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Inhibition by Nitrobenzylthioinosine of Adenosine Uptake by Asynchronous HeLa Cells

ALAN R. P. PATERSON, LORI R. BABB, JOHN H. PARAN, AND CAROL E. CASS

Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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SUMMARY

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Initial rates of adenosine uptake by HeLa cell monolayers were measured by a replicate culture technique. Under the assay conditions, the cellular content of adenosine was almost entirely in the form of metabolites, primarily ATP, ADP, and AMP, and thus the time course of adenosine uptake was essentially that of the formation of the adenosine phosphates; both time courses were linear and could be extrapolated through zero time. Adenosine uptake was mediated by a mechanism that appeared to be distinct from the thymidine, uridine, and guanosine uptake mechanisms. In the presence of nitrobenzylthioinosine (NBMPR), mediated entry of adenosine was eliminated and a nonsaturable component of uptake, evidently simple diffusion, was apparent. In the presence of partially inhibitory concentrations of NBMPR, the apparent K_m values of the uptake process were increased and V_{max} values were unchanged. In a structure-activity study, comparisons were made of inhibition of adenosine uptake by graded concentrations of various nucleoside derivatives related to NBMPR. Inhibitors more potent than NBMPR were identified, and it was apparent that the 2'-hydroxyl group of NBMPR and related compounds was not involved in the cell-inhibitor interaction. NBMPR and its 5'-monophosphate were comparable inhibitors of adenosine uptake.

INTRODUCTION

The kinetics of adenosine uptake, and inhibition of this process by related permeants and by specific inhibitors, have demonstrated clearly the existence of a saturable uptake mechanism[1] in erythrocytes (1-5), cultured cells (6-8), polymorphonuclear leukocytes (9), and myocardium (10, 11). At high concentrations, adenosine may enter cells by simple diffusion (4, 5, and present study).

Specific mechanisms also mediate the uptake of nucleosides other than adenosine by animal cells (12, 13). The nucleoside uptake mechanism of human red cells (1, 2) and that of rabbit polymorphonuclear leukocytes (9) each accept a variety of substrates, whereas in cultured Novikoff hepatoma cells the uptake mechanisms for adenosine, uridine, thymidine, and inosine-guanosine have properties suggesting that each is distinct (7, 12); the

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[1] The term "uptake mechanism" in the present context includes both the transport and metabolic events involved in nucleoside uptake.

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present report and other data from this laboratory (14, 15)[2] indicate that HeLa cells also possess several distinct mechanisms for nucleoside uptake. Whether this specificity is imparted at the level of transport or at the level of nucleoside metabolism is not yet apparent.

Uridine and thymidine transport by human erythrocytes, cells which do not metabolize either permeant, have the characteristics (1, 16) of classical facilitated diffusion processes (17). In cells that metabolize nucleosides, internalized permeant does not accumulate as free nucleoside but as nucleotide metabolites, as the present study illustrates. In such cells, nucleoside transport has been studied primarily by measuring initial rate kinetics of permeant uptake. Nucleoside permeation may be studied under special circumstances that preclude anabolism (18, 19), but the permeation of adenosine, or of other nucleosides, has not been established as a facilitated diffusion process operating independently of nucleoside anabolism.

Previously we reported that nitrobenzylthioinosine and various S^6 derivatives of 6-thiopurine nucleosides were potent inhibitors of uridine and thymidine transport in human erythrocytes and were bound tightly, but reversibly, to particular membrane sites (2, 16, 20, 21). These sites, evidently on the nucleoside transport mechanism, appear to be distinct from the permeation sites (22). NBMPR[3] and related compounds are also potent inhibitors of nucleoside uptake in other cell types, including HeLa cells (15, 23), canine myocardium (10), 3T3 cells (24), and MCT cells (25, 26). NBMPR binds with high affinity to MCT cells (a cultured cell line derived from a hamster tumor), which possess 4.7–7.7 $\times$ 10^4 binding sites per cell (25, 26). NBMPR inhibits the uptake of thymidine, uridine, inosine, and guanosine[2] by HeLa cells, which possess sites (about 10^5/cell at 20°) that bind NBMPR with high affinity (K_{diss} = 10^{-9} at 20°) (27).

In the present study, initial rates of uptake of adenosine by HeLa cells were assayed with a replicate monolayer technique. NBMPR was employed as a probe of certain aspects of adenosine transport, and the ability of NBMPR to inhibit adenosine tranport was compared with those of various related compounds.

MATERIALS AND METHODS

HeLa cell stocks were maintained as monolayer cultures in antibiotic-free Eagle's minimal essential medium supplemented with 10% calf serum; after seven or eight weekly transfers, such cultures were restarted from frozen stocks. Inocula from stock monolayers were expanded in spinner cultures and then in suspension cultures kept under continuous agitation with vibrating mixers (Vibro-Mixer, model E1, Chemapec, Hoboken, N. J.). The medium (MEM-S) for the spinner and suspension cultures consisted of calcium-free MEM supplemented with 5% calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM HEPES (pH 7.4.). In the suspension cultures, cell concentrations were kept below 5 $\times$ 10^5 cells/ml by dilution, and cell proliferation was exponential, with doubling times of about 20 hr.

For the determination of adenosine uptake rates, replicate cultures were prepared in 2-ounce prescription bottles (Brockway Glass, Brockway, Pa.) "conditioned" by 24 hr of exposure at 37° to MEM containing 20% calf serum. Bottles were inoculated with 10^6 cells from Vibro-Mixer cultures in MEM-S with final concentrations of calf serum and calcium salts adjusted to 7.5% and 0.9 mM, respectively, and were incubated at 37° for 24 hr in air with 5% CO_2. Cell attachment was 50–65%, and after a 4-hr lag cell proliferation was exponential (see Fig. 3). The replicate cultures were cooled to 20° just prior to assays of adenosine uptake.

A previously described procedure (15)

[2] A. R. P. Paterson, and C. E. Cass, unpublished observations.

[3] The abbreviations used are: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBMP, 6-[(4-nitrobenzyl)thio]purine; NBTG, 2-amino-6-[(4-nitrobenzyl)thio]purine; MEM, Eagle's minimal essential medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; PCA, perchloric acid; PEI, polyethyleneimine.

was employed to assay adenosine uptake from transport medium (MEM-T), which consisted of $NaHCO_3$-free MEM with 20 mM HEPES (pH 7.4 at 20°) plus a 12 mM increment in NaCl. Three monolayer cultures were employed per assay, and each was processed individually as follows. To initiate an interval of uptake, the monolayer was rapidly immersed (15) in 4.0 ml of MEM-T containing [2-^{3}H]adenosine (2 μCi/bottle). The latter was removed by rapid suction 5 sec before the end of the uptake interval by flooding the cell sheet with 60 ml of ice-cold 0.154 M NaCl: after 15 sec, the bottle was thoroughly drained. NCS digests of cell sheets (NCS tissue solubilizer, Amersham/Searle) were dissolved in Bray's counting solution (28) and assayed for ^{3}H by liquid scintillation counting. From each replicate set, four to eight cultures were assayed for cell number using an electronic particle counter.

To assess the conversion of adenosine to cellular metabolites, monolayer cultures were exposed at 20° to MEM-T containing [2-^{3}H]adenosine for intervals initiated and terminated exactly as in the assays for adenosine uptake. Bottles were processed individually, and immediately after removal of the cold NaCl each was placed on ice and the monolayer was extracted with 1.5 ml of cold 0.5 M PCA for 2 min. This PCA extract was passed successively over four additional replicate monolayers, and therefore each extract ultimately represented five monolayers. Extracts were neutralized, freeze-dried, and taken up in 200 μl of 1.0 mM phosphate buffer (pH 6.5), and 20-μl samples were applied as 5-cm strips on MN300 thin layers of PEI-cellulose (Macherey-Nagel and Company, Brinkmann Instruments), or on Eastman 6064 cellulose thin-layer sheets (Eastman Kodak) along with 50-nmole portions of appropriate carriers. After chromatographic development, carrier areas were assayed for ^{3}H by combustion (Packard Model 306 Sample Oxidizer) and liquid scintillation counting. In chromatographic system I, PEI-cellulose chromatograms were developed with 0.5, 2.0, and 4.0 M sodium formate solutions (pH 3.4), employed in that order, without drying between transfers from one to another. When solvents fronts had moved 2.5 and 7.0 cm above the origin, chromatograms were moved to 2.0 and 4.0 M sodium formate, respectively; development in the latter proceeded until the front had moved 17 cm. System I resolved the mono-, di-, and triphosphates. Cellulose thin-layer chromatograms (system II) were developed in ethyl acetate–isopropyl alcohol–water (65:22.5:12.5 by volume); in this system nucleoside phosphates were unresolved and remained near the origin.

Cell culture materials were purchased from Grand Island Biological Company. Dr. S. R. Naik of this laboratory prepared NBMPR (29) from 6-thioinosine generously provided by Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md. The latter agency also provided 2'-deoxycoformycin. Isotopically labeled bases and nucleosides were commercial products.

RESULTS AND DISCUSSION

Adenosine uptake. Monolayer cultures were employed in this study because the rapidity with which medium changes could be effected afforded a means of measuring initial rates of adenosine uptake; individually processed, replicate monolayer cultures were employed. Time courses for uptake from medium containing 1.0 μM adenosine could be extrapolated through zero time and were initially linear (Fig. 1A). However, when the adenosine concentration was 10 μM, the time course of uptake was not linear (Fig. 1B). At adenosine concentrations in excess of 2 μM, uptake during the first 15 sec of permeant exposure was employed as a measure of initial rate; otherwise the uptake interval was 30 sec. The change in rate of adenosine uptake seen in Fig. 1 was also clearly apparent in two other experiments; similar rate changes have also been found in the time courses of guanosine and inosine uptake by HeLa cells monolayers.[2] The significance of these changes in uptake rate is unknown. All measurements of uptake were corrected for the small amount of adenosine (less than 100 cpm) taken up during exposure to the cold NaCl stopping solution.

Adenosine taken up by the monolayer

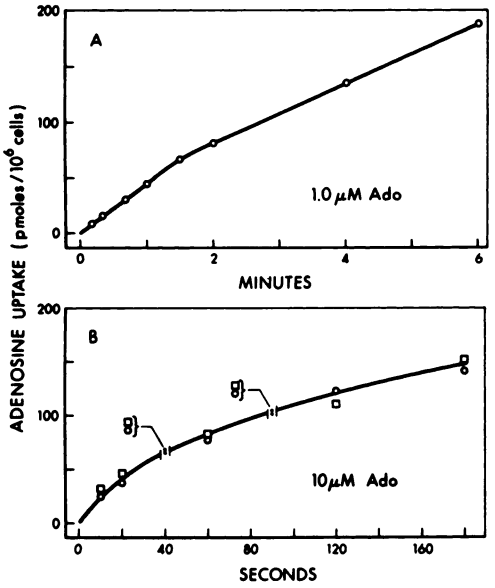

FIG. 1. *Time course of adenosine uptake by HeLa cells, and failure of 2'-deoxycoformycin to influence adenosine uptake*

The uptake of adenosine by replicate monolayer cultures incubated at 20° for various intervals was determined. The means of triplicate assays are plotted. A. Cultures were incubated individually in MEM-T containing 1.0 μM [2-^{3}H]adenosine for the intervals indicated and then processed for determination of the cellular content of adenosine as in MATERIALS AND METHODS. B. Cultures were incubated in MEM-T containing 10 μM [2-^{3}H]adenosine with (□) or without (○) 5 μM 2'-deoxycoformycin.

cells was extensively anabolized, with ATP as the principal product. The time course of adenosine phosphate formation could be extrapolated through zero time and was linear (Fig. 2). Since the conditions for initiating and ending exposure to [2-^{3}H]adenosine were identical with those of the uptake assay, the conversion of adenosine to its metabolites, illustrated in Fig. 2 and Table 1, is representative of that which occurred during uptake assay intervals. The relative proportions of AMP, ADP, and ATP were similar after all intervals of uptake and were unaffected by the presence of the nucleoside transport inhibitor NBMPR. The latter compound did not inhibit the adenosine kinase activity of Ehrlich ascites carcinoma cells (30).

The linear time course of ATP formation and its extrapolation through zero time (Fig. 2) would appear to indicate either (a) that intracellular pools of AMP and ADP were very small (and therefore isotopic dilution of [2-^{3}H]adenosine-derived AMP and ADP did not significantly delay appearance of ^{3}H in ATP) or (b) that adenosine metabolism associated with the uptake process is compartmentalized, perhaps in the manner suggested by Rapaport and Zamecnik (31).

Small but significant conversion of adenosine to inosine, hypoxanthine, and

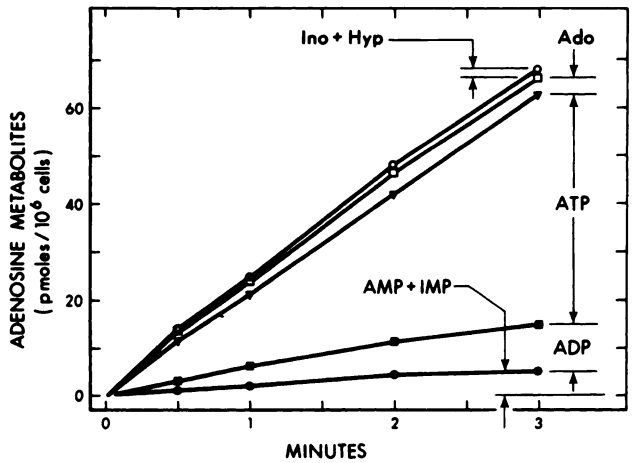

FIG. 2. *Time course of adenosine metabolite formation*

Monolayer cultures were incubated at 20° in MEM-T containing 1.0 μM [2-^{3}H]adenosine for the intervals indicated, which were terminated exactly as in assays for simple uptake of adenosine. PCA extracts of the monolayer cultures were analyzed by thin-layer chromatography to determine the intracellular distribution of adenosine metabolites. These data and those of Table 1 are from the same experiment.

TABLE 1

Metabolites of adenosine in perchloric acid extracts of HeLa monolayer cells

Replicate monolayer cultures were incubated at 20° in MEM-T containing 1.0 μM [2-^{3}H]adenosine with and without 5 μM NBMPR for the intervals specified; these intervals were started and ended exactly as in assays of adenosine uptake. For each interval three cultures were assayed for adenosine uptake (as in Fig. 1) and five cultures were extracted with 0.5 μM PCA. Neutralized PCA extracts, each representing five cultures (MATERIALS AND METHODS), were analyzed by thin-layer chromatography to determine the percentage distribution of radioactivity among the metabolites of adenosine (this table). Such distributions were then applied to the estimates of cellular adenosine content (uptake assay) to obtain the data of Fig. 2. Distributions of radioactivity within the "unresolved phosphates" area of system II chromatograms were obtained from system I chromatograms of samples from the same extract. This table presents a sample of the chromatographic data from which the metabolite distributions plotted in Fig. 2 were obtained.

Metabolite	Adenosine metabolites in cells incubated as follows:					
	Radioactivity			Distribution		
	+NBMPR	−NBMPR		+NBMPR	−NBMPR	
	30 sec	30 sec	180 sec	30 sec	30 sec	180 sec
	cpm/20-μl extract			%	%	%
Unchromatographed extract	2,132	6,780[a]	35,589[a]	100	100	100
System II						
Adenosine	354[b]	914	1,719	16.6	13.5	4.8
Hypoxanthine	42	56	115	2.0	0.8	0.3
Inosine	76	158	771	3.6	2.3	2.2
Unresolved phosphates	1,465	5,168	31,329			
System I						
AMP	126	319	2,089	5.6	4.8	6.0
IMP	35	145	277	1.5	2.2	0.8
ADP	287	859	4,712	12.3	12.8	13.5
ATP	1,119	3,782	23,401	49.4	56.5	66.8
Guanosine phosphates		53	339			
Total				91.0	92.9	94.4

[a] Acid-insoluble residues of the cultures from which these extracts were prepared contained 160 cpm (30 sec) and 510 cpm (180 sec) per amount equivalent to 20 μl of extract.

