0.5	1
1	58	30	20	43	20	13	53	25	48	16
2	72	48	28	50	26	20	57	26	48	23
3	90	56	41	56	33	25	64	30	48	23
5	100	82	59	60	42	35	73	39	50	25
7	100	93	80	66	53	46	78	42	48	30

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