Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells

Differences in sensitivity to nitrobenzylthioinosine and thiol reagents

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The characteristics of nucleoside transport were examined in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. In Walker 256 cells the initial rates of uridine, thymidine and adenosine uptake were insensitive to the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) ($1\ \mu\text{M}$), but were partially inhibited by dipyridamole ($10\ \mu\text{M}$), another inhibitor of nucleoside transport. In contrast, the transport of these nucleosides in S49 cells was completely blocked by both inhibitors. Nucleoside transport in Walker 256 and S49 cells also differed in its sensitivity to the thiol reagent *p*-chloromercuribenzenesulphonate (*p*CMBS). Uridine transport in Walker 256 cells was inhibited by *p*CMBS with an IC_{50} (concentration producing 50% inhibition) of less than $25\ \mu\text{M}$, and inhibition was readily reversed by β -mercaptoethanol. In S49 cells uridine transport was only inhibited at much higher concentrations of *p*CMBS ($\text{IC}_{50} \approx 300\ \mu\text{M}$). In other respects nucleoside transport in Walker 256 and S49 cells were quite similar. The K_m and V_{max} values for uridine transport were nearly identical, and the transporters of both cell lines appeared to accept a broad range of nucleosides as substrates. Uridine transport in Walker 256 cells was non-concentrative and did not require an energy source. These studies demonstrate that nucleoside uptake in Walker 256 cells is mediated by a facilitated-diffusion mechanism which differs markedly from that of S49 cells in its sensitivity to the transport inhibitor NBMPR and the thiol reagent *p*CMBS.

INTRODUCTION

The uptake of nucleosides by cultured mammalian cells involves the permeation of the nucleoside across the plasma membrane, followed by metabolism of the nucleoside within the cell (Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1980a). The permeation step is very fast, requiring the use of rapid sampling techniques (Wohlhueter *et al.*, 1978b; Harley *et al.*, 1982) to isolate this step kinetically from the subsequent metabolism of the nucleoside. Through the use of such techniques it has been demonstrated in a number of cell lines that permeation is mediated by nucleoside-specific transporters which accept a broad range of nucleosides as substrates.

NBMPR has proved to be a specific and useful probe of the nucleoside-transport mechanism in erythrocytes (Cass *et al.*, 1974) and many cultured mammalian cells (Paterson *et al.*, 1980a). NBMPR binds reversibly to these cells with a dissociation constant of 0.1–1 nM, and occupancy of the high-affinity binding sites by NBMPR is associated with loss of nucleoside-transport activity (Cass *et al.*, 1974; Lauzon & Paterson, 1977; Paterson *et al.*, 1980b). In some cells, however, the relationship between NBMPR binding and inhibition of transport is complex. In HeLa cells, the dose-response curve for inhibition of uridine transport is biphasic, with some 40% of the transport activity remaining when the high-affinity NBMPR-binding sites are saturated (Paterson *et al.*, 1980b). In transformed hamster fibroblasts, although

uridine uptake is inhibited by NBMPR with a K_i of 4 nM, 20–30% of the uptake activity remains insensitive to 100 nM-NBMPR (Eilam & Cabantchik, 1977a,b). Similar observations have also been made in Chinese-hamster ovary cells (Wohlhueter *et al.*, 1978a).

We have reported (Belt, 1983b) that the dose-response curve for NBMPR inhibition of uridine transport in L1210 cells is also biphasic, and that the NBMPR-insensitive component of uptake is mediated by a transport mechanism with kinetic properties and substrate specificity similar to that of the NBMPR-sensitive mechanism. In these studies an NBMPR-insensitive component of transport was also noted in L5178Y and P388 mouse leukaemia cells (Belt, 1983b) and RPMI 6410 human lymphoblastoid cells (Belt, 1983b). Plagemann & Wohlhueter (1984a) have confirmed the presence of an NBMPR-insensitive component of nucleoside transport in L1210 and P388 cells and have demonstrated that Chinese-hamster ovary cells and mouse L929 cells also have biphasic dose-response curves for NBMPR inhibition of uridine transport.

Although many cell lines appear to have both NBMPR-sensitive and insensitive transport activity, only one cell line, Novikoff rat hepatoma, has previously been shown to have only the NBMPR-insensitive activity (Plagemann & Wohlhueter, 1984b). Nucleoside transport has been extensively characterized in Novikoff cells and is similar to that of other cells with respect to substrate specificity and the kinetics of transport, but is resistant

Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; *p*CMBS, *p*-chloromercuribenzenesulphonate.

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to NBMPR, with micromolar concentrations required to inhibit transport of uridine (Plagemann *et al.*, 1978) or thymidine (Wohlhueter *et al.*, 1979).

In preliminary studies (Belt, 1983c; Paterson *et al.*, 1983), we and others have identified a second cell line, Walker 256 rat carcinosarcoma, that appears to have only the NBMPR-insensitive nucleoside-transport mechanism. In the present study we characterize the properties of this transporter and compare them with the NBMPR-sensitive transporter of S49 mouse lymphoma cells.

MATERIALS AND METHODS

Cell lines and growth conditions

S49 cells were provided by Dr. A. R. P. Paterson, University of Alberta, Edmonton, Alberta, Canada, and Walker 256 cells by Dr. P. M. Schwarz and Dr. R. E. Handschumacher, Yale University, New Haven, CT, U.S.A. Stock cultures were maintained as static suspension cultures in Fischer's medium supplemented with 10% (v/v) heat-inactivated horse serum in a humidified atmosphere of 5% CO₂ in air. Cells used for transport studies were grown in roller bottles (1.5 rev./min) gassed with 5% CO₂ in air.

Nucleoside uptake

Nucleoside uptake was determined by a modification of the method described by Harley *et al.* (1982). Cells were harvested during exponential growth, washed twice with cold buffer A (130 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 5 mM-NaH₂PO₄, 10 mM-glucose and 25 mM-Hepes, adjusted to pH 7.4 with NaOH), resuspended at 4×10^7 cells/ml in the same buffer and kept on ice. Immediately before determination of each uptake curve, a portion of the cell suspension was mixed with an equal volume of buffer A (at 20 °C) containing twice the indicated concentration of inhibitor and incubated in a shaking water bath for 5 min at 20 °C. At the end of the incubation, 100 μ l portions of the cell suspension were layered over 100 μ l of silicone oil (Nyosil 50; William F. Nye, New Bedford, MA, U.S.A.) in 12 1.5 ml microcentrifuge tubes and the tubes placed in a Beckman Microfuge B equipped with an Eppendorf 12-place 45°-fixed-angle rotor. Nucleoside uptake was initiated by rapidly adding 100 μ l of a reaction mixture (buffer A containing twice the indicated concentration of nucleoside and 5–10 μ Ci of [³H]nucleoside/ml) to each tube at 2 s intervals. Uptake was terminated by turning on the centrifuge 2 s after addition of the reaction mixture to the last tube, and the tubes were centrifuged for 30 s. When nucleoside uptake was determined over periods of 1 min or longer, incubations were carried out in a shaking water bath, and at the times indicated 200 μ l of the cell suspension were removed, layered over oil in a micro-centrifuge tube and centrifuged for 30 s. After centrifugation, samples were set aside until all assays were completed, and then processed as follows. The supernatant fractions were aspirated and the tubes washed twice with water. The oil layer was removed with the last wash and the cell pellet was dissolved in 0.5 ml of 1% Triton X-100. The dissolved sample and tube were transferred to a scintillation vial, mixed with 10 ml of Budget-Solve (Research Products International Corp., Mount Prospect, IL, U.S.A.), and radioactivity was determined in a liquid-scintillation counter.

Harley *et al.* (1982) have previously demonstrated that methodology similar to that used here provides sufficiently rapid mixing to obtain accurate uptake rates over time intervals of a few seconds.

Determination of intra- and extra-cellular space

In order to quantify the contribution of nucleoside in the extracellular space to the total radioactivity in the cell pellet, the space occupied by [¹⁴C]sucrose was determined. [¹⁴C]Sucrose (10 μ Ci/ml) was substituted for [³H]nucleoside in the reaction mixture and, after a 5–10 s exposure of cells to the reaction mixture, the centrifuge was turned on and the cells were pelleted under the layer of oil. To determine the total water space of the cell pellet, ³H₂O (10 μ Ci/ml) was substituted for [³H]nucleoside in the reaction mixture, and 1–2 min was allowed for equilibration with the intracellular water. Radioactivity in the cell pellets was determined as described above, and the intracellular space was determined as the difference between the total ³H₂O space and the [¹⁴C]sucrose space.

Estimation of the zero time

There is a time between turning on the centrifuge and the effective removal of the cells from contact with the incubation medium which is not negligible with respect to the rate of nucleoside transport (Wohlhueter *et al.*, 1978b). To determine the effective zero time, [³H]uridine uptake was determined in cells treated with a concentration of NBMPR or dipyrindamole sufficient to produce a linear rate of uptake for at least 20 s. Uptake was corrected for the amount of ³H in the extracellular space, and the best-fit line was determined by linear regression analysis. The effective zero time was taken as the intercept on the abscissa. In several experiments this value varied from –1 to –4 s, with a mean of approx. –2.5 s. This is in reasonable agreement with the lag time of 2 s reported by Wohlhueter *et al.* (1978b) for an Eppendorf microcentrifuge. In calculation of uptake rates 2.5 s was added to the sampling time to obtain the actual incubation time.

Data analysis

Since the time courses of nucleoside uptake in the absence of inhibitors were not linear, initial rates of uptake were determined from the coefficients of the first-order term in parabolas fitted to the uptake data as described by Harley *et al.* (1982). Uptake values were corrected for ³H in the extracellular space, and the parabolas were constrained to pass through zero. K_m and V_{max} values for transport were calculated by direct computer fit of the non-linear Michaelis–Menten equation as described by Cleland (1979).

Materials

Fischer's culture medium was obtained from K.C. Biologicals (Lenexa, KS, U.S.A.). Horse serum and other cell culture supplies were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). [5-³H]Uridine, [2-³H]adenosine, [6-³H]thymidine and ³H₂O were obtained from New England Nuclear (Boston, MA, U.S.A.), and [U-¹⁴C]sucrose was from Schwarz/Mann (Spring Valley, NY, U.S.A.). Unlabelled nucleosides were obtained from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.) or Sigma Chemical Co. (St Louis, MO, U.S.A.). NBMPR was prepared from 6-mercaptapurine riboside by Dr Josef Nemec of this Department, as described by Paul *et al.* (1975). 6-Mercaptapurine riboside was generously

provided by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.), and dipyrindamole {2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine} was from Boehringer-Ingelheim (Ridgefield, CT, U.S.A.).

Results

The effect of the nucleoside-transport inhibitor NBMPR on uridine transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells is shown in Fig. 1. At a concentration of $1\ \mu\text{M}$, NBMPR had no effect on uridine transport in Walker 256 cells, but completely blocked transport in S49 cells. Even when the uptake time course in S49 cells was extended to 10 min there was no discernible uptake of uridine in the presence of NBMPR (results not shown). Although uridine transport in Walker 256 cells was not affected by $1\ \mu\text{M}$ -NBMPR, it was inhibited by dipyrindamole, another inhibitor of nucleoside transport (Plagemann & Wohlhueter, 1980). Even dipyrindamole, however, appeared to be less effective in Walker 256 cells than in S49 cells (Fig. 1).