[b] Attributable to adenosine carried forward from the cold NaCl stopping step: zero-time values in the assays for simple uptake would account for 420 cpm.

inosinate occurred; only trace amounts of adenosine were converted to guanosine phosphates within the brief incubation times employed here. Free adenosine was present in cells after all intervals of incubation. Since the presence of the nucleoside transport inhibitor NBMPR reduced the adenosine content of PCA extracts to an amount attributable to carryover from the stopping step, adenosine appeared to be a real intracellular constituent and not an artifact of the assay procedure. The low levels of ^{3}H activity remaining in cellular material after PCA extraction (Table 1) indicate that incorporation of adenosine into polynucleotides was insignificant under the conditions of the uptake assay.

A potent inhibitor of adenosine deaminase, 2′-deoxycoformycin (32), had no effect on adenosine uptake (Fig. 1B), suggesting, in agreement with the results of Fig. 2, that deamination was not an important metabolic fate of adenosine under the conditions of the uptake assay. In other experiments (data not shown), prior treatment for 1 or 10 min with 0.5 or 5.0 μM 2′-deoxycoformycin had no appreciable effect on adenosine uptake during a 30-sec interval. As well, another potent inhibitor of adenosine deaminase, *erythro*-9-(2-hydroxy-3-nonyl)adenine (33), had no significant effect on adenosine uptake in similar experiments (Table 4).

Adenosine uptake experiments routinely employed 24-hr monolayer cultures

containing about 10^6 cells in an area of 28 cm^2. As seen in Fig. 3A, plating efficiences measured after 4 hr at 37° were 50–65%, and cells were proliferating exponentially 24 hr after plating, at which time cultures were cooled to 20° for the assay of nucleoside uptake. The experiment of Fig. 3B demonstrated that adenosine uptake rates were proportional to cell number in the cultures up to at least 1.2×10^6 cells/monolayer. Thus uptake rates (picomoles per 10^6 cells per minute) were independent of population density in the monolayers under the conditions of these experiments.

Kinetic studies. In the experiments of Fig. 4, adenosine uptake rates were measured over a range of concentrations in the presence and absence of other nucleosides in order to determine whether the latter might compete with adenosine for entry. Reciprocal plots of uptake rate and adenosine concentration in the absence of additives were straight lines, indicating

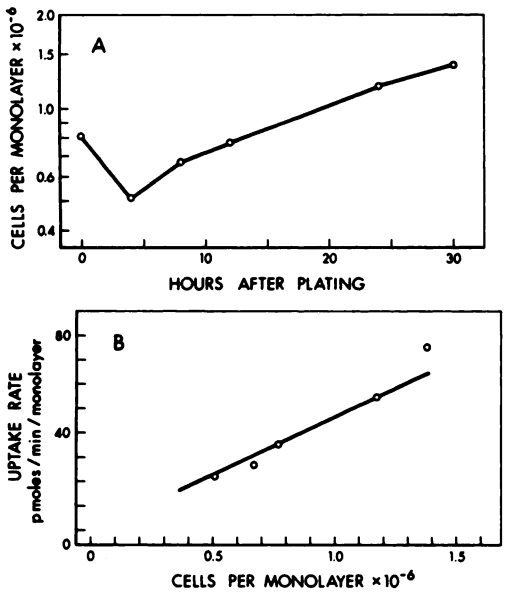

FIG. 3. *Proportionality between adenosine uptake rate and cell number in HeLa cell monolayers*

Replicate monolayer cultures, in a set inoculated with 8×10^5 cells/culture bottle, were incubated at 37° for the intervals shown, at which times cultures were withdrawn for determination of cell number (A) and assay of adenosine uptake (B). Adenosine uptake from MEM-T containing 1.0 μM [2-^{3}H]adenosine during a 30-sec interval was measured at 20°, as in Fig. 1.

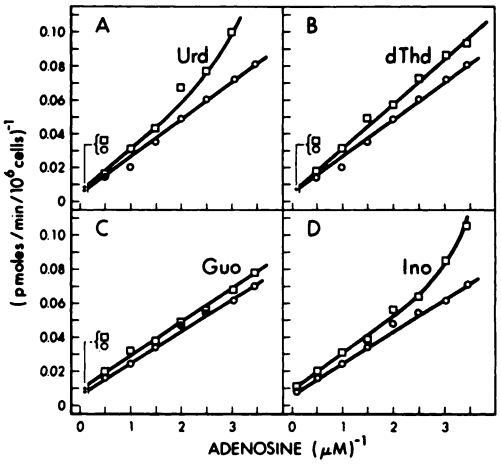

FIG. 4. *Weak inhibition of adenosine uptake by guanosine, inosine, uridine, and thymidine*

Replicate monolayer cultures were incubated for 30-sec intervals at 20° in MEM-T containing graded concentrations of [2-^{3}H]adenosine in the absence (○) or presence (□) of 25 μM guanosine, inosine, uridine, or thymidine. Uptake of adenosine was assayed as described in MATERIALS AND METHODS and Fig. 1. Adenosine uptake was measured in the presence of uridine or thymidine in an experiment summarized in Fig. 4A and B, and in the presence of guanosine or inosine in a separate experiment (C and D).

