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Nucleoside Transport in Mammalian Cell Membranes

IV. Organomercurials and Organomercurial-Mercaptonucleoside Complexes as Probes for Nucleoside Transport Systems in Hamster Cells

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Summary. Organomercurials form stable stoichiometric complexes with thiolated nucleosides. The complexes inhibited uptake of ribonucleosides and cytosine arabinoside (CAR) in various types of normal and transformed cells. The inhibition was competitive and reversible ($K_i = 3-6$ μM). The interaction between complexes and transport system displayed a 1:1 stoichiometry. Chemical factors which contributed to the inhibitory power were evaluated with a series of S-alkylated derivatives and S–Hg–R complexes of mercaptonucleosides. The inhibitory potency was not determined exclusively by the hydrophobic nature of either the S-alkylated or the S–Hg–R moieties.

Chemical modification of cells with penetrating and nonpenetrating organomercurials lead to stimulation of nucleoside uptake and to an increase in its susceptibility to inhibition by S–Hg–R complexes or S-alkylated derivatives of mercaptopurine ribosides.

The kinetic and chemical data obtained with nucleoside analogs and with chemical modifiers suggested complex features of nucleoside transport systems. Four distinct classes of sites were implied: (i) a substrate binding site susceptible directly to competitive inhibition by organomercurial-mercaptonucleoside complexes, (ii) an additional site susceptible either to S-arylalkylated or S-mercuriated derivatives of 6-mercaptopurine ribosides, (iii) SH-containing modifier sites which *stimulate* uridine uptake upon binding of organomercurials, and (iv) SH-containing modifier sites which *inhibit* the function upon binding of organomercurials. From the observation that only SH sites related to stimulation were susceptible to modification by macromolecular-SH modifier probes, some conclusions can be drawn regarding the disposition of the various sites in the cell membrane in general and among membrane components in particular.

A series of S-substituted derivatives of 6-mercaptopurine ribosides have been used to probe nucleoside transport systems in mammalian cells [4–7, 14, 15, and 22]. Some of these probes inhibited the transport

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system of hamster cells competitively and reversibly, displaying binding affinities which were more than 10,000-fold higher than those of the corresponding substrates, i.e., $0.2–5 \times 10^9$ M^{-1} *vs.* $1–5 \times 10^4$ M^{-1} [4, 5, 6, and 7]. The high affinity binding properties and specificity of the probes for nucleoside transport entities have allowed the estimation of the number of nucleoside carriers present in red blood cell membranes [4, 5] and in hamster fibroblasts in culture [6, 7]. Chemical and kinetic studies performed on hamster cells have also revealed some complex features of nucleoside transport and of chemical probing with high affinity probes and SH reactive agents [9]. Organomercurials caused inhibitory effects in the presence of S-6 benzylated derivatives of mercaptonucleosides [7]. In the course of determining the reasons for these synergistic effects, the possibility was considered that mercurials might cleave the S-alkylated bond and complex the liberated thiol to form a new highly inhibitory species. Although no cleavage of the R−S bond was detected, it was found that mercurials indeed formed stable complexes with the free thiolated bases [10], and that these complexes inhibited nucleoside uptake in hamster and other animal cell lines in culture and in human red blood cells. The similarity in chemical features and inhibitory properties between these complexes and S-alkylated derivatives prompted us to further study their structure-activity relationships and their mode of action on nucleoside transport. The specific binding of metal ions with thiolated nucleosides had been previously used to study structure and base sequence of polynucleotides, which normally contain limited amounts of SH modified nucleosides [2, 3, 10, 17, 18, and 21].

The high inhibitory power of mercaptonucleoside derivatives has been attributed, in the past, mainly to the hydrophobic nature of the S-6 substituting moiety of the SH compounds [4, 15]. However, it appeared as though other chemical features present in the probes might also have contributed to the inhibitory power [14, 15]. In the present work we attempted to evaluate these and other features, by comparing the inhibitory profiles of ribonucleoside analogs and of S-6 substituted derivatives of mercaptonucleosides. We have found that while neither free organomercurials nor mercaptonucleosides impaired nucleoside transport, the thiolated derivatives formed stable mercurial complexes which were specific and potent inhibitors. Modification of the hydrophobic properties of either the S-6 substituting or S-6 complexing moieties of mercaptonucleosides allowed us to assess the extent to which these features might be responsible for inhibitory potency. The mechanism of

inhibition of these probes and the localization of their effects within the membrane have also been studied with the aid of selective chemical modifiers and of nonpenetrating macromolecular analogs of nucleoside probes.

Materials and Methods

The mercaptonucleosides and organomercurials were purchased from Sigma Chemical Company. The nitrobenzyl, hydroxynitrobenzyl and methoxynitrobenzyl bromides were obtained from either Pierce Chemical Company, Aldrich Chemicals, or Fluka (Switzerland). N-ethylmaleimide was obtained from Calbiochem. *p*-hydroxymercuribenzoamido-ethyl-Dextran (10,000 mol wt); *p*MBAE: Dextran ratio 1:1 was a gift from Dr. Rolf Kinne (Max Planck Institut für Biophysik, Frankfurt) [20]. The benzylated derivatives of mercaptopurine ribosides were prepared in freshly distilled N,N-dimethylformamide according to [13] and were crystallized from methanolic solutions. The chemical and physical properties of the nitrobenzyl and hydroxynitrobenzyl derivatives were the same as those previously presented [4, 5] and in accordance with [13] and [15]. The compounds MNBMI[1] and MNBMG showed the following properties: molar absorptivity in PBS, pH 7.4, 23,700 (292 nm) and 19,300 (313 nm), respectively; R_f values in thin layer chromatography plates (Silica Gel, Machery-Nagel) of 0.37 and 0.40 in chloroform/methanol (85:15, v/v), respectively, of 0.67 and 0.68 in water-saturated *n*-butanol), respectively, and of 0.70 and 0.71 in butanol/acetic acid/water (60:15:20, v/v/v), respectively. The compounds NEM-MI and NEM-MG were prepared by reacting either MI or MG with a molar excess of NEM in methanol or dimethylformamide solutions for 72 hr at room temperature. The solvent was removed by repeated evaporations in ethanol and the excess NEM was removed by ether-extractions. The final compounds were crystallized from methanol-ether. The concentration of these compounds was determined spectrophotometrically in PBS, pH 7.4 using molar absorptivities similar to those shown for the methoxynitrobenzyl derivatives. In chloroform/methanol (1:1; v/v), the R_f values of MNBMI and MNBMG were 0.88 and 0.85, and of NEM-MI and NEM-MG 0.75 and 0.72, respectively.

Unless specified otherwise, all the tritiated nucleosides were purchased from the Israel Atomic Energy Agency, NRC-Negev., Beer Sheba, at 1 mCi/ml, solutions (10 Ci/mmole).

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1 *Abbreviations:* MI (mercaptoinosine), 6-mercapto-9-β-D-ribofuranosylpurine; MG (mercaptoguanosine), 2-amino-6-mercapto-9-β-D-ribofuranosylpurine; TU (thiouridine), 4-thiouridine; NEM, N-ethylmaleimide; MB, hydroxymercuribenzene; *p*MBC, *p*-hydroxymercuribenzoate; *p*MBS, *p*-hydroxymercuribenzenesulfonate; *p*MBAED, *p*-hydroxymercuribenzoamido-ethyl-Dextran (mol wt 10,000); NBMI, S-(*p*-nitrobenzyl)-6-mercapto-9-β-D-ribofuranosylpurine; HNBMI, S-(2-hydroxy-5-nitrobenzyl)-6-mercapto-9-β-D-ribofuranosylpurine; MNBMI, S-(2-methoxy-5-nitrobenzyl)-6-β-D-ribofuranosylpurine; HNBMG, 2-amino-S-(2-hydroxy-5-nitrobenzyl)-6-mercapto-9-β-D-ribofuranosylpurine; MNBMG, 2-amino-S (2-methoxy-5-nitrobenzyl)-6-mercapto-9-β-D-ribofuranosylpurine; NEM-MI, S-(N-ethylmaleimido)-6-mercapto-9-β-D-ribofuranosylpurine; NEM-MG, 2-amino-S-(N-ethylmaleimido)-6-mercapto-9-β-D-ribofuranosylpurine; PBS, phosphate-buffered saline, pH 7.4; MCT, 20-methylcholanthrene transformed; BHK, baby hamster kidney; RSV, Rous Sarcoma Virus (Schmidt-Ruppen).

Complexation of Organomercurials with Mercaptonucleosides

Spectrophotometric titration of 6-MI, 6-MG, 8-MG and 4-TU with organomercurials (i.e., MB, *p*MBC and *p*MBS) were carried out in PBS at 22 °C. Solutions of 18–50 μM mercaptonucleosides dissolved in PBS were titrated by addition of small aliquots of 1–5 mM solutions of $HgCl_2$ or *p*MBS (in PBS) and of MB or *p*MBC (in ethanol). The spectra were recorded in a Cary 14 spectrophotometer. The dissociation constants of the various complexes were evaluated either by the method of Armor and Haim [1], or by the method of Scheit and Faerber [17]. The molar absorption values of free nucleosides used for these calculations were as follows: 6-MI, 22,900 ($\lambda = 322$ nm), 6-MG, 21,800 ($\lambda = 342$ nm), 8-MG, 20,800 ($\lambda = 300$ nm) and 4-TU, 21,200 ($\lambda = 330$ nm). The concentration of organomercurial solutions were determined gravimetrically. The concentration of the complexed nucleosides was calculated after subtracting the absorption values of free mercaptonucleosides at the above-mentioned wavelengths from those obtained after complexation with mercurials. Corrections were made for the small absorption of complexed mercaptonucleosides at these wavelengths (normally not more than 5 %). The molar absorptivity of the fully complexed *p*MBS-mercaptonucleosides were as follows: *p*MBS-6-MI, 19,000 ($\lambda = 311$ nm); *p*MBS-6-MG, 15,500 ($\lambda = 320$ nm); *p*MBS-8-MG, 19,500 ($\lambda = 280$ nm); *p*MBS-TU, 21,000 ($\lambda = 312$ nm). These and the aforementioned values were also used for the preparation of known solutions of either free or mercurial complexed mercaptonucleosides, all prepared in PBS medium.

Cell Cultures

MCT is a cell line derived from a tumor in golden hamsters after inoculation of 10^6 Hamster cells (20-methyl-cholanthrene transformed embryo cells, Huberman *et al.* [11]). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum (Gibco), 10 % triptose-phosphate, 2 mM glutamine and antibiotics (streptomycin, 400 μg/ml; penicillin, 400 U/ml; neomycin, 4 μg/ml and kanamycin, 5 μg/ml). BHK-21 cells obtained from Dr. I. MacPherson (Imperial Cancer Research Fund Laboratories, London) were cultured in RPMI 1640 medium (Biolab), supplemented with the same ingredients as those described above with the exception of fetal calf serum which was used at a final concentration of 10 %. RSV (Rous Sarcoma Virus) transformed hamster tumor cells (Flow Lab.) were grown in the same conditions as described for the MCT hamster cells.

Uptake Studies

Cells were plated in 5-cm plastic petri dishes (Nunc) at $8–12 \times 10^5$ cells per dish. They were grown to densities ranging from $8–30 \times 10^5$ cells per dish, unless otherwise specified. Prior to flux measurements they were washed twice with 5-ml aliquots of PBS at room temperature. For uptake studies (^{3}H) uridine or other (^{3}H)-labeled nucleosides at defined concentrations ($1–2 \times 10^6$ cpm/ml) were offered to each plate for 5 min at 20 °C. CAR uptake was measured for 10 sec at 20 °C. Fluxes were usually performed in duplicate. The uptake was terminated by quickly placing the cells on ice and washing 5 times with 5-ml portions of ice cold PBS. Ice cold TCA (5 %) was then added and the plates incubated for 5–30 min at 5 °C. The TCA-soluble fraction was counted for (^{3}H) in Triton-toluene based scintillation fluid using a Tricarb Scintillation Spectrometer (Packard).

Chemical modification of cells with mercurials was performed at room tmperature with freshly prepared PBS solutions of compounds and with minimal exposure to light.

At the end of either a 10 or 20-min incubation period, the cells were washed thrice with 5-ml portions of PBS at room temperature, and uridine uptake was subsequently measured, as previously described. Uridine uptake was also measured in the presence of defined concentrations of either NBMI, free mercaptonucleosides, or mercurial-mercaptonucleoside complexes in a slight (10–20 %) molar excess of mercaptonucleoside. The interaction between either (^{203}Hg) *p*MBS (Amersham, 8 Ci/mole) or (^{203}Hg) *p*MBS-MG (20 % molar excess of 6-MG) and MCT cells was followed by offering 20 μM concentrations of these compounds to cells for different periods of time (0.5–10 min). At the end of the interaction period, the cells were washed as described for uridine uptake and dissolved in 1 N NaOH (4 hr, 60 °C) and counted in a Gamma Scintillation Spectrometer (Packard).

The nucleoside-free space of MCT cells was measured by equilibrating cells at room temperature with (^{3}H) cytosine arabinoside (Amersham) at 2–4 μCi/ml (100 μM) and measuring the intercellular radioactivity after washing five times with 5-ml portions of ice cold PBS as recently described [6, 7]. This method is described in more detail in the accompanying paper [9]. All the kinetic constants, Hill coefficients and association-dissociation constants were computed from linear regression analysis performed on a Wang 2200 computer.

The effects of complex on uridine phosphorylation by broken cell preparations were performed as previously described for NBMI [6, 7].

Results

Interaction between Mercurials and Thiolated Nucleosides

The interaction between the organomercurial *p*-hydroxymercuribenzene sulfonate (*p*MBS) and four different thiolated ribonucleosides was studied spectrophotometrically (Fig. 1). The addition of stoichiometric amounts of organomercurials (R_2HgOH) to mercaptonucleosides (R_1-SH) leads to a complete hypsochromic shift of the base absorption band, suggesting the formation of $R-S-Hg-R_2$ species (Fig. 2; shown only for 6-mercaptopurine ribonucleosides). Similar results were obtained with either $HgCl_2$, MB, *p*MBC, or *p*MAED indicating that the common element, the $Hg-R_2$, moiety is the predominant factor in this interaction. The absorption spectra of 6-MI or 6-MG titrated with *p*MBS (or the other organomercurials) exhibited isosbestic points (Fig. 3). Similar spectral data obtained by titration of 6-MG with NaOH yielded a pK of 8.34 for the $R-SH$ group [8]. No hydrolysis to inosine or guanosine occurred upon addition of mercurial or base (up to pH 9) as judged by lack of spectral changes below 270 nm (at pH 7.4). The titration of mercaptonucleosides with organomercurials revealed strong interactions between the two species which resulted in the formation of 1:1 complexes (Fig. 4). Evaluation of the dissociation constants of the complexes K_D from data shown in Fig. 4 was possible

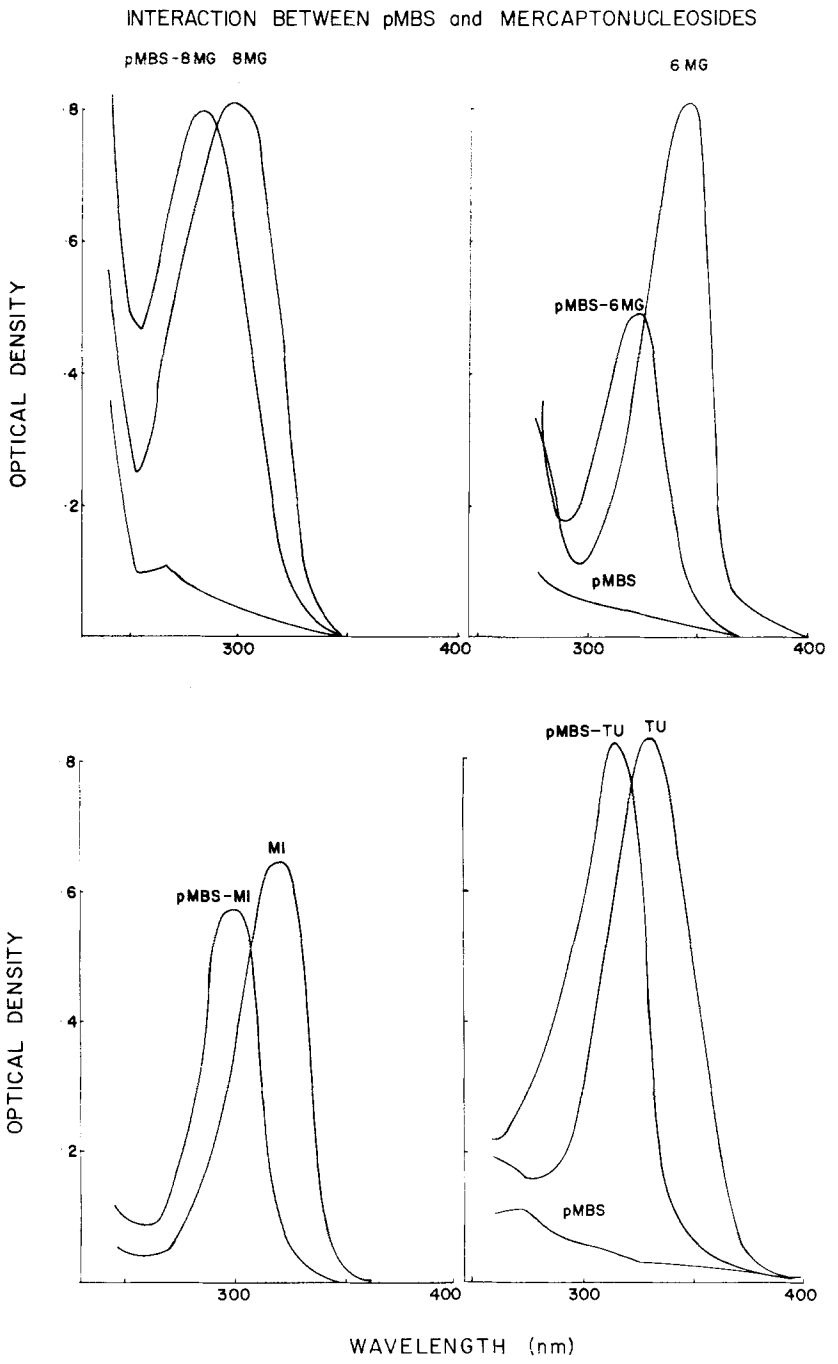

Fig. 1. Interaction between *p*-hydroxymercuribenzene sulfonate (*p*MBS) and mercaptonucleosides. Absorption properties of free and *p*MBS-complexed nucleosides in phosphate buffered saline (PBS) at pH 7.4 at 20 °C. Concentration, 40 μM. Chemical names of compounds are given in text

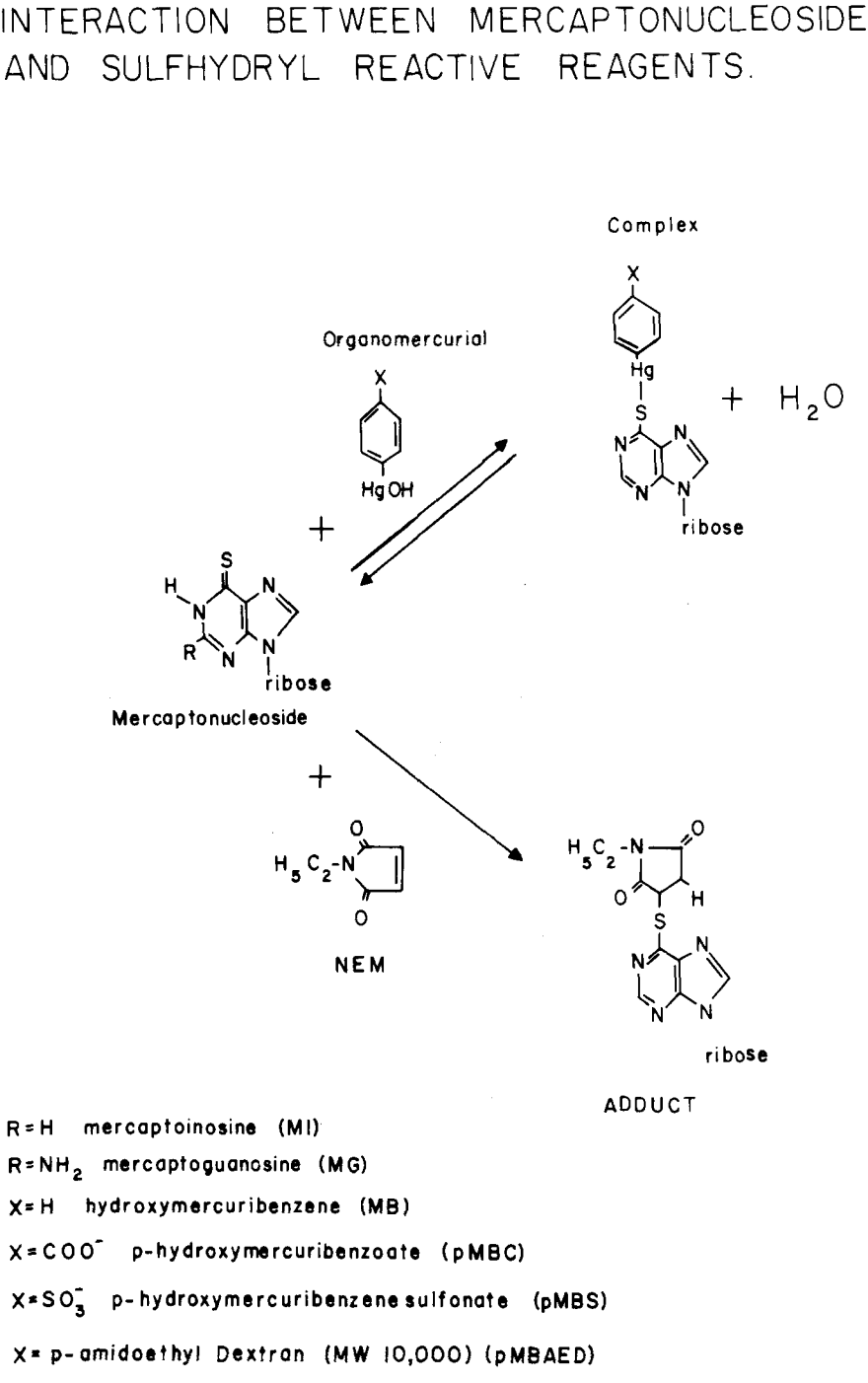

Fig. 2. Reaction of 6-mercaptonucleosides with organomercurials and with N-ethylmaleiimide (NEM)

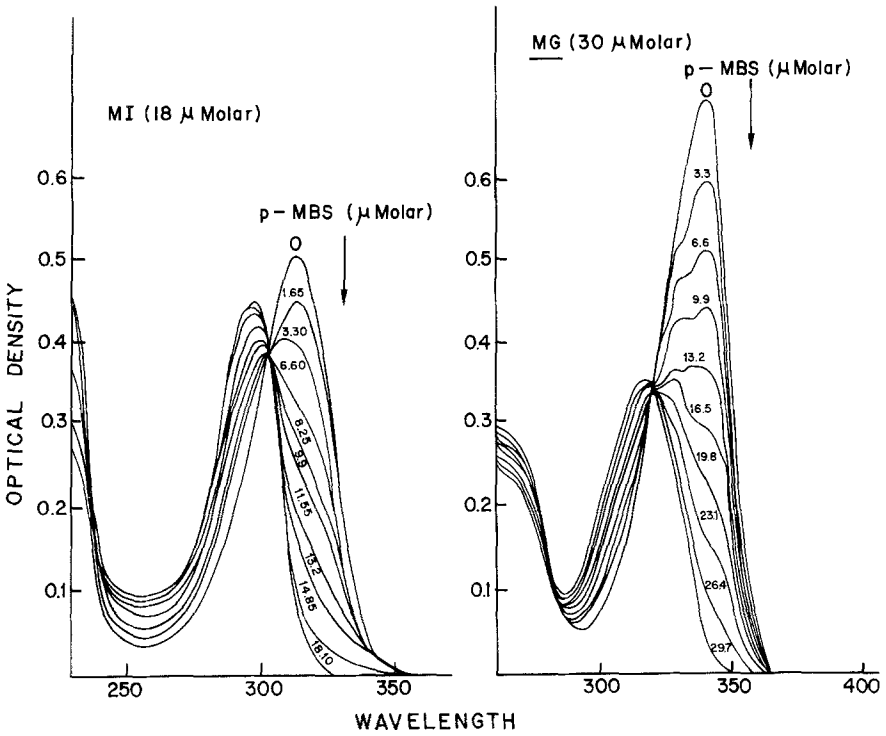

Fig. 3. Complexation of *p*MBS and 6-mercaptonucleosides. Solutions of 6-MI and 6-MG were titrated with *p*MBS in phosphate buffered saline (PBS) at pH 7.4 at 20 °C as indicated and the absorption spectra recorded

only toward the end of the titration curve due to the strong complexations displayed. The K_D values were computed from Armor and Haim [1] or Scheitt and Faerber [17] plots, but were obtained with relatively large standard errors (Table 1). It could be demonstrated that organomercurials also interacted strongly with 4-thiouridine (K_D about 0.15 μM), but relatively weaker with 8-MG ($K_D = 2.2$ μM).

Inhibitory Effects of Mercurials, Mercaptonucleosides and Complexes on Nucleoside Transport Systems

The uptake of (^{3}H) uridine by MCT hamster cells in culture was measured in the presence of either free mercaptonucleosides, free mercurials, or mercurial-complexed mercaptonucleosides (Fig. 5). Free mercaptonucleosides had only slight inhibitory effects on uridine uptake (PBS series), while free organomercurials stimulated it. However, the ad-

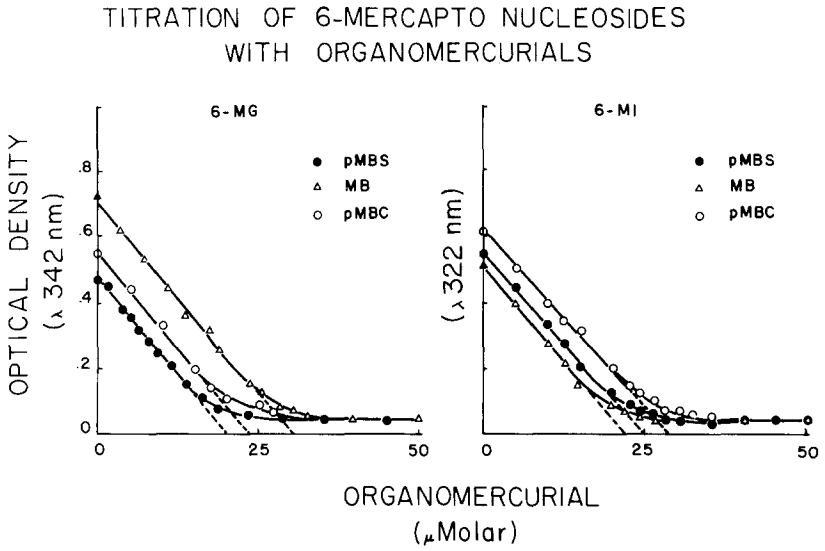

Fig. 4. Spectrophotometric titrations of 6-MI and 6-MG with various organomercurials. The initial concentrations of 6-MG were: 21 (●), 30 (△), and 24 (○) μM; those of 6-MI were: 25 (●), 22 (△), and 27 (○) μM. Small aliquots of 1–5 mM solutions of organomercurials (*p*MBS in PBS, MB and *p*MBC in ethanol) were added to a 3-ml solution of mercaptonucleosides (20 °C) and the optical absorption was measured 5 min later (6-MI at 322 nm and 6-MG at 342 nm) at 20 °C

Table 1. Dissociation constants (K_D) of mercaptonucleoside derivatives and their inhibition constants (K_i) related to uridine uptake in MCT hamster cells

Mercaptonucleoside derivative	K_D[a] (μM)	K_i^1[b] (μM)[b]	K_i^2[b] (μM)
*p*MBS-6-MG	0.15±0.06	2.7±0.6	3.2±0.8
*p*MBS-8-MG	2.2 ±0.9	ND[c]	>200
*p*MBS-6-MI	0.27±0.13	5.8±1.2	6.5±1.1
*p*MBS-4-TU	0.5 ±0.2	ND	>200
NEM-6-MG	–	ND	9.6±3.2
NEM-6-MI	–	ND	41 ±15

[a] K_D values were obtained by spectrophotometric titration of mercaptonucleosides with organomercurials as shown in Fig. 4 and others as described in [10]. The data were calculated according to [1] or (17).

[b] K_i^1 and K_i^2 refer to the inhibition constant K_i obtained from S/v *vs.* S plots (Fig. 7) and from v_o/v_I ("modified Dixon") plots (Fig. 8) respectively.

[c] ND (not determined).

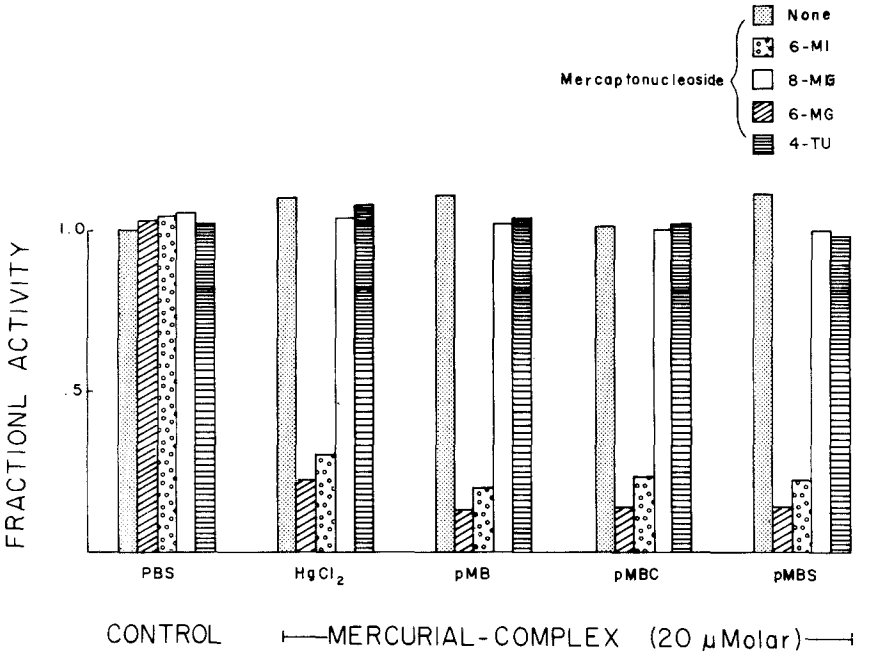

Fig. 5. Comparative effects of mercurials, free mercaptonucleosides and mercurial-mercaptonucleoside complexes on uridine uptake in MCT hamster cells. Uridine uptake was measured for 5 min at 20 °C (2×10^6 cells/plate) in the presence of free mercaptonucleosides (20 μM) in PBS medium (control) or in the presence of mercurial-mercaptonucleoside complexes (20 μM). The left column of each set represents the uptake obtained in the absence of mercaptonucleosides. All values are given relative to the control (PBS) in the absence of either mercurials or mercaptonucleosides. Details are given in *Materials and Methods*

dition of organomercurial-mercaptonucleoside complexes caused marked inhibitions of uridine uptake. The relative potency of the complexes depended primarily on the mercaptonucleoside moiety. Only the 6-mercaptopurineriboside-Hg$-X$ complexes elicited significant effects, the most prominent was displayed by 6$-$MG$-$Hg$-$R.

In order to ascertain whether the inhibition of the complex could be ascribed to the modified electronic structure of the nucleoside induced by Hg$-X$ binding rather than to the bulk presence of th X group, we have studied pH effects on MG structure and inhibition of transport. It was found that while the relative concentration of ionized and unionized MG changed considerably between pH 6 and 9 [8], uridine uptake remained constant in that pH range either in the absence or presence of 25 μM MG. Thus, the anionic form of MG did not afford inhibition. The studies shown in Fig. 5 also indicated that the hydrophobic/hydrophilic nature

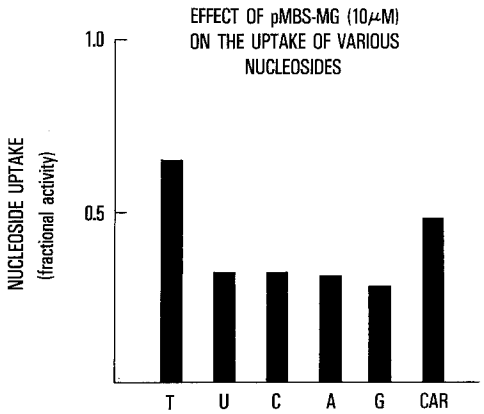

Fig. 6. Comparative effects of *p*MBS−MG on the uptake of various nucleosides. Nucleoside uptake was measured at 10 μM substrate except for CAR (60 μM) and in the presence of 10 μM of *p*MBS−MG. Data are given in terms of fractional activity relative to the control (no *p*MBS−MG)

of the R substituent (i.e., phenyl, benzoic or benzene sulfonic) in the R−Hg−MG of R−Hg−MI complexes did not significantly contribute to the inhibitory potency of the complexes. Thus, the major structure determinant of probe efficacy is probably associated with the structural alterations brought about exclusively by metal binding to thione groups.

The effects of the most potent of these complexes, *p*MBS−MG, were observed on the uptake of thymidine, cytidine, uridine, adenosine, and guanosine and on the transport of the nonmetabolizable cytosine-arabinoside (Fig. 6). The levels of inhibition attained on the various ribonucleoside uptake systems were very similar. The effects on thymidine and CAR, however, were much less pronounced. In broken cell preparations, neither *p*MBS−MG (20 μM) nor *p*MBS (20 μM) affected uridine conversion to uridine monophosphate.

The inhibitory effects of the complexes and of NBMI on nucleoside uptake were also evaluated in kinetic terms. Uridine uptake was first measured in the presence of constant concentrations of complexes (20 μM) and increasing concentrations of substrate (Fig. 7). The inhibition by *p*MBS−MG and *p*MBS−MI was competitive (K_i values were 3 and 6 μM, respectively; K_m was 50 μM). In order to determine whether the inhibitions were fully or partially competitive, uptake was measured at constant concentrations of uridine and increasing concentrations of complexes (Fig. 8). From the rates of uptake in the absence and presence of a competitive inhibitior, v_0 and v_I, respectively (19, 23, 24):

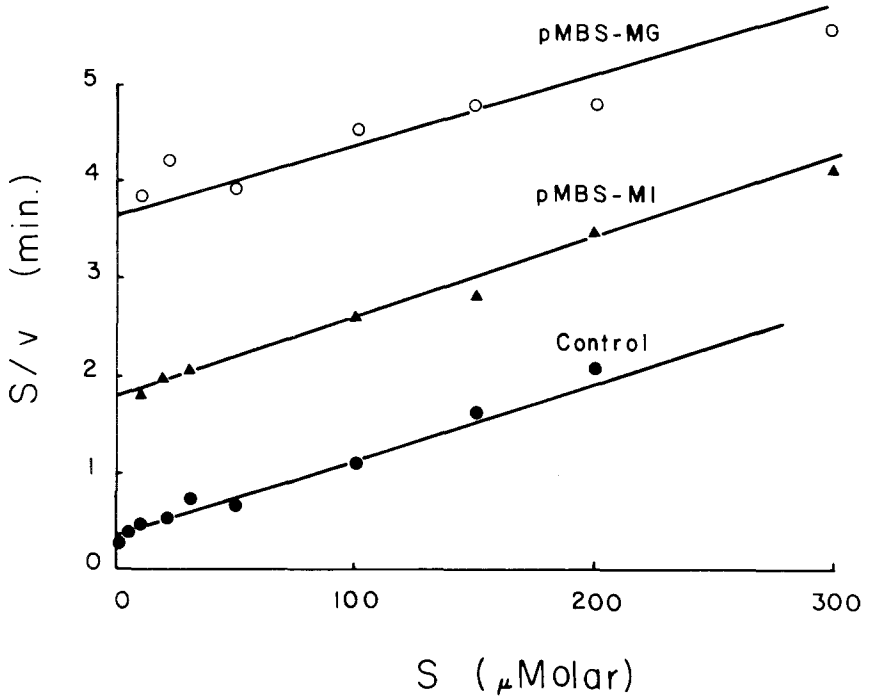

Fig. 7. Kinetic profiles of uridine uptake in the presence of *p*MBS−MI and *p*MBS−MG (Hanes-Woolf plot). Uridine uptake (v) was measured at different concentrations of substrate for 5 min at 20 °C in the presence of either *p*MBS−MI or *p*MBS−MG (at 20 μM). The values of v were calculated with the assumption that 1×10^6 cells have a 2-μl nucleoside free space. The maximal uptake, V, obtained either in the presence or absence of complexes $1.2 \pm 0.2 \times 10^{-4}$ M · min^{-1} or 2.4×10^{-10} moles 10^{-5} cells min^{-1}). The K_m of uridine was 50 ± 12 μM, and the K_i values of *p*MBS−MI and *p*MBS−MG were 5.8 ± 1.2 μM and 2.07 ± 0.6 μM, respectively. The plot is based on the equation: $[S]/v = [(1 + [I]/K_i) K_m/V] + [S]/V$ [19, 23, 24]

$$v_o = \frac{V \cdot [S]}{K_m + [S]} \quad \text{and} \quad v_I = \frac{V \cdot [S]}{K_m(1 + [I]/K_i) + [S]} \tag{1}$$

we obtain

$$\frac{v_o}{v_I} = \frac{K_m(1 + I/K_i) + [S]}{K_m + [S]} \tag{2}$$

and at $[S] \ll K_m$

$$\frac{v_o}{v_I} = 1 + [I]/K_i. \tag{3}$$

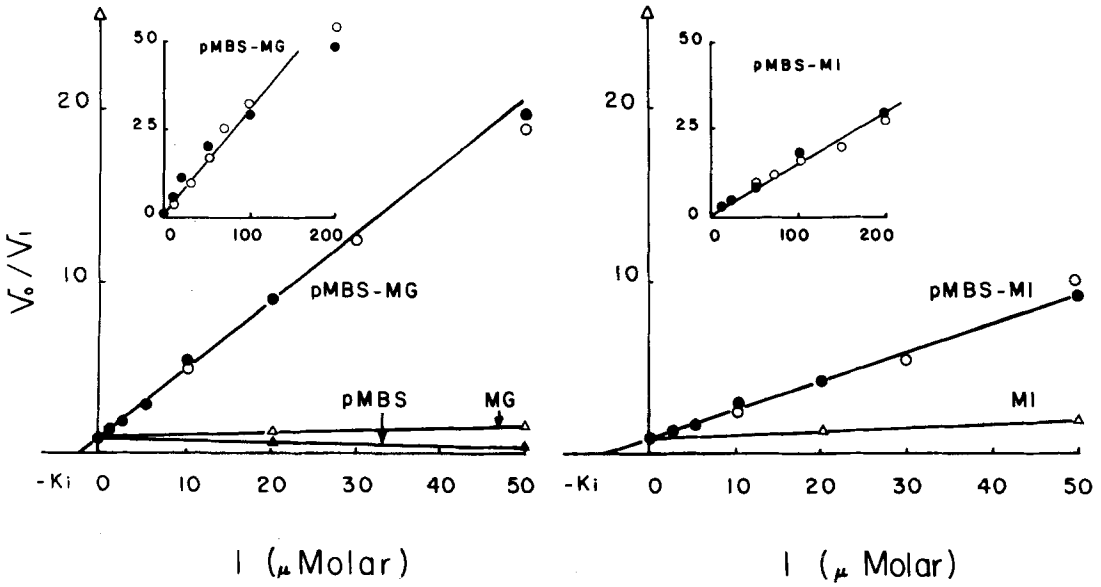

Fig. 8. Inhibitory profiles of *p*MBS−MG and of *p*MBS−MI on uridine uptake (Dixon plots). Uridine uptake was measured at 1 μM of substrate in the presence of either *p*MBS, MG, MI, *p*MBS−MG, or *p*MBS−MI. Data are given in terms of a modified Dixon plot as described in the text. Computed K_i values are shown in Table 1. Filled and open circles denote data points obtained from two separate experiments

By plotting v_o/v_I *vs.* I, we obtained the normalized Dixon plot, as seen in Fig. 8. The intercept on the I axis yielded directly the $-K_i$ value. Inhibition by either *p*MBS−MG or *p*MBS−MI went to completion (more than 90%, i.e., $v_o/v_I = 10$), unlike the S-benzylated derivatives of MI and MG which were shown to cause only partially competitive inhibitions [6 and 7]. The K_i values obtained for *p*MBS−MG and *p*MBS−MI, 3.2 and 6.5 μM, respectively, were very similar to those obtained by the former method (S/V *vs.* S, Fig. 7) (*see* Table 1 for compilation of data).

In Fig. 8, it was shown that neither 6-MG nor 6-MI have significant inhibitory effects on uridine uptake, whereas *p*MBS alone stimulated it. Table 1 shows data from similar studies with *p*MBS−TU and *p*MBS−8MG. The effects observed in MCT cells were also obtained in human red blood cells, BHK-21 cells, in secondary hamster embryo fibroblasts and in RSV Rous-sarcoma (Schmidt-Ruppen) virus transformed tumor cells in culture. In all of these cases, the inhibition by the *p*MBS-6-mercaptonucleoside complexes were fully competitive, displaying K_i

values in the lower μM range. Essentially equivalent results were obtained with MB and *p*MBC complexes of 6-MG and 6-MI.

Interaction of Organomercurials and of pMBS – MG Complexes with the Uridine Transport System

A. Reversibility. The competitive nature of *p*MBS – MG inhibition of uridine uptake suggested and data in Table 1 showed that *p*MBS – MG inhibition is reversible. They also indicated that inhibition was produced by *p*MBS – MG as a unit and not by the *p*MBS moiety alone as a result of affinity labeling (i.e., if *p*MBS would have been left covalently bound while MG would have been released).

