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DEPHOSPHORYLATION OF NITROBENZYLTHIOINOSINE 5′-MONOPHOSPHATE BY ECTO 5′-NUCLEOTIDASE OF HeLa CELLS

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Abstract—HeLa cells as well as human and mouse erythrocytes possess membrane sites which bind the inhibitor of nucleoside transport, nitrobenzylthioinosine (NBMPR), reversibly but tightly (K_D, 10^{-9}–10^{-10} M). Site-specific binding of the ligand correlates with inhibition of nucleoside transport. The present study showed that the 5′-phosphate of NBMPR, NBMPR-P, was not transport inhibitory. Upon exposure to [^{35}S]NBMPR-P or [G-^{3}H]NBMPR-P, HeLa cells retained the isotopic labels virtually exclusively in the form of NBMPR. The dephosphorylation of [G-^{3}H]NBMPR-P by HeLa cells, assayed by the production of extracellular [G-^{3}H]NBMPR, was competitively inhibited by AMP, but was not affected by the presence of 5 μM NBMