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Binding of the Nucleoside Transport Inhibitor Nitrobenzylthioinosine to HeLa Cells

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SUMMARY

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Nitrobenzylthioinosine (NBMPR), a potent inhibitor of nucleoside transport, was bound tightly but reversibly to HeLa cell membrane sites associated with the nucleoside transport mechanism. Site-specific binding was assayed with [^{35}S]NBMPR and a competing, nonisotopic congener. Mass law analysis of the binding data indicated that each HeLa cell possessed about 10^5 binding sites of a single class which bound NBMPR tightly; the bound inhibitor had a dissociation constant of about 0.1 nM. Occupancy of these binding sites by NBMPR correlated with inhibition of uridine and thymidine uptake; however, the relationship between these parameters was not simple because, as binding saturation was approached (at about 5 nM NBMPR), a substantial fraction (25-30%) of the transport capability remained active but inhibitable by 5 μM NBMPR.

INTRODUCTION

The passage of nucleosides across the plasma membrane of animal cells is mediated by specific elements of the membrane (1-6). The transport[1] of uridine and thymidine by human erythrocytes has been identified as a classical "facilitated diffusion" process; because of the inability of these cells to metabolize uridine or thymidine, it has been possible to demonstrate transport phenomena that involve internal nucleoside pools [such as equilibrium exchange diffusion and accelerative exchange diffusion (2, 3)] and are characteristic of facilitated diffusion. The naturally occurring nucleosides are metabolized upon entering most cells, and it is not known whether transport mechanisms, such as facilitated diffusion, ordinarily function independently of enzymes involved in nucleoside metabolism. The principal approach to the study of nucleoside permeation has been through measurement of initial rates of uptake; kinetic studies have shown that a rate-limiting step in the uptake process is mediated, and it has been assumed frequently that transport is that step.

Accelerative exchange diffusion data have indicated that human erythrocytes have a single type of nucleoside transport mechanism that accepts ribosides and deoxyribosides of both purines and pyrimidines (1-3). In contrast, HeLa cells appear to have several distinct nucleoside uptake mechanisms; the uptake processes for uridine, thymidine, adenosine, and guanosine are distinguishable by kinetic and

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[1] "Transport" refers to the mediated passage of permeant across the plasma membrane of cells; the "uptake" process includes both transport and intracellular metabolism of permeant.

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other criteria[2] (4), and therefore each is separate or, at least, has a step that is distinct from the others.

NBMPR,[3] NBTGR, and related compounds are potent inhibitors of nucleoside transport in erythrocytes (2, 3, 7). Erythrocytes were found to have high-affinity binding sites of a single type on the plasma membrane (8, 9); these sites, which were present to the extent of $1.0–1.5 \times 10^4$/cell, bound NBMPR with an apparent dissociation constant of 1 nM (9). The sites were presumed to be part of the nucleoside transport mechanism, since inhibition of uridine transport was proportional to the number occupied by NBMPR (9). While transport permeants compete with NBMPR for occupancy of the erythrocyte binding sites (10), there are cogent reasons for believing that the NBMPR binding sites are distinct from the nucleoside permeation sites (10).

NBMPR is also a potent inhibitor of nucleoside uptake in various types of cells which metabolize nucleosides, including HeLa cells (4, 11). Since the thymidine and uridine kinase activities of HeLa cell extracts are unaffected by NBMPR at concentrations well in excess of those effective in blocking nucleoside transport (4), the latter effect is evidently due to impairment of transport. The present report describes the binding of [^{35}S]NBMPR to HeLa cells and explores relationships between binding and inhibition of uridine and thymidine uptake.

METHODS

Chemicals. NBMPR and NBTGR were prepared by established methods (12), using thioinosine and thioguanosine generously provided by Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md.

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

[3] The abbreviations used are: NBMPR (or nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; thioinosine, 6-thio-9-β-D-ribofuranosylpurine; thioguanosine, 2-amino-6-thio-9-β-D-ribofuranosylpurine; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

In the preparation of [^{35}S]NBMPR, the sulfur atom of thioinosine was first labeled by exchange (13) and then alkylated with 4-nitrobenzyl bromide (12). In a typical preparation, 10 μmoles of thioinosine, 0.36 mg of elemental ^{35}S (600 mCi/milliatom, Amersham/Searle, Oakville, Ont.), and 7 ml of freshly distilled, dry pyridine were refluxed with stirring under nitrogen for 1 hr. The ^{35}S exchange was complete by 30 min. The pyridine was evaporated under a stream of nitrogen, and the product was dissolved in 0.5 ml of freshly distilled, dry dimethylformamide containing 20 μmoles of 4-nitrobenzyl bromide; the alkylation reaction (10 min, 20°) was essentially quantitative. NBMPR was isolated by chomatography on 250-μm layers of silica gel (G-HR, Macherey, Nagel and Company). Prior to use, thin-layer plates were washed once with 15% (v/v) methanol in chloroform and heated at 120° for 12 hr. Chromatograms were developed in 15% methanol–chloroform, and the NBMPR band was eluted from the silica gel with methanol. In a typical preparation, [^{35}S]NBMPR (specific activity, 6.6×10^8 cpm/μmole) was obtained in 69% yield (in terms of thioinosine). The labeled product co-chromatographed with NBMPR on paper in these solvent systems: isopropyl alcohol–ammonia–water (70:5:25, v/v), R_f 0.79; 5% (w/v) disodium phosphate in water, R_f 0.05; and isobutyl alcohol–acetic acid–water (120:30:50, v/v), R_f 0.82. When stored in absolute methanol at −20°, [^{35}S]NBMPR preparations were stable for 5 months.

Cell culture. HeLa S3 cells were maintained by weekly passage of monolayer cultures in Eagle's minimal essential medium supplemented with 10% calf serum and 2 mM HEPES buffer (pH 7.4) at 37° in 5% CO_2–air. After six to eight serial passages, cultures were restarted from stocks kept in liquid nitrogen. Spinner cultures, started weekly from trypsinized monolayers, employed calcium-free minimal essential medium supplemented with 5% calf serum and 2 mM HEPES buffer (spinner medium); cell concentrations were kept below 6×10^5 cells/ml and, under these conditions, cell proliferation was exponential

with doubling times of 22–24 hr. Cell culture materials were purchased from Grand Island Biological Company, Calgary.

Binding studies. Binding studies employed cells from spinner cultures collected by centrifugation (150 × *g*, 5 min) and resuspended in fresh spinner medium at 4 × 10^6 cells/ml. Binding assays were initiated by mixing equal volumes of cell suspension and calcium-free minimal essential medium containing [^{35}S]NBMPR; assay intervals were terminated by centrifuging (500 × *g*, 3 min) samples containing at least 10^7 cells. Supernatants were reserved for determination of ^{35}S content using Bray's counting solution (14) and liquid scintillation counting. Cell pellets were thoroughly drained, dissolved in 1.5 ml of NCS tissue solubilizer (Amersham/Searle), and transferred with rinsing into 18 ml of Bray's counting solution. Specific binding was defined as the difference between [^{35}S]NBMPR bound to cells in the absence and presence of NBTGR, the latter at a concentration which displaced bound NBMPR from the cellular binding sites. All assays of ^{35}S activity were performed under the same conditions as those for determination of cell-bound ^{35}S.

Uptake studies. In these experiments, the effect of prior incubation with [^{35}S]NBMPR on the cellular uptake of uridine and thymidine was studied. Cell suspensions, 1–2 × 10^6 cells/ml in "uptake medium" (calcium-free minimal essential medium containing 2.5% calf serum and 1 mM HEPES, pH 7.4), were incubated at 20° for 5 min with and without [^{35}S]NBMPR. Uptake assays were then initiated by the addition of [^{3}H]nucleoside (Amersham/Searle) and terminated by transferring 1.0-ml samples of the incubation mixture into 40 ml of cold buffered NaCl (15) containing 5 μM NBTGR. When uptake of [*methyl*-^{3}H]thymidine was assayed, the buffered NaCl contai ed 100 μM thymidine. After centrifugation (500 × *g*, 3 min), cell pellets from assay samples were dissolved in 0.3 ml of NCS tissue solubilizer for assay of ^{3}H, using Bray's solution and liquid scintillation counting. Permeant uptake by cell samples added directly to 40 ml of cold stopping solution containing permeant was 30–80 cpm. The [^{35}S]-NBMPR content of samples assayed for uptake of ^{3}H-labeled permeant was small (less than 80 cpm) under the conditions of the liquid scintillation assay for ^{3}H activity and was corrected for by subtracting appropriate blanks. In parallel samples, the amount of [^{35}S]NBMPR specifically bound to 1–2 × 10^7 cells was determined as described above.

RESULTS

Binding of NBMPR to HeLa cells. The present study showed that HeLa cells possess binding sites with high affinity for NBMPR, resembling those on erythrocytes (8, 9). When these binding sites are occupied by NBMPR, nucleoside transport is impaired in either cell type. The distribution of [^{35}S]NBMPR between HeLa cells and the suspending medium was studied as follows. Cells were assayed for ^{35}S content without washing: the difference between the ^{35}S content of cell pellets from incubation mixtures with and without NBTGR (a tightly bound homologue of NBMPR) measured site-specific binding of NBMPR and, at the same time, corrected for ^{35}S attributable to the medium content of the pellets. The specific activity of our [^{35}S]NBMPR preparations, together with the low number of specific binding sites present on HeLa cells, required that at least 10^7 cells be present in each binding assay.

Binding of NBMPR at 37° was almost complete by 1 min (Fig. 1). These data also illustrate the large reduction in NBMPR binding which resulted from prior treatment of cells with NBTGR; this difference was the basis of the assay for site-specific NBMPR binding. NBTGR and NBMPR are both potent, firmly bound inhibitors of nucleoside transport with similar concentration-effect relationships for inhibition of uridine and thymidine uptake[2] (16).

Dissociation of NBMPR from cellular binding sites was examined in the experiment of Fig. 2. Cells were first labeled by incubation with [^{35}S]NBMPR; NBTGR was then added to the incubation mixture, and cells were assayed at intervals for ^{35}S. At 37°, displacement of [^{35}S]NBMPR from

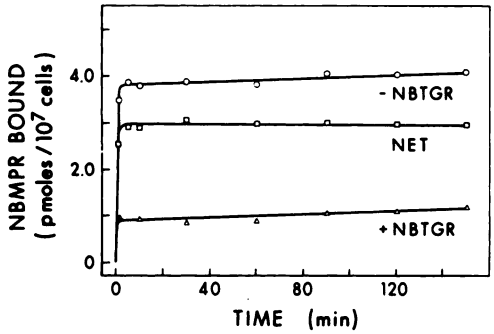

FIG. 1. *Binding of NBMPR by HeLa cells at 37°*

Cells from a spinner culture were resuspended in fresh medium (3.6×10^6 cells/ml) with and without 3.6 μM NBTGR. After 5 min at 37°, each culture was diluted with an equal volume of warmed medium containing [^{35}S]NBMPR (final concentration, 7.4 nM). At intervals samples were assayed for cell number and the ^{35}S content of the cell pellet. The difference between the ^{35}S content of cells incubated in the absence and presence of NBTGR (NET) was employed throughout this work to determine specifically bound NBMPR.

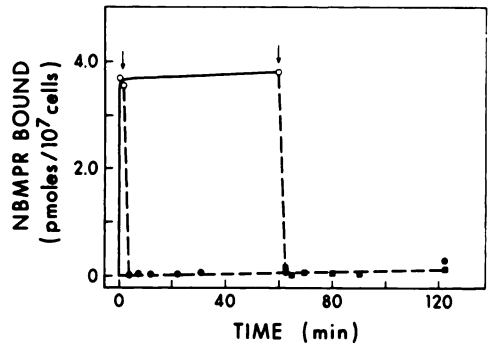

FIG. 2. *Displacement of bound NBMPR from HeLa cells by NBTGR at 37°*

As in Fig. 1, [^{35}S]NBMPR was added (final concentration, 6.4 nM) to replicate cell suspensions at 37°, and cell samples taken thereafter were assayed for ^{35}S. NBTGR (final concentration, 3.2 μM), was added (arrow) to one group of the replicate suspensions 1.0 min (●) after addition of [^{35}S]NBMPR, and to another group (■) 59 min later (arrow); NBTGR was not added to the control group (○). Each of the replicate cell suspensions so treated had an NBTGR-treated control. To the latter, NBTGR (final concentration, 2 μM) was added 5 min before the addition of [^{35}S]NBMPR, and each was processed in parallel with its untreated counterpart. Differences between the ^{35}S contents of cells with and without NBTGR treatment are plotted.

the cells by NBTGR was rapid whether latter was added early or late after contact of the cells with [^{35}S]NBMPR (Fig. 2). These data demonstrate the reversibility of NBMPR binding. Similar experiments conducted at 20° (Fig. 3) showed that association of [^{35}S]NBMPR with the binding sites was rapid (84% complete after 1 min), but displacement by NBTGR was slower at 20° than at 37° (Fig. 2). The half-life of the binding site–NBMPR complex was about 4.5 min at 20° under the displacement conditions specified in Fig. 3.

Displacement of cell-bound NBMPR by its congener, NBTGR, demonstrated reversibility of the binding. Another aspect of this reversibility is illustrated in Table 1, which shows that [^{35}S]NBMPR dissociated from cells during incubation under culture conditions in the absence of a displacing agent: successive dilutions caused the loss of successive increments of cell-bound [^{35}S]NBMPR to the medium. It is apparent in these data that NBMPR con-

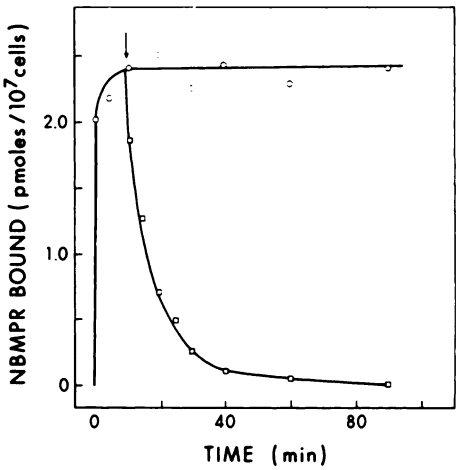

FIG. 3. *Displacement of bound NBMPR from HeLa cells by NBTGR at 20°*

As in Fig. 2, [^{35}S]NBMPR was added (final concentration, 5.9 nM) to replicate cell suspensions at 20°, and at various times thereafter cells were assayed for ^{35}S (○). NBTGR was added (final concentration, 2 μM) 10 min (arrow) after addition of [^{35}S]NBMPR (□). Suspensions of cells pretreated with 2 μM NBTGR as in Fig. 2 were also assayed in this way. Differences between the ^{35}S contents of cells with and without NBTGR pretreatment are plotted.

TABLE 1

Dissociation of bound [^{35}S]NBMPR

HeLa cells were incubated at 37° in spinner medium containing [^{35}S]NBMPR, and the ^{35}S contents of pelleted cells and of medium samples were determined separately. For suspension A, cells (10^6/ml) were incubated for 5 min. Suspension B consisted of suspension A cells resuspended in warmed, NBMPR-free medium and incubated for 60 min prior to assay of cells and medium for ^{35}S. For suspension C, a portion of suspension B was diluted with 3 volumes of medium, incubated for 60 min, and assayed for ^{35}S. For suspension D, a portion of suspension C was diluted with 2 volumes of medium, incubated for 60 min, and assayed for ^{35}S. NBMPR was absorbed from medium samples onto 100 mg of charcoal (grade AU-4, Barnebey-Cheney, Ltd., Columbus, Oh.), from which it was eluted with 5 ml of 1,4-dioxane (56°, 30 min) prior to assay of radioactivity.

Suspension	NBMPR in medium	Cell-bound NBMPR
	nM	*pmoles/10^7 cells*
A	4.7	1.95
B	0.12	0.41
C	0.04	0.27
D	0.02	0.26

centrations in the cell pellet were two to three orders of magnitude higher than in the medium; a portion of the cell-associated NBMPR could be intracellular.

The relationship between the site-specific binding of NBMPR and the extracellular concentration of NBMPR was explored in the experiments of Figs. 4–6. Figure 4 illustrates the basis of the binding assay; it is apparent that the ability of HeLa cells to bind NBMPR in the presence of 3.7 μM NBTGR was greatly reduced over a wide range of NBMPR concentrations. NBTGR was added to the assay system before [^{35}S]NBMPR and was present at a concentration greatly in excess of free NBMPR, essentially excluding the latter from the cellular binding sites. [^{35}S]-NBMPR binding in the presence of NBTGR did not exceed 10% of that in its absence for concentrations of [^{35}S]NBMPR below 5 nM. Site-specific NBMPR binding was defined as the difference between the cellular content of ^{35}S in the presence and absence of NBTGR under these conditions.

Figure 5 describes the relationship between bound and free NBMPR, the latter presumably in equilibrium with the former. These data illustrate saturability of the site-specific binding of NBMPR and also show that (a) more NBMPR is bound at 37° than at 20°, and (b) binding saturation at 37° occurs at a higher concentration than at 20°. Half-maximal values for site-

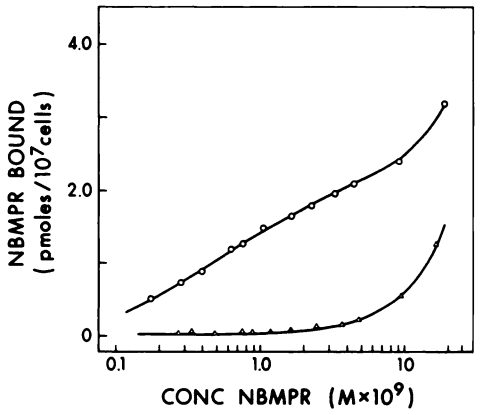

FIG. 4. *Binding of NBMPR by HeLa cells*

Portions (10.0 ml) of a cell suspension (1.4×10^6 cells/ml in growth medium) were mixed with 10.0 ml of calcium-free minimal essential medium containing [^{35}S]NBMPR with (△) or without (○) NBTGR (final concentration, 3.7 μM) and were incubated at 37° for 5 min. The ^{35}S contents of cells and medium from each incubation mixture were then determined.

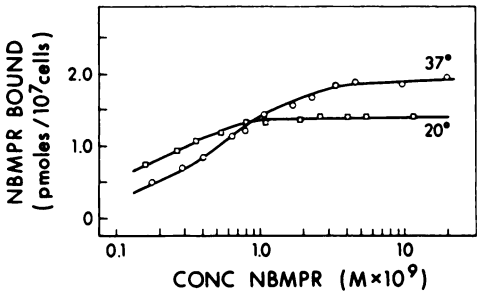

FIG. 5. *Site-specific binding of NBMPR by HeLa cells at 20° and 37°*

As in Fig. 4, replicate cell suspensions were incubated for 5 min at 20° (□) or 37° (○) in calcium-free minimal essential medium containing [^{35}S]NBMPR at various concentrations with and without 3.7 μM NBTGR. The ^{35}S contents of cells and medium from each suspension were measured. Specifically bound NBMPR was determined as the difference between the [^{35}S]NBMPR contents of cells in the presence and absence of NBTGR.

specific binding were reached at NBMPR concentrations of 0.47 nM (37°) and 0.14 nM (20°). Reciprocals of the data from Fig. 5 gave straight-line plots, from which were obtained saturation values for site-specific NBMPR binding of 2.13 (37°) and 1.48 (20°) pmoles/10^7 cells. Site-specific binding of NBMPR at concentrations above saturation of binding in 14 independent assays averaged 2.49 ± 0.53[4] (37°) and 1.93 ± 0.38 (20°) pmoles/10^7 cells.

Binding data from Fig. 5 were subjected to mass law analysis (Fig. 6) by the method of Scatchard (17). Because straight lines fit the data, it would appear that a single type of receptor was responsible for the site-specific binding of NBMPR by HeLa cells. These data indicate that 1.3 × 10^5 and 0.9 × 10^5 sites per HeLa cell bound NBMPR at 37° and 20°, respectively, and that NBMPR dissociation constants were 0.59 nM (37°) and 0.14 nM (20°). Table 2 summarizes results from similar experiments, which also yielded straight-line Scatchard plots at both temperatures.

To determine whether the particular NBMPR binding sites detected here were part of (or perhaps interacted with) the nucleoside transport mechanism, the ability of HeLa cells to take up thymidine (0.1 μM) or uridine (4 μM) was measured in the presence of graded concentrations of NBMPR. Figure 7 shows that time courses for the uptake of [*methyl*-^{3}H]thymidine were linear for at least 2 min at 20°. The low rate of thymidine uptake in the presence of 5 μM NBMPR is attributed to passive diffusion; other communications from this laboratory have reported that NBMPR at this concentration eliminated mediated uptake of uridine and thymidine by HeLa cells (4, 16). Use of [^{35}S]NBMPR enabled the simultaneous determination of both NBMPR binding and inhibition of nucleoside uptake. For example, when the uptake of [*methyl*-^{3}H]thymidine was measured, 1–2 × 10^6 cells/assay were employed; the contribution to the observed ^{3}H radioactivity of cell-associated [^{35}S]-NBMPR was not significant with cell samples of this size. Parallel assays with a 10-fold larger number of cells (and nonisotopic thymidine) enabled determination of NBMPR binding. The data of Fig. 7 show that partial occupancy of the NBMPR receptor sites resulted in partial inhibition of thymidine uptake. However, in the presence of 4.6 nM NBMPR, 2.14 pmoles of ligand were bound per 10^7 cells, indicating nearly total occupancy of binding sites (see comments above relating to Fig. 5), yet the inhibition (75%) of thymidine uptake was well short of complete. Similar results were obtained in other experiments including that of Fig. 8. Partial occupancy of the NBMPR binding sites also resulted in partial inhibition of uridine uptake (data not shown), and, as with thymidine up-

[4] Average deviation from the mean.

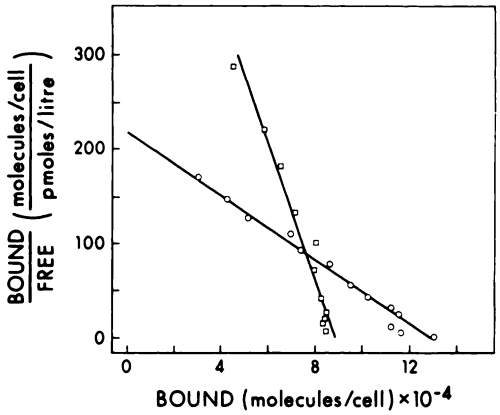

FIG. 6. *Binding of NBMPR by HeLa cells at 20° and 37°*

Data from the experiment of Fig. 5 are presented here in the form of a mass law (Scatchard) plot (17). Lines were fitted to the data by the method of least squares.

TABLE 2

Binding of [^{35}S]NBMPR

The site-specific binding of NBMPR to HeLa cells was measured as in Fig. 5; Scatchard plots were linear and yielded the constants listed.

Expt.	Dissociation constant		Binding sites per cell	
	20°	37°	20°	37°
	nM		*×10⁻⁵*	
1[a]	0.14	0.59	0.9	1.3
2		0.51		1.5
3		0.37		1.6
4	0.15		1.1	

[a] Figure 6.

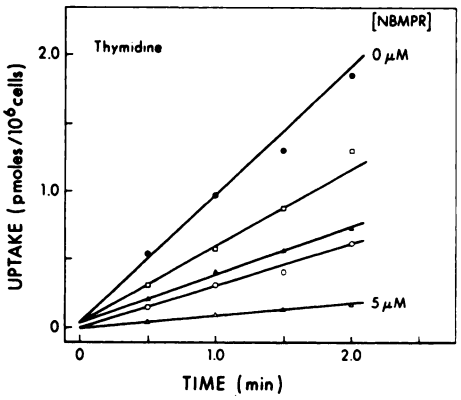

FIG. 7. *Effect of bound NBMPR on uptake of thymidine by HeLa cells*

The time course of thymidine uptake from medium containing 0.1 μM [*methyl*-^{3}H]thymidine was assayed as described in METHODS at 20° in the absence (●) and presence of [^{35}S]NBMPR (□, ▲, ○). Thymidine uptake was also assayed in the presence of 5 μM nonisotopic NBMPR (△) to measure thymidine entry by diffusion. The binding of [^{35}S]NBMPR under conditions identical with those of the thymidine uptake assay was determined at the same time in parallel experiments which employed larger (10-fold) cell numbers and nonisotopic thymidine. After 2 min of incubation under conditions identical with those of the thymidine uptake assay, the [^{35}S]-NBMPR contents of cells and medium were determined with these results:

Free (medium) NBMPR	Bound NBMPR
*n*M	*pmoles/10⁷ cells*
0.2 (□)	1.10
0.7 (▲)	1.35
4.6 (○)	2.14

take, as saturation values of binding were approached, uridine uptake was able to proceed at rates about 25% of control (uninhibited) values.

Thus, although different degrees of inhibitor site occupancy correlated with nucleoside transport inhibition, the relationship between these parameters was not simple. When the high-affinity sites studied here were almost fully occupied, a substantial transport capacity (25%) for uridine and thymidine remained active, yet this was inhibited when NBMPR concentrations were increased 1000-fold to about 5 μM. This result suggests the existence of NBMPR-transporter interactions distinct from those which the present binding assay detected. For example, it is possible that the transport of a particular nucleoside may be mediated by several types of transporter, which may differ in affinity for NBMPR. In this context, it may be noted that (a) the cell populations employed were asynchronous and therefore included cells at all stages of the mitotic cycle, and (b) some nucleoside transport activities, notably those for thymidine and deoxycytidine, vary at different stages of the cell cycle (4, 18).

The experiment of Fig. 8 demonstrated that the binding of NBMPR (and the resultant inhibition of thymidine uptake) was reversible. In this experiment, cells which had bound NBMPR to the extent of

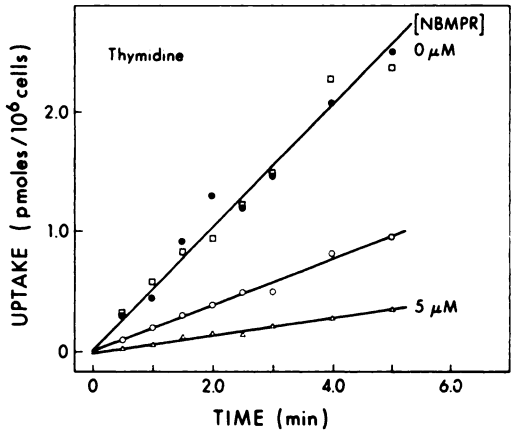

FIG. 8. *Reversibility of NBMPR inhibition of thymidine uptake*

Time courses of thymidine uptake by HeLa cells from medium containing 0.1 μM [*methyl*-^{3}H]-thymidine at 20° were determined in the absence (●) and presence (○) of 5.5 nM [^{35}S]NBMPR. Thymidine uptake was also assayed in the presence of 5 μM nonisotopic NBMPR (△) to measure the diffusional entry of thymidine. As in Fig. 7, the binding of [^{35}S]NBMPR was determined under conditions identical with those of the thymidine uptake assay in parallel experiments; in this assay, 2.2 pmoles of NBMPR were bound per 10^7 cells. The release of cell-bound NBMPR under incubation conditions at 37° was examined as follows. After incubation in the presence of 5.5 nM [^{35}S]NBMPR for 5 min at 20°, HeLa cells were resuspended in warmed growth medium (3.2×10^5 cells/ml) and incubated at 37° for 30 min. The cells were then collected by centrifugation, resuspended in growth medium (1.3×10^6 cells/ml), and assayed for uptake of [*methyl*-^{3}H]thymidine (□) as above and for their content of [^{35}S]NBMPR; the latter value was 0.21 pmol/10^7 cells.