B. Effects of preincubation with organomercurials. The preincubation of cells with *p*MBS led to a modification of the uridine transport system, as manifested in the following properties. On one hand, uridine uptake was stimulated to a significant degree (Table 2 and Fig. 9). The stimu-

Table 2. The effect of probes in preincubation media on the subsequent inhibition of uridine uptake by various probes

Preincubation media	Wash (×5)	Flux media	Uptake (cpm/5 min/10^6 cells)
PBS	±	control	4980
PBS	±	*p*MBS – MG	650
*p*MBS – MG	+	control	4930
PBS	±	+ MG	4010
pMBS	±	control	5760
*p*MBS	±	+ *p*MBS – MG	230
*p*MBS	–	+ MG	4570
*p*MBS – MG	+	+ *p*MBS – MG	4660
*p*MBS + *p*MBS – MG	+	control	5800
*p*MBS	+	+ MG	4890
*p*MBAED	±	control	5950
*p*MBAED	±	+ *p*MBS – MG	210
*p*MBAED – MG	+	+ *p*MBS – MG	4830

Cells (0.8×10^6/plate) were preincubated for 10 min at 37 °C in phosphate-buffered saline (PBS) containing either organomercurials (*p*MBS or PMBAED) or organomercurial-mercaptoguanosine complexes (at 20 μM). The cells were washed subsequently (+) 5 times with 5-ml aliquots of PBS and (^{3}H) uridine (10 μM, 4×10^5 cpm/ml) uptake was measured in the absence (control) or in the presence of various compounds (all at 20 μM), as described in *Materials and Methods.* All samples were run in duplicate (SE: ±10%). In cases where washings after preincubation had no effect on the final values of uptake, the symbol ± was used, and the four data points (two duplicates) were pooled and averaged.

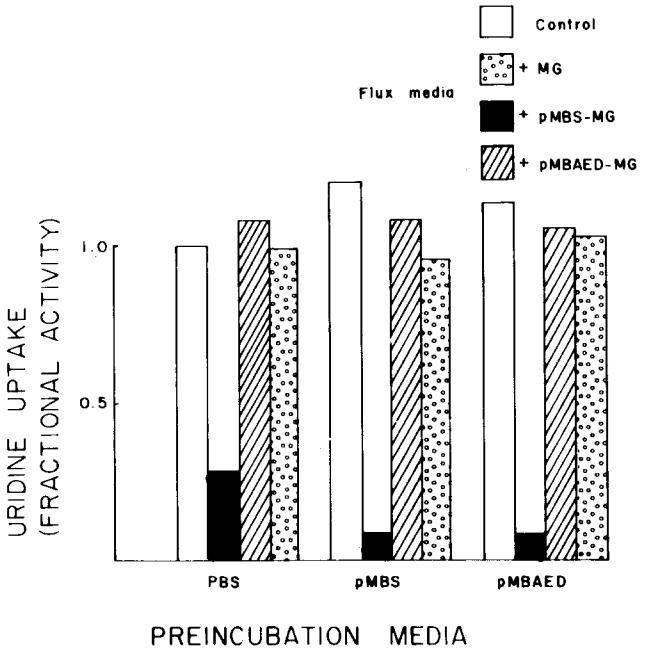

Fig. 9. The effect of preincubation with organomercurials on the subsequent inhibition by *p*MBS – MG. Cells (3.4×10^6/plate) were preincubated for 10 min at 20 °C in the presence of 10 μM of either *p*MBS or *p*MBAED (10,000 mol wt) in PBS media, and washed thrice with 5 ml PBS. Uridine uptake was measured in the presence of 10 μM of either free or organomercurial-complexed MG in PBS medium. Data are given in terms of fractional activity relative to control (preincubation in PBS and flux media in PBS)

lation varied between 20 and 80 percent, depending on the time of preincubation, concentration of *p*MBS, and density of cells *(to be published)*. Other organomercurials, like the macromeolcular *p*MBAED (10,000 dalton), produced similar stimulatory effects. On the other hand, preincubation with either *p*MBS or *p*MBAED resulted in an irreversible potentiation of *p*MBS – MG inhibition (Table 2 and Fig. 9). In order to assess whether the latter was evoked by *p*MBS binding to cells as compared to complexation of *p*MBS remaining in the cells with traces of free MG present in the solution of complex (e.g., approximately 2 μM complex), the following observations were considered:

(1) Addition of free MG to cells preincubated with *p*MBS and washed had no significant inhibitory effects (Table 2).

(2) Preincubation of cells with *p*MBS – MG did not potentiate the inhibitory effects of *p*MBS – MG present during uridine uptake.

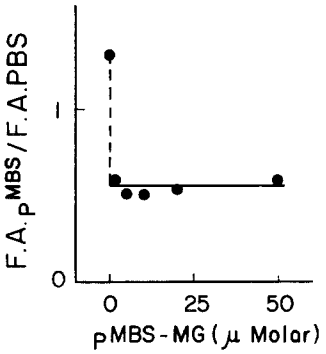

Fig. 10. Relative inhibitory effects of *p*MBS – MG after preincubation with either *p*MBS or buffer. Cells (3.5×10^6/plate) were preincubated with buffer (PBS) or *p*MBS 50 μM for 10 min at 22 °C and then washed. Uptake of uridine (10 μM) was measured in the presence of *p*MBS – MG. Data are given as the ratio of fractional activity (*F.A.*) of uridine uptake in cells treated with *p*MBS relative to control (PBS)

(3) Although complexes of *p*MBAED – MG had no inhibitory effects on uridine uptake (Fig. 9), *p*MBAED potentiated the effect of *p*MBS – MG (Table 2).

The inhibitory effects of *p*MBS – MG after preincubation with *p*MBS relative to the inhibition caused by *p*MBS – MG alone (i.e., preincubation with buffer) are given in Fig. 10. The effects of free organomercurial and complex were not additive; they remained constant in the range of concentrations of complex used, except at zero *p*MBS – MG, where *p*MBS caused stimulation. *p*MBS reduced the V of uridine uptake by half in the presence of *p*MBS – MG, while the apparent K_m remained essentially the same as that obtained in the presence of *p*MBS – MG (no *p*MBS) (Fig. 7). These results suggested that free *p*MBS and complex act at distinctly different sites.

Inhibition by *p*MBS could not be elicited unless the complex *p*MBS – MG was present in the flux medium (Table 2). Preincubation with *p*MBS – MG in the presence of excess *p*MBS and subsequent washing had essentially the same stimulatory effects as preincubation with *p*MBS alone, indicating that the complex did not interfer with *p*MBS binding.

C. Site of interaction of pMBS – MG and its relationship to NBMI. Although inhibition by *p*MBS – MG was distinctly different from that of NBMI [7] (the first was fully competitive, whereas the second was only partially competitive), similarities in *p*MBS synergistic effects suggested that both compounds interact with the uridine transport system at

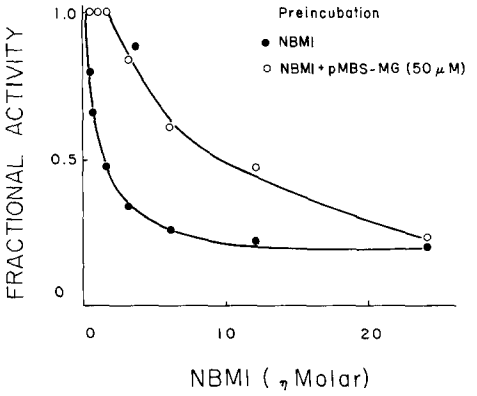

Fig. 11. Cells (2.5×10^6/plate) were preincubated for 10 min at room temperature (22 °C) with NBMI either in the absence or presence of *p*MBS – MG (50 μM) and washed thrice with 5 ml of PBS. Uridine uptake was measured for 5 min at room temperature (10 μM substrate). Data are given as fractional activity relative to the control (preincubation with no NBMI either in the presence or absence of *p*MBS – MG)

equivalent sites. This was tested by preincubating cells with increasing concentrations of NBMI either in the presence or in the absence of *p*MBS – MG. The cells were subsequently washed to remove *p*MBS – MG and free NBMI, and uridine uptake was then measured (Fig. 11). Preincubation with NBMI alone followed by extensive washing caused the typical partial inhibition; *p*MBS – MG itself followed by washing did not cause any detectable effects on uridine uptake. Similar experiments carried out with *p*MBAED – MG complexes failed to show protection from NBMI. However, from the above data alone, it could not be resolved whether *p*MBS – MG interacted exclusively with NBMI sites. To accomplish that, uridine uptake was measured in the presence of NBMI (at saturating inhibitor concentration, i.e., 20 nM) and of *p*MBS – MG at concentrations which did not offer protection from NBMI, i.e., 20 μM (based on data presented in Fig. 11). The results shown in Table 3 indicated that *p*MBS – MG and NBMI effects were additive and suggested that *p*MBS – MG complexes interacted also at sites not directly affected by NBMI. Nonetheless, binding/inhibition relationships as manifested in the Hill plots [19] (Fig. 12) showed a similar 1:1 stoichiometry for either NBMI, *p*MBS – MG or *p*MBS – MI.