To compare nucleoside transport in Walker 256 and S49 cells further, the kinetics of uridine transport were examined (Figs. 2 and 3). In these studies uridine uptake was determined at 2 s intervals for 26 s over the concentration range $40\text{--}1000\ \mu\text{M}$ -uridine. Since uptake was not linear even over this short time period, initial rates of uptake were calculated from parabolas fitted to the uptake data as described by Harley *et al.* (1982). As shown in Figs. 2(a) and 3(a), the calculated parabolas provided a good fit to the uptake data for both cell lines. The initial rates of uptake were calculated from the fitted parabolas and plotted against the uridine concentration in Figs. 2(b) and 3(b). These data demonstrate that uridine uptake in both cell lines was saturable and that the two transporters had similar kinetic constants. In Walker 256 cells the K_m was $196 \pm 14\ \mu\text{M}$ and the $V_{\max}$ was $21 \pm 5\ \text{pmol/s per } 10^6\ \text{cells}$ (mean $\pm$ s.d. for four experiments). The corresponding values in S49 cells were $172 \pm 20\ \mu\text{M}$ and $19 \pm 5\ \text{pmol/s per } 10^6\ \text{cells}$ (mean $\pm$ s.d. for three experiments). The K_m values were slightly higher than those reported by Cass *et al.* (1981) for S49 cells, but

were well within the range of values reported for uridine transport in other cultured mammalian cells (Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1980a).

Uridine transport in Walker 256 cells was also similar to nucleoside transport in other cells in that it did not require an energy source and was non-concentrative. In the experiment shown in Fig. 4 a comparison was made between uridine uptake in cells depleted of ATP by incubation with azide (Cybulski *et al.*, 1981) and in untreated cells. The initial rate of uridine uptake was the same in ATP-depleted and untreated cells (inset), but uptake rapidly reached a plateau in the azide-treated cells, with the intracellular concentration of ^3H approximately equal to that in the medium. In contrast, ^3H continued to accumulate in the untreated cells, presumably as uridine nucleotides, and a new steady-state rate of uptake was established. This slower rate of uptake most likely reflects the rate of phosphorylation of uridine by uridine kinase (Wohlhueter & Plagemann, 1980). The observations that the initial rate of uridine uptake was saturable (Fig. 2), that it was not affected by ATP depletion (Fig. 4) and that uptake was non-concentrative indicated that uridine permeation in Walker 256 cells, like that of other cells (Plagemann & Wohlhueter, 1980; Paterson *et al.*, 1980a), was mediated by a facilitated-diffusion mechanism.

It has previously been reported (Cohen *et al.*, 1979; Cass *et al.*, 1981) that the nucleoside transporter in S49 cells has a broad substrate specificity and mediates the entry of a number of different nucleosides into these cells. To examine the question of substrate specificity in Walker 256 cells, the uptakes of [^3H]adenosine and [^3H]thymidine were examined and compared with those in S49 cells (Fig. 5). The patterns of uptake were similar to those for uridine (Fig. 1). NBMPR had no effect on adenosine or thymidine transport in Walker 256 cells, but inhibited the transport of these two nucleosides in S49 cells. Dipyrindamole inhibited adenosine and thymidine transport in both cell lines, although again it may have been less effective in Walker 256 cells than in S49 cells. The specificity of nucleoside transport in Walker 256 cells was examined further through inhibition studies. As shown in Fig. 6, the transport of uridine in these cells was inhibited

Fig. 1. Effect of nitrobenzylthioinosine and dipyrindamole on the initial rate of uridine uptake in S49 and Walker 256 cells

Cells were incubated with the indicated inhibitor for 5 min at 20 °C. At the end of this period, uridine ($100\ \mu\text{M}$) uptake was determined as described in the Materials and methods section. The data plotted are the means of duplicate determinations and are representative of experiments repeated four or more times. ○, No additions; ●, $1\ \mu\text{M}$ -NBMPR; □, $10\ \mu\text{M}$ -dipyrindamole (DP).

Fig. 2. Kinetics of uridine transport in Walker 256 cells

Uridine uptake was determined at 20 °C as described in the Materials and methods section. The concentration of [5-³H]uridine in the assays was held constant at 5 μCi/ml and the uridine concentration varied from 40 to 1000 μM. Final uridine concentrations (μM) are shown in parentheses at the right side of panel (a). The curves drawn are least-squares parabolas constrained to pass through zero. The uptake curves for 45 μM- and 70 μM-uridine were omitted from panel (a) for clarity, but were included in calculation of the kinetic constants. Initial rates of uptake calculated from the fitted parabolas (a) are plotted against the uridine concentration in (b).

Fig. 3. Kinetics of uridine transport in S49 cells

Initial rates of uridine transport and kinetic constants were determined as described in the legend to Fig. 2.

by several other nucleosides. These included purine and pyrimidine nucleosides as well as ribo- and deoxyribonucleosides. Thus it appears that the nucleoside transporter of Walker 256 cells, like that of S49 and other cells, may accept a wide range of nucleoside as substrates.

Although the nucleoside transporters of Walker 256 and S49 cells appeared to be quite similar in some respects, they differed markedly in their response to the thiol reagent *p*CMBS. Uridine transport in Walker 256 cells was inhibited by *p*CMBS, with an IC₅₀ (concentration producing 50% inhibition) < 25 μM (Fig. 7), whereas much higher concentrations of *p*CMBS were required to inhibit transport in S49 cells (IC₅₀ ≈ 300 μM). Inhibition of uridine transport by *p*CMBS in Walker 256 cells was readily reversed by incubation with β-mercaptoethanol (Table 1), indicating that *p*CMBS was reacting with an essential thiol group in this transporter. It was not possible to determine whether *p*CMBS inhibition in S49 cells was also due to interaction with a thiol group, because cells treated with high concentrations (400 μM) of *p*CMBS were fragile, and incubation of these cells at 37 °C with or without β-mercaptoethanol (or dithio-

threitol) resulted in non-specific membrane damage, as evidenced by an increase in the sucrose-accessible space (results not shown). The requirement for the participation of a thiol group in nucleoside transport in S49 cells could, however, be demonstrated by its sensitivity to *N*-ethylmaleimide (Fig. 8). Uridine transport in Walker 256 cells was also inhibited by *N*-ethylmaleimide (IC₅₀ ≈ 250 μM), although these cells were slightly less sensitive to this reagent than were S49 cells. The large differences in the sensitivity of Walker 256 and S49 cells to *p*CMBS suggested that the local environment about the thiol group(s) involved in nucleoside transport may be different in the two transport proteins.

DISCUSSION

The studies presented here indicate that the nucleoside transporters of Walker 256 and S49 cells differ markedly in their sensitivity to the transport inhibitor NBMPR and to the thiol reagent *p*CMBS. Uridine transport in S49 cells is completely blocked by 1 μM-NBMPR, but is relatively insensitive to *p*CMBS (IC₅₀ ≈ 300 μM). In contrast,

Fig. 4. Uridine uptake in ATP-depleted and untreated Walker 256 cells

Cells were harvested and divided into two equal portions. One portion of cells was washed and resuspended in buffer A; the other portion was washed with glucose-free buffer A and resuspended in the same buffer containing 10 mM- NaN_3 . Both cell suspensions were incubated for 15 min at 37 °C and then cooled to 20 °C for determination of uridine uptake. Uridine (100 μM) uptake was determined as described in the Materials and methods section at 2 s intervals over the initial portion of the uptake curve and at 1, 2, 5 and 10 min. The data plotted represents the means of duplicate determinations. For purposes of clarity, every fourth point is shown in the initial part of the curves: all points are shown in the inset. ○, Untreated cells; ●, NaN_3 -treated cells. The dashed line indicates the level at which the intracellular ^3H content is equal to the concentration of ^3H in the medium.

uridine transport in Walker 256 cells is not affected by 1 μM -NBMPR, but is inhibited by $p\text{CMBS}$ ($\text{IC}_{50} < 25 \mu\text{M}$). In other respects nucleoside transport in the two cell lines is quite similar. The K_m and V_{max} values for uridine transport are the same, and both cell lines appear to accept a variety of nucleosides as substrates. If there are any differences in the substrate specificity of transport between these two cell lines, they have yet to be discovered.