2.2 pmoles/10^7 cells (medium concentration, 5 nM), and in which the thymidine uptake was reduced by 75%, lost bound NBMPR during a subsequent incubation at 37°; the ability to take up thymidine was regained concomitantly with this loss. This experiment demonstrated that association of NBMPR with and release from the cellular binding sites correlated with inhibition and restoration of thymidine uptake, respectively.

Identity of cell-bound NBMPR. To determine whether NBMPR underwent chemical transformation during cellular binding, HeLa cells with bound [^{35}S]-NBMPR were extracted with ethanol, and the extracted material was subjected to analysis by thin-layer chromatography. HeLa cell suspensions (10^6 cells/ml) were incubated for 10 min at 20° or 37° in growth medium containing 5 nM [^{35}S]NBMPR. Cell pellets (10^8 cells) were frozen at −76°, dried under vacuum over P_2O_5, and then extracted with 2 ml of 70% aqueous ethanol for 10 min at 56°. The ethanolic extracts were dried, and the residues were re-extracted with absolute methanol; this procedure recovered more than 95% of the ^{35}S initially associated with the cell pellet. The methanolic extracts were chromatographed on paper with carrier NBMPR in three solvent systems: (a) water-saturated 1-butanol; (b) 1-butanol–glacial acetic acid–water (120:30:50, v/v/v); and (c) 95% ethanol–1 M ammonium acetate, pH 7.5 (70:30, v/v). In these systems, R_F values for NBMPR were 0.59, 0.80, and 0.93, respectively. With each solvent system, the extracted ^{35}S activity co-chromatographed with NBMPR, whether extracted from cells incubated at 20° or 37°. It was concluded that NBMPR remained unchanged when bound to HeLa cells.

DISCUSSION

This study has shown that HeLa cells possess high-affinity receptor sites for NBMPR. The method employed to determine site-specific binding of NBMPR measured the difference between cell-associated [^{35}S]NBMPR (a) under experimental conditions and (b) when the binding sites were saturated with NBTGR, a tightly bound homologue of NBMPR (4). The following properties of the NBMPR binding sites were apparent in this study.

1. The sites bound NBMPR tightly, but reversibly. The reversible nature of the binding was apparent in the displacement of cell-bound [^{35}S]NBMPR by NBTGR (Figs. 1 and 2) and the dissociation of bound [^{35}S]NBMPR under culture conditions at 37° (Table 1 and Fig. 8). The recovery of chemically unchanged [^{35}S]NBMPR from cells to which it had been bound is consistent with a reversible binding process. Dissociation constants for the cell-bound NBMPR determined by mass law calculations were about 0.1 nM, indicating tight binding; dissociation constants of about 1 nM for NBMPR bound to human erythrocytes were reported previously (8).

2. The NBMPR binding sites were evidently of a single type, because of the linearity of mass law (Scatchard) plots derived from the binding data.

3. HeLa cells possess about 10^5 NBMPR binding sites; in comparison, human erythrocytes have about 10^4 such sites per cell.

4. Fractional occupancy of the binding sites by NBMPR resulted in fractional inhibition of the transport of thymidine and uridine (Figs. 7 and 8); however, the relationship between occupancy and inhibition was not a simple proportionality as with human erythrocytes (9). It would appear that interactions between the nucleoside transport mechanism and NBMPR took place (at higher concentrations of NBMPR) that were not perceived by the binding assay.

5. The maximum number of NBMPR binding sites was larger at 37° than at 20°. It is possible that this observation may have an explanation in temperature-related transitions in nucleoside uptake activity, such as those seen in Novikoff hepatoma cells (19); the latter occurred in the 15–18° range and were probably due to changes in the physical state of the membrane lipids (19).

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