The interactions between *p*MBS and *p*MBS – MG and hamster cells were also studied using (^{203}Hg) labeled compounds (Fig. 13). Uptake of either free or complexed (^{203}Hg) *p*MBS displayed a fast component operative even at 0 °C, which could probably be equated with surface

Table 3. Additive inhibitory effects of NBMI and *p*MBS−MG on uridine uptake

Flux media	Uridine uptake (cpm/5 min/10^6 cells)
Control	5120±95
+NBMI	1740±80
+*p*MBS−MG	650±55
+NBMI+*p*MBS−MG	270±30

Uptake of (^{3}H) uridine (10 μM, 5×10^5 cpm/ml) was performed in the presence of either NBMI (20 μM), *p*MBS−MG (20 μM), or both inhibitors, as described in *Materials and Methods*. Samples were run in triplicate and values were averaged. The value of radioactivity associated with cells after 20 sec incubation with (^{3}H) uridine at 0 °C was 250±50 cpm/10^6 cells.

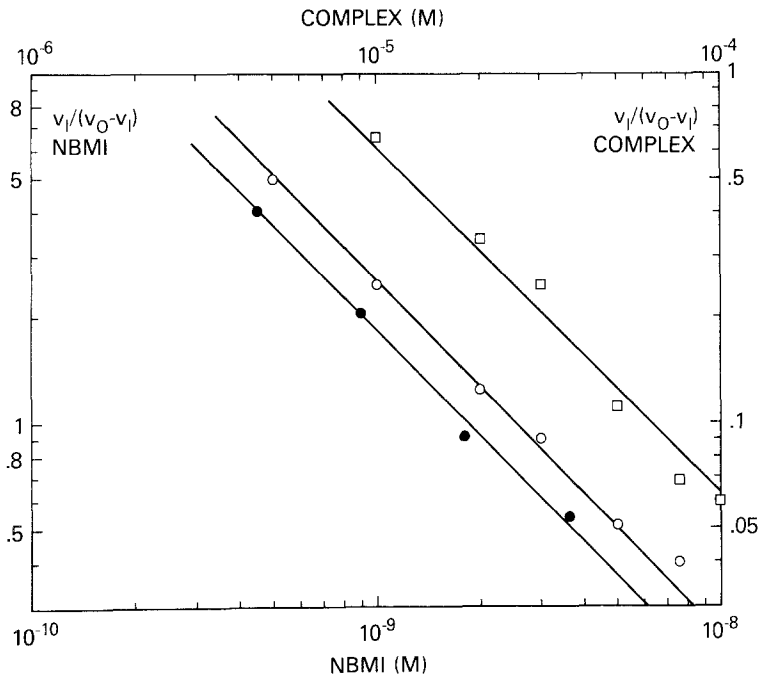

Fig. 12. Hill plots of inhibition of uridine uptake by various probes. Plots were done as in reference [19] with data given in Fig. 8 and reference [6]

binding. The latter was much larger for free *p*MBS (10^7 sites per cell) than for *p*MBS−MG (6×10^6 sites per cell) after pretreatment with NBMI (4×10^6 sites per cell). A second component of uptake observable between 0–10 min at 20 °C shows slower rate constants but similar

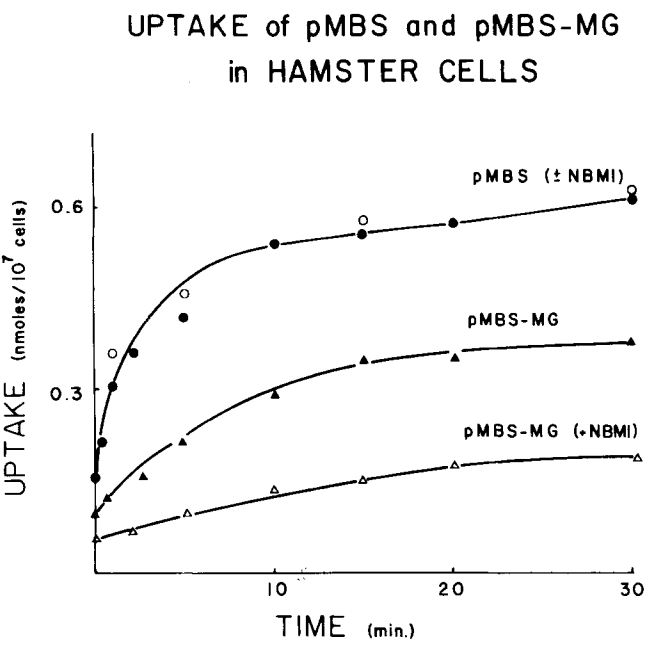

Fig. 13. Cells (8×10^6/plate) were preincubated with either PBS or NBMI (60 nM) for 10 min at 22 °C (treatment given in brackets). The uptake of either (^{203}Hg) *p*MBS or (^{203}Hg) *p*MBS – MG (20 μM, 1.5×10^3 cpm/mole) was followed at 22 °C. Zero time values were obtained by exposing cells to the compounds for 1 min at 0 °C. At indicated times, cells were washed 5 times with 5 ml of PBS at 0 °C. The (^{203}Hg) radioactivity was extracted with 1 ml of NaOH for 4hr at 50 °C and counted

behavior as the initial component. Pretreatment with NBMI (60 nM) reduced uptake of *p*MBS – MG, but not of *p*MBS. The amount of material incorporated into the cells (binding and transport) during this phase (first 10 min) was 25, 15 and 7 μM for *p*MBS, *p*MBS – MG and *p*MBS – MG (NBMI pretreated cells), respectively. The third component observable between 10–30 min displayed even slower rate constants but similar differences in uptake rates of free and complexed mercurials. Washing of cells at 20 °C instead of at 0 °C resulted in no reduction of (^{203}Hg) radioactivity associated with cells, indicating that the mercurial-containing moiety of either free or complexed *p*MBS interacted irreversibly (i.e., it cannot be reversed by washing with warm buffer) in conditions where nucleoside transport activity was fully restorable.

D. Chemical specificity of inhibitors: Structural-functional relationships. Mercurial complexes of MI and of MG inhibited uridine uptake at

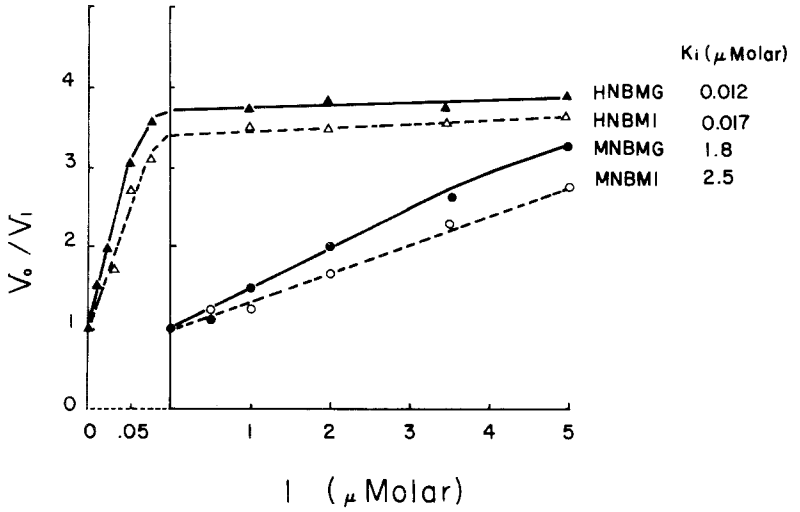

Fig. 14. Comparative inhibition profiles of 2-hydroxy and 2-methoxy analogs of S-nitrobenzyl derivatives of 6-mercaptopurine ribosides. (^{3}H) Uridine uptake was followed in the presence of constant concentration of substrate (10 μM) and increasing concentrations of inhibitor (Dixon plot)

least 100-fold more efficiently than free mercaptonucleosides (Fig. 5). Although the hydrophobic nature of the S-substituent did not seem to confer these probes with extra inhibitory potency, this possibility was further tested with hydroxynitrobenzyl (HNB) and methoxynitrobenzyl (MNB) derivatives of MG and MI. As demonstrated in Fig. 14, the more hydrophobic MNBMI and MNBMG exhibited 100-fold higher K_i values than the respective hydroxynitrobenzyl derivatives (2 μM *vs.* 0.02 μM), thus arguing against lipid solubility as the determining factor of probe potency.

Discussion

A variety of high affinity probes obtained by S-arylalkylation of 6-mercaptonucleosides have previously been shown to inhibit uptake of ribonucleosides by acting directly at the transport level, without affecting the intracellular metabolic conversion of these nucleosides [5, 6, 7, 9 and 14]. In an analogous fashion we could demonstrate that the probes used in the present work also affected the transport component of ribonucleoside uptake and of CAR.