Most of the cells that we have examined (Belt, 1983a, b) have a small, but measurable, component of nucleoside-transport activity that is not inhibited by NBMPR; however, in S49 cells we have been unable to detect any NBMPR-insensitive transport. This is in keeping with studies from other laboratories (Cohen *et al.*, 1979; Cass *et al.*, 1981) suggesting that nucleoside uptake in S49 cells is mediated by a single NBMPR-sensitive nucleoside transport mechanism. Cohen *et al.* (1979) have isolated a nucleoside-transport-deficient clone (AE_1) from a mutagenized population of S49 cells by a single-step selection for resistance to adenosine. These cells are resistant to a number of cytotoxic nucleosides and have an impaired capacity to take up several physiological nucleosides. Cass *et al.* (1981) have shown that nucleoside transport in the parent S49 cells is sensitive to NBMPR and that the loss of nucleoside-transport activity in AE_1 cells is accompanied by loss of the high-affinity

NBMPR-binding sites. Thus it appears that S49 cells provide a good model of NBMPR-sensitive nucleoside transport.

Walker 256 cells, on the other hand, appear to provide a model of NBMPR-insensitive nucleoside transport. These cells have no detectable NBMPR-sensitive transport (Fig. 1) nor any high-affinity NBMPR-binding sites (Paterson *et al.*, 1983). Only one other cell line, Novikoff N1S1-67 rat hepatoma, has been reported to have an NBMPR-insensitive nucleoside-transport mechanism without also having an NBMPR-sensitive transporter (Plagemann & Wohlhueter, 1984a). The kinetics and substrate specificity of nucleoside transport in Walker 256 cells are similar to those of Novikoff N1S1-67 cells, which have been extensively characterized by Plagemann & Wohlhueter (1980). Since it has been observed that some strains of Novikoff cells possess high-affinity NBMPR-binding sites (Plagemann & Wohlhueter, 1984a; J. A. Belt, E. S. Jakobs & A. R. P. Paterson, unpublished results), we prefer the Walker 256 cell line as a model of NBMPR-insensitive nucleoside transport. The use of Walker 256 cells and the NBMPR-sensitive S49 cell line should provide a means further to compare and contrast these two types of nucleoside-transport activity.

The observation that dipyridamole inhibits nucleoside transport in both S49 and Walker 256 cells merits comment. Paterson *et al.* (1980b) have previously observed that dipyridamole competes for NBMPR binding at high-affinity sites on HeLa cells and conclude that inhibition is, at least in part, due to the interaction of dipyridamole at these sites. The observation that dipyridamole inhibits nucleoside transport in Walker 256 (Figs. 1 and 5) cells and Novikoff N1S1-67 cells (Plagemann & Wohlhueter, 1980), both of which lack high-affinity NBMPR-binding sites (Paterson *et al.*, 1983; Plagemann & Wohlhueter, 1984a), indicates that dipyridamole must also act at a site distinct from the NBMPR-binding site. As suggested previously by Plagemann & Wohlhueter (1980), dipyridamole and other lipophilic inhibitors that have no structural resemblance to nucleosides may partition into the membrane and interact with hydrophobic domains of the transporter.

The role of thiol groups in nucleoside transport is not well understood. In two cell lines (Eilam & Cabantchik, 1977b; Bibi *et al.*, 1978; Dahlig-Harley *et al.*, 1981), which exhibit significant nucleoside uptake in the presence of 0.1 μM -NBMPR, it has been observed that $p\text{CMBS}$ acts in an apparently synergistic manner with NBMPR to inhibit uptake completely. It has been suggested that $p\text{CMBS}$ may act to increase the sensitivity of the transport mechanism to NBMPR (Eilam & Cabantchik, 1977a; Dahlig-Harley *et al.*, 1981), or alternatively that there may be two transport systems which differ in their sensitivity to NBMPR and thiol reagents (Bibi *et al.*, 1978). We have examined NBMPR-sensitive and -insensitive nucleoside transport in L1210 cells (Belt, 1983a, b) and find that these two transport activities differ in their sensitivity to $p\text{CMBS}$. The data presented in Fig. 7 demonstrate that the nucleoside transporter of Walker 256 cells is approx. 10-fold more sensitive to $p\text{CMBS}$ than is that of S49 cells, again suggesting that NBMPR-sensitive and -insensitive nucleoside transport differ in their response to $p\text{CMBS}$. These results are not in agreement with the reports by Plagemann & Wohlhueter (1984a, b) that the two transport mechanisms do not differ in their