that the uptake process was saturable. Apparent K_m and V_{max} values for adenosine uptake, averaged from six independent experiments, including those of Fig. 4, were 2.5 μM and 133 pmoles/10^6 cells/min, without correction for adenosine entry by diffusion (see below). Several nucleosides were only weakly inhibitory toward adenosine uptake; mixed inhibition by 25 μM uridine, guanosine, and inosine is apparent in Fig. 4A, C, and D, and that by thymidine (Fig. 4B) appeared to be competitive. Inosine and guanosine appear to be taken up by the same mechanism, which is distinct from the mechanism(s) for thymidine and uridine in cultured Novikoff hepatoma cells (7) and in HeLa cells.[2] Thus, in HeLa cells, either the adenosine uptake mechanism is a separate entity or, in the sequence of events comprising the adenosine uptake process, at least one event is adenosine-specific.

Inhibition of adenosine uptake by NBMPR. NBMPR inhibits the transport of thymidine and uridine across the

plasma membrane of human erythrocytes in the absence of metabolism of those permeants (1, 16). Thus the NBMPR inhibition of nucleoside uptake shown here and elsewhere (14, 15) would appear to be effected also at the level of membrane transport. Rates of adenosine uptake (1 μM, 20°) by HeLa cells were measured in the presence of graded concentrations of NBMPR (Table 2); in this experiment the permeant and inhibitor were added simultaneously to the cells. The rate of adenosine uptake was inhibited by 50% in the presence of 0.05 μM NBMPR, suggesting high affinity of NBMPR for its receptor sites. Concentrations of the aglycone, NBMP, required to effect inhibition to the same extent were about 100-fold higher, illustrating the contributions of the pentosyl moiety of NBMPR to the interaction between the inhibitor and its cellular binding site.

The experiment of Fig. 5 measured the influence of adenosine concentration on uptake rates in the absence and presence of 5 μM NBMPR. These data show that in the presence of NBMPR the mediated component of uptake was abolished and adenosine uptake rates were directly proportional to adenosine concentration; it would appear that simple diffusion was responsible for adenosine permeation under these circumstances. A similar nonsaturable component of thymidine uptake by HeLa cell monolayers has been reported (15). The inset graph of Fig. 5 is a plot of the reciprocals of adenosine concentrations and uptake rates; the latter were corrected for the diffusional component of uptake. The metabolic fate of adenosine which diffused into cells (i.e., uptake in the presence of 5 μM NBMPR) was evidently the same as that for which entry was mediated (see Table 1).

The data of Fig. 6 indicate that in the presence of NBMPR the apparent K_m of adenosine uptake was increased, but the V_{max} value was unchanged. This is the classical pattern of competitive inhibition; however, because of the following considerations, it is unlikely that this result indicates competition between adenosine and NBMPR for the permeation site. (a) Similar kinetic characteristics are seen in the inhibition by NBMPR of the uptake of thymidine, uridine, inosine, and guanosine; the structural differences between

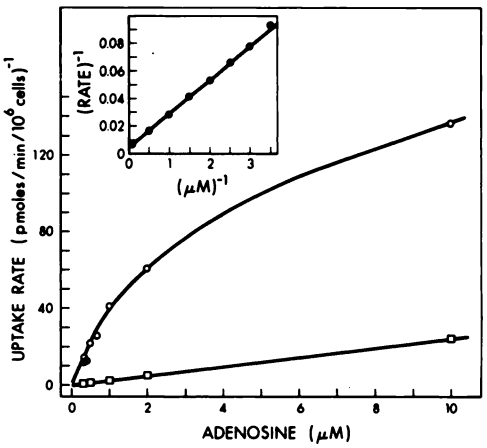

FIG. 5. *Diffusion component of adenosine uptake*
Replicate monolayer cultures were incubated at 20° in MEM-T containing graded concentrations of [2-^{3}H]adenosine with (□) or without (○) 5 μM NBMPR. The uptake intervals were 60 sec in the presence of NBMPR, and 15 sec (10 μM adenosine) and 30 sec (all other adenosine concentrations) in the absence of NBMPR.

TABLE 2

Inhibition of adenosine uptake

In the determination of adenosine uptake rates, replicate monolayer cultures were individually incubated for 30 sec at 20° in MEM-T containing 1.0 μM [2-^{3}H]adenosine and the indicated concentrations of inhibitor.

Inhibitor concentration[a]	Adenosine uptake rate in the presence of:		
	NBMPR 5'-phosphate	NBMPR	NBMP
μM	% control		
0[b]	100[c]	100[d]	100[e]
0.001		95	109
0.01	80	82	96
0.1	40	40	83
1.0	24	26	62
5.0			44
10.0	16	13	

[a] Inhibitor concentrations that reduced adenosine uptake rates to 50% of control values were 0.05 μM NBMPR 5'-phosphate, 0.05 μM NBMPR, and 3 μM NBMP.

[b] Control.

[c] Equivalent to 47.3 pmoles/10^6 cells/min.

[d] Equivalent to 30.9 pmoles/10^6 cells/min.

[e] Equivalent to 39.1 pmoles/10^6 cells/min.

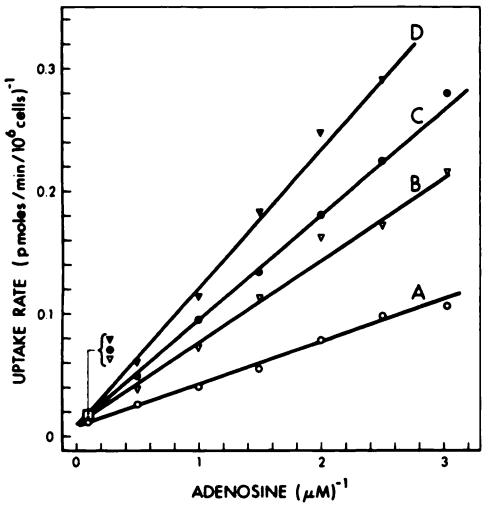

FIG. 6. *Inhibition of adenosine uptake by prior treatment with NBMPR*

Prior to assay for adenosine uptake, replicate monolayer cultures were treated individually at 20° as follows: (a) growth medium was replaced with 4.0 ml of fresh growth medium with (*C* and *D*) or without (*A* and *B*) 0.01 μM NBMPR, and (b) after 1.0 min of contact with the culture, the pretreatment medium was replaced with 4.0 ml of fresh NBMPR-free growth medium, which was removed after 30 sec. The rate of adenosine uptake in these cultures was then assayed at 20° as follows. Cultures were incubated individually in MEM-T containing graded concentrations of [2-^{3}H]adenosine with (*B* and *D*) or without (*A* and *C*) 0.01 μM NBMPR; in incubations with 10 μM adenosine, uptake intervals were 15 sec; all other uptake intervals were 30 sec. Adenosine uptake was then determined as in Fig. 1.

these permeants and NBMPR argue against the likelihood of a competition for permeation sites. (b) Other instances are known in which compounds unrelated to nucleosides [for example, Persantine or *p*-chloromercuribenzoate (12)] cause nucleoside transport inhibition of this kinetic type. (c) Treatment of the monolayers with NBMPR, followed by washing and assay for adenosine uptake in the absence of NBMPR, produced inhibition of the same kinetic character (Fig. 6, curve *C*).

The effect of prior treatment of cultures with a partially inhibitory concentration of NBMPR (0.01 μM) is illustrated in Fig. 6. Cultures were incubated in medium with (Fig. 6, curves *C* and *D*) and without (curves *A* and *B*) 0.01 μM NBMPR and then washed, and rates of uptake from graded concentrations of adenosine were determined in the presence and absence of the same concentrations of NBMPR. Treatment with 0.01 μM NBMPR for 10 min prior to the assay of adenosine uptake (curve *C*) was more inhibitory than the same concentration of NBMPR added simultaneously with the permeant (curve *B*). When cells previously treated with NBMPR were assayed in the presence of NBMPR, the inhibition was further enhanced (curve *D*), again suggesting the firm binding of NBMPR to its receptor sites.

Structure-Activity Experiments

Tables 3 and 4 summarize structure-activity experiments in which various relatives of NBMPR were assayed for their ability to inhibit adenosine uptake. To initiate the uptake assay, test compounds and permeant were added together. In the following comparisons of IC_{50} values (concentrations of test compound that reduced the adenosine uptake rate to 50% of the control rate), reference numbers for test compounds from Tables 3 or 4 are cited. Such comparisons led to conclusions about the contributions to inhibitory activity of structural features of NBMPR and related compounds, discussed here as various substituents on the purine ring.

Pentosyl group at purine 9-position. The importance of the 9-pentosyl group to the activity of these inhibitors of nucleoside transport was apparent in previous reports (15, 21) and is shown here by comparison of the inhibitory activity of NBMPR (554) and its arabinosyl homologue (947) with that of the aglycone, 924; the activities of 957 and 958 may also be compared. 2′-Hydroxyl groups are evidently not involved in the cell-inhibitor interaction, because NBMPR (554), its 2′-*O*-methyl derivative (861), and the 2′-deoxyribosyl and arabinosyl homologues of NBMPR (844 and 947, respectively) are potent inhibitors of adenosine uptake (also compare 218 and 914). The inhibitor binding site is less tolerant of variations at the pentosyl 3′-position than at the 2′-position [2′-*O*-methyl-NBMPR (861) vs. 3′-*O*-methyl NBMPR (862)]. Of the two possible configurations of the *N*-glycosidic linkage at the

TABLE 3

Inhibitors of adenosine uptake

Rates of adenosine uptake by replicate monolayer cultures were determined in the absence and presence of at least three dilutions of test compounds. Cultures were incubated at 20° for 30 sec in MEM-T containing 1.0 μM [2-^{3}H]adenosine with and without (control) inhibitor in 10-fold dilutions. Concentrations of test compounds that reduced uptake rates by 50% of control values (IC_{50}) were estimated from semilogarithmic plots. Compounds were tested in groups of three, each with controls.

Ref. No.	Compound	Source[a]	IC_{50}
			μM
852	2-Amino-6-[(4-nitrobenzyl)seleno]-9-β-D-ribofuranosylpurine	H	0.005
861	6-[(4-Nitrobenzyl)thio]-9-β-D-2′-*O*-methyl-ribofuranosylpurine	G	0.005
947	6-[(4-Nitrobenzyl)thio]-9-β-D-arabinofuranosylpurine	D	0.006
844	6-[(4-Nitrobenzyl)thio]-9-β-D-2′-deoxyribofuranosylpurine	G	0.01
914	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-2′-deoxyribofuranosylpurine	E	0.02
218	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine	E	0.03
120	6-(Benzylthio)-9-β-D-ribofuranosylpurine	A	0.03
554	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine	E	0.05
936	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine 5′-monophosphate	C	0.05
783	6-(Cyclohexylthio)-9-β-D-ribofuranosylpurine	F	0.07
951	6-(4-Nitrobenzyl)-9-β-D-ribofuranosylpurine	I	<0.1
938	6-(Benzylthio)-9-β-D-arabinofuranosylpurine	D	0.1
558	N^6-Benzyladenosine	G	0.2
862	6-[(4-Nitrobenzyl)thio]-9-β-D-3′-*O*-methyl-ribofuranosylpurine	G	0.2
201	2-Amino-6-(benzylthio)-9-β-D-ribofuranosylpurine	A	0.3
940	6-[(3-Pyridylmethyl)thio]-9-β-D-arabinofuranosylpurine	D	0.3
217	2-Amino-6-(phenylethyl)thio-9-β-D-ribofuranosylpurine	A	0.4
963	N^6-Benzyladenosine 5′-phosphate	I	0.4
961	6-(β-Phenethyl)-9-β-D-ribofuranosylpurine	I	0.4
915	2-Amino-6-[(4-nitrobenzyl)thio]-9-α-D-2′-deoxyribofuranosylpurine	E	0.4
445	4-Benzylthio-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine	A	0.5
878	6-(Benzylamino)-9-β-D-arabinofuranosylpurine	D	1.0
957	N^6-Benzyloxyadenosine	B	0.9
946	6-(Allylthio)-9-β-D-arabinofuranosylpurine	D	1.0
926	6-(Benzylthio)-9-β-D-ribofuranosyl, cyclic 3′,5′-monophosphate	D	1.0
928	6-(Methylthio)-9-β-D-ribofuranosyl, cyclic 3′,5′-monophosphate	D	1.2
937	6-[(3-Methylbut-2-enyl)amino]-9-β-D-arabinofuranosylpurine	D	2.0

TABLE 3—*Continued*

Ref. No.	Compound	Source[a]	IC_{50}
			μM
962	N^6-(β-3,4-Dihydroxyphenethyl)adenosine	I	2.0
958	N^6-Benzyloxy-9-methyladenine	B	2.5
924	6-[(4-Nitrobenzyl)thio]purine	E	3.0
944	6-(Ethylthio)-9-β-D-arabinofuranosylpurine	D	5.0
959	N^6-(*p*-Methoxybenzyloxy)-9-methyladenine	B	6.4
942	6-(Methylthio)-9-β-D-arabinofuranosylpurine	D	30
943	6-(Thiocyanato)-9-β-D-arabinofuranosylpurine	D	30
117	6-Thio-9-β-D-ribofuranosylpurine	A	48
941	6-(β-Hydroxyethylamino)-9-β-D-arabinofuranosylpurine	D	50
954	N^1-(*p*-Methoxybenzyloxy)-9-methyladenine	B	<50

[a] Chemicals were obtained from the following sources: A, Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md.; B, T. Fujii, Kanazawa University, Kanazawa, Japan; C, G. J. Lauzon and T. P. Lynch, this laboratory; D, J. P. Miller and R. A. Long, ICN Nucleic Acid Research Institute, Irvine, Cal.; E, S. R. Naik, this laboratory; F, B. Paul, this laboratory (20); G, M. J. Robins, University of Alberta, Edmonton; H, L. R. Townsend, University of Utah, Salt Lake City; I, H. Vorbrüggen, Schering A. G., Berlin; J, H. J. Schaeffer, Wellcome Research Laboratories, Research Triangle Park, N.C.

pentosyl 1-position, the β-configuration is preferred (compare 914 and 915). NBMPR (554) and its 5′-monophosphate derivative (936) had equivalent inhibitory activity toward adenosine uptake (see also Table 2); it is not known whether NBMPR 5′-phosphate per se is the inhibitor, or whether this compound interacts with the same cellular sites as NBMPR. It would appear that the inhibitor binding site is less able to accept cyclic 3′,5′-monophosphate derivatives than homologous active nucleoside inhibitors (compare 558, 963, and 935; 957 and 934).

Purine 2-amino group. The purine 2-amino group is evidently not involved in the interaction between inhibitors and the receptor site, because compounds differing only in the presence or absence of the 2-amino group had comparable, potent inhibitory activities (compare 844 and 914, 218 and 554, 120 and 201).

Purine ring system. The relatively low inhibitory activities of 445 and 950 (which may be regarded as 7-deaza analogues of 6-substituted purine ribosides) toward adenosine uptake may indicate involvement of the nitrogen atom at the purine 7-position in the cellular binding of these inhibitors (compare 445 and 120, and 950 with 852).

Substituent groups at purine 6-position. Previous reports have illustrated the important contribution of substituents at the purine 6-position in NBMPR homologues to inhibition of nucleoside transport in erythrocytes (20, 21). This point is also apparent in the data of Table 5 for the inhibition of adenosine transport in HeLa cells by a series of S^6 derivatives of 6-thiopurine nucleosides. Table 5 demonstrates that the number of carbon atoms in the S^6 substituent is an important determinant of the cell-inhibitor interactions, suggesting that hydrophobic binding forces are involved; evidently the aromaticity of the S^6 substituent does not contribute to binding (compare 783 and 120).

R—X groups at purine 6-position. The majority of active compounds listed in Table 3 are 6-(R—S)-purine derivatives (i.e., S^6 derivatives); however, 6-(R—N)- and 6-(R—C)-purine ribosides were also potent inhibitors. While the N^6-benzyl (558) and C^6-benzyl (961) homologues of S^6-benzyl-6-

TABLE 4

Compounds ineffective as inhibitors of adenosine uptake

The following compounds, when present at 10 μM in assays for inhibition of adenosine uptake (Table 3), were ineffective or did not reduce adenosine uptake below 80% of that in control culture (absence of test compound).

Ref. No.	Compound	Source[a]
945	6-Aminomercapto-9-β-D-arabinofuranosylpurine	D
921	9-(2',3'-Anhydro-β-D-ribofuranosyl)adenine	G
935	N^6-Benzyladenosine cyclic 3',5'-monophosphate	D
934	N^6-Benzyloxyadenosine cyclic 3',5'-monophosphate	D
953	N^1-Benzyloxy-9-methyladenine	B
956	N^1-Cyclohexylmethoxy-9-methyladenine	B
931	N^6-Ethyladenosine cyclic 3',5'-monophosphate	D
927	6-(Ethylthio)-9-β-D-ribofuranosylpurine cyclic 3',5'-monophosphate	D
933	N^6-Hydroxyadenosine cyclic 3',5'-monophosphate	D
922	9-(2-*O*-Methanesulfonyl)-β-D-xylofuranosyladenine	G
932	6-Methoxy-9-β-D-ribofuranosylpurine cyclic 3',5'-monophosphate	D
930	N^6-Methyladenosine cyclic 3',5'-monophosphate	D
955	N^1-*p*-Nitrobenzyloxy-9-methyladenine	B
950	4-[(-Nitrobenzyl)selenol]-7-β-D-ribofuranosylpyrrolo[2,3-*d*]-pyrimidine	H
948	6-Thio-9-β-D-arabinofuranosylpurine	D
929	6-Thio-9-β-D-ribofuranosylpurine cyclic 3',5'-monophosphate	D
868	*Erythro*-9-(2-hydroxy-3-nonyl)-adenine	J

[a] Compound sources are listed in Table 3.

thioinosine (120) were less inhibitory than the latter, they were nevertheless potent inhibitors of adenosine uptake. Also listed in Table 3 are two pairs of inhibitors that differ only in respect to the atom linking R to the purine 6-carbon (961 and 120, 878 and 938); again the S^6 derivative is the more potent inhibitor of each pair, suggesting that the sulfur atom may contribute to inhibitor binding [as the selenium atom apparently does (compare 218 and 852)]. The nitro group is evidently not a prime determinant of inhibitor binding (compare 120 and 554).

Other aspects of the specificity of the cell-inhibitor interaction may be noted. (a) In contrast to the profound inhibition

TABLE 5

Inhibition of adenosine uptake by S^6 derivatives of 6-thiopurine nucleosides

The data listed are from Tables 3 and 4; the test compounds compared differ only in respect to their S^6 substituents.

Class	Ref. No.	S^6 Substituent	IC_{50}
			μM
6-Thiopurine ribosides	117	H	48
	783	Cyclohexyl	0.07
	120	Benzyl	0.03
	554	4-Nitrobenzyl	0.05
6-Thiopurine arabinosides	948	H	–[a]
	942	Methyl	30
	944	Ethyl	5
	946	Allyl	1.0
	940	3-Pyridylmethyl	0.3
	938	Benzyl	0.1
	947	4-Nitrobenzyl	0.006

[a] Not active.

TABLE 6

Failure of NBMPR and related aglycones to exert substantial inhibition of purine uptake by HeLa cells

Rates of purine uptake by replicate monolayer cultures were determined in the absence and presence of NBMPR, NBMP, and NBTG. Cultures were incubated at 20° for 60 sec in MEM-T containing 20 μM [8-^{14}C]adenine, 1.0 μM [8-^{3}H]guanine, or 2.0 μM [8-^{14}C]hypoxanthine with or without (control) each test compound at the indicated final concentrations.

Test compound	Rate of uptake[a]		
	Adenine	Guanine	Hypoxanthine
	% control		
NBMPR (5 μM)	111	79	106
NBMP (5 μM)	108	78	82
NBTG (4 μM)	96	52	90[b]

[a] Control rates (absence of test compound) for uptake of adenine, guanine, and hypoxanthine were 105, 42, and 108 pmoles/10^6 cells/min, respectively.

[b] The final concentration of NBTG was 1.2 μM.

of nucleoside uptake by NBMPR, the uptake by HeLa cells of glucose, amino acids, or several nucleic acid bases was inhibited by NBMPR to a minor extent or not at all (14) (Table 6). (b) The aglycones of NBMPR and NBTGR (NBMP and NBTG, respectively) had little inhibitory activity toward the uptake of adenine, guanine, and hypoxanthine by HeLa cells monolayers (Table 6).

In summary, the potent inhibitory activity of NBMPR, and presumably of related compounds, toward the mediated entry of nucleosides is class-specific in that entries of thymidine (15), uridine (14), guanosine,[2] inosine,[2] and adenosine are similarly inhibited, but those of the corresponding bases are much less affected. The inhibitor aglycones have only minor effects on base or nucleoside uptake.

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