Chemical Features of Inhibition of Ribonucleoside Transport in Hamster Cells

A common feature displayed by ribonucleoside transport systems in MCT hamster cells was a susceptibility to inhibition by analogs of inosine (I) and guanosine (G) obtained either by substitutions or additions at the 6-position of the purine ring. The inhibitory power and the "modus operandi" of these probes depended on the nature of the S-substituting group. In an extensive study of structure-activity relationships of probes for nucleoside transport systems in red blood cells [15] and Hela cells [14], it was shown that either S-, O-, or N-arylalkylation at the 6-position of the purine riboside rings conferred the probes with an enormous inhibitory power. The inhibitory potency correlated to a large extent with the hydrophobicity of the substituting moiety. We observed in hamster cells in culture that either S-nitrobenzylation or S-hydroxynitobenzylation of 6-MI and of 6-MG also led to probes which display unusually high affinities ($K_i = 0.25 - 5\,\text{nM}$) for ribonucleoside transport entities [6, 7]. However, the inhibitory power did not correlate with the hydrophobic properties of the S-substituents. In fact, the studies with 2-hydroxynitrobenzyl, 2-methoxynitrobenzyl and NEM derivatives of 6-MG and 6-MI indicated, that the opposite might be the case (Fig. 4, Table 1), suggesting that factors other than hydrophobicity confer inhibitory potency to the thionucleoside core or, alternatively, that above some critical molecular size the above property is no longer the determining factor [15]. The effects obtained with organo-mercurial substituents are also in line with those views. For the specific case of the macromolecular PMBAED – MG, factors such as limited acceptibility to the inhibitory sites could explain the poor inhibitory effects (Table 2).

Properties of Ribonucleoside Transport Systems as Studied with Mercurial-Mercaptonucleoside Complexes

Complexation of mercaptonucleosides with mercurials was reversible and stoichiometric (Figs. 3 and 4). The computed dissociation constants showed strong interactions by 6-mercapto-purine ribosides, particularly 6-MG (Table 1). This may result from alleged interactions between the mercury atom and N-7 of the purine ring, leading to formation of an additional five-membered ring (10, 12, 18).

Ribonucleoside transport systems displayed chemical selectivity properties in their interactions with different kinds of mercaptonucleosides and their mercurial complexes (Figs. 5 and 6). Similar to S-alkylated nucleosides [7, 14 and 15], the mercuriated derivatives of S-6-purine ribosides were the most prominent inhibitors of nucleoside transport in either hamster cells in culture or in red blood cells. Neither 8-mercaptopurine riboside complexes nor thiopyrimidine complexes had significant effects on nucleoside transport. Thus, the inhibitory potency of complexes was not related to the presence of R−Hg per se, but to the actual position of complexation. Interestingly, the K_i of inhibitors of uridine transport correlated with the K_D of complexation (Table 1) and suggested that factors which apparently determine the stability of the complexes, might also contribute to their "affinity" for nucleoside transport systems. Another aspect of specificity was related to the lipophilic character of the R group in the R−Hg substituting moiety, which apparently had no effect on the inhibitory property of MG and MI complexes.

The interaction between *p*MBS−MG and cells could be separated into a fast and slow phase (Fig. 13). The first probably represented binding to sites located on the cell exterior. These sites were probably related to inhibition of nucleoside transport on the basis that the latter was elicited within seconds following addition of *p*MBS−MG. In addition, the specific inhibitor NBMI reduced the above interaction by 2 picomoles *p*MBS−MG/10^5 cells, suggesting that about this number of sites could be involved in nucleoside transport system. The slow phase of interaction represented penetration into cells. This component was also reduced by NBMI and by uridine, although not completely. However, a substantial fraction of *p*MBS−MG penetration could conceivably have proceeded by nucleoside transport systems. However, neither free *p*MBS nor free *p*MBAED could be shown to affect any of the above components of interaction.

Unlike S-arylakylated analogs, inhibition of uridine uptake by complexes was fully describable in terms of a single competitive mechanism (Figs. 7, 8 and 12), thus suggesting that a single complex molecule acted on the nucleoside transport systems by affixing directly at the substrate site. This, in turn, has been shown to protect the sites from NMBI binding (i.e., inhibition) (Fig. 11), and suggested commonality of substrate, complex, and NMBI sites. Furthermore, the fraction of nucleoside transport refractive to NBMI inhibition could be fully inhibited by *p*MBS−MG at concentrations which did not affect NBMI binding

(Table 3). Thus, NBMI and *p*MBS – MG produced additive effects at low concentration of complexes, but competed with each other at higher concentrations of the latter. These phenomena were apparently in line with the suggestion that uridine transport in MCT hamster cells [9] was heterogeneous. Thus, one system was inhibited by either NBMI or *p*MBS – MG, while the other was inhibited only by *p*MBS – MG.

Heterogeneity was also demonstrated in the effects of SH reagents. Penetrable and nonpenetrable organomercurials had dual effects on nucleoside transport. While they stimulated transport in the absence of externally added probes, they supported inhibition in the presence of the probes (Figs. 5 and 9, Table 2). Removal of probes, however, restored the initial stimulation (Table 2). On the other hand a lipophylic SH reagent as N-ethylmaleimide only elicited inhibitory effects on nucleoside transport [9].

Most of the data presented in this and the accompanying work [9] were rationalized in terms of the schematic model shown in Fig. 15. Kinetic evidence for the existence of multiple transport systems which provide parallel routes of entry for nucleosides was presented elsewhere (Cabantchik & Eilam, *submitted for publication*). System *I* is the predominant entry route. The two systems have different susceptibilities to analogs of MG and MI and to SH reactive reagents. While NBMI inhibited only System *I*, *p*MBS – MG inhibits both *I* and *II*. The inhibitory profiles of nucleoside transport by these compounds, the additivity of their effects, and the competitive nature of their inhibition

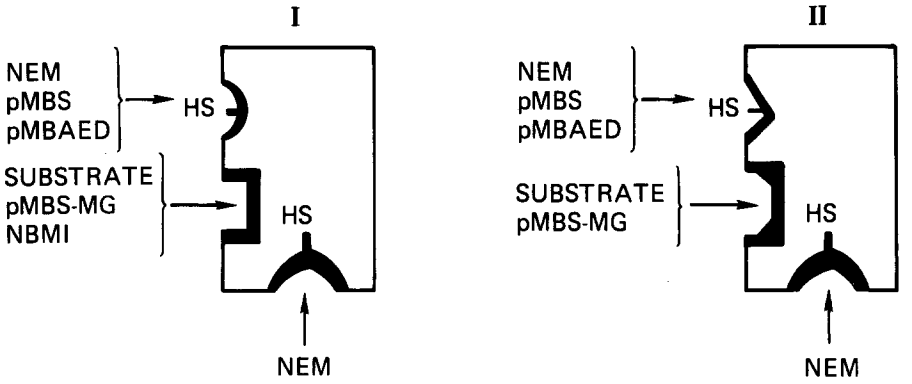

Fig. 15. Schematic model of nucleoside transport systems, *I* and *II* represent two different types of transport systems for the same nucleoside which contain substrate binding sites with distinct chemical characteristics and distinct SH sites involved either in stimulation or inhibition of the function. The site reactive to NEM alone is considered to be the inhibitory site

were fully in line with the above hypothesis. The various pieces of evidence presented earlier in this work would also rule out the possibility that the two types of analogs affect uptake of nucleosides in series rather than in parallel. Failure of *p*MBAED – MG to affect the systems suggested that substrate binding sites are either inaccessible or unreactive to macromolecular probes. Differences between Systems *I* and *II* could also be demonstrated in their response to chemical modifiers, particularly after addition of NBMI. At least one of the two systems was stimulated either by *p*MBAED, *p*MBS, or even by NEM at low concentrations, but both systems were definitely inhibited by NEM alone. Thus, at least two types of SH groups are likely to be related to modulation of transport activity by SH-reactive reagents, one for stimulation and the other for inhibition. The two sites differ topographically, although they are indirectly related insofar as either *p*MBS or *p*MBAED supported rather than eliminated inhibition by NEM [9]. Some relationship between the stimulatory site and the substrate site in System *I* was also reflected in the increased susceptibility to either *p*BMS – MG or NBMI after reaction with organomercurials. In this case neither the apparent K_m for substrate nor the K_i of the probe were altered. Only V was susceptible to modification by organomercurials [6, 7]. Preliminary work indicates that the number of NMBI sites did not change in the presence of *p*MBS, so that the factor affected by the latter is likely to be the turnover rate of the transport system.

Some molecular details of the stimulatory mechanism are presently being explored in the context of the work herewith presented and in relation to the question of modulation of transport activities by ligand membrane interaction.

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