Fig. 5. Adenosine and thymidine transport in Walker 256 and S49 cells

Cells were incubated for 5 min at 20 °C with the indicated additions and then assayed for adenosine (100 μM) or thymidine (100 μM) uptake as described in the Materials and methods section. $\circ$, No additions; $\bullet$, 1 μM -NBMPR; $\square$, 10 μM -dipyridamole (DP).

Fig. 6. Inhibition of uridine transport in Walker 256 cells by other nucleosides

Uridine uptake was determined over 12.5 s intervals as described in the Materials and methods section. Unlabelled nucleosides were added simultaneously with [^3H]uridine in the reaction mixtures. The final concentration of uridine in the assay was 100 μM , and the final concentration of unlabelled nucleoside is indicated on the abscissa. The data plotted are the means of duplicate determinations and are representative of experiments repeated three or more times.

Fig. 7. Effect of *p*-chloromercuribenzenesulphonate on uridine transport in Walker 256 and S49 cells

Cells were incubated for 20 min at 20 °C with the indicated concentration of *p*CMBS. Uridine (100 μM) uptake was then determined over 12.5 s intervals as described in the Materials and methods section. The data plotted are the means of duplicate or triplicate determinations and are representative of experiments repeated twice or more. □, S49 cells; ○, Walker 256 cells.

Fig. 8. Effect of *N*-ethylmaleimide on uridine transport in Walker 256 and S49 cells

Incubations and assays were carried out as described in the legend to Fig. 7, but with *N*-ethylmaleimide. □, S49 cells; ○, Walker 256 cells.

sensitivity to *p*CMBS, or to the more membrane-permeant thiol reagent *p*-hydroxymercuribenzoate. These authors, however, treated cells with the thiol reagents at 37 °C, whereas our studies have been done at 20 °C. The IC_{50} for *p*CMBS inhibition of uridine transport in S49 cells is dependent on temperature, and decreases about 10-fold between 20° and 37 °C, whereas that of Walker 256 cells is independent of temperature (J. A. Belt & L. D. Noel, unpublished work). Thus we also see no difference in the sensitivity of the two cell lines to *p*CMBS if the incubations are carried out at 37 °C. A possible explanation of these results is that the thiol groups involved in nucleoside transport in Walker 256 and S49 cells differ in their orientation in the membrane, with the latter being less accessible to *p*CMBS from the outside of

Table 1. Reversal of *p*CMBS inhibition by β-mercaptoethanol in Walker 256 cells

Cells were incubated with or without 100 μM-*p*CMBS for 10 min at 20 °C (Expt. A) or 0 °C (Expt. B) and then washed at 0 °C, resuspended in buffer A, and incubated with 1 mM-β-mercaptoethanol at 37 °C. At the time indicated, samples of the cell suspension were removed and cooled to 20 °C, and uridine uptake was determined over a 12.5 s interval. Data presented are means of duplicate assays.

Expt.	Time with β-mercaptoethanol (min)	Uridine uptake (pmol/12.5 s per 10 ⁶ cells)	
		Control cells	<i>p</i> CMBS-treated cells
A	0	57.4	5.4
	10	57.2	36.4
	30	65.0	54.8
B	0	61.6	14.4
	10	70.4	50.6
	30	67.4	64.7

the cell. Increasing the temperature of the incubation may increase permeation of *p*CMBS across the membrane and result in better inhibition of transport in S49 cells. This would agree with the findings by Jarvis & Young (1982) that *p*CMBS interacts with the NBMPR-sensitive nucleoside transporter of sheep erythrocytes only at the cytoplasmic side of the plasma membrane. The hypothesis that the thiol groups involved in NBMPR-sensitive and -insensitive nucleoside transport differ in their orientation in the membrane requires investigation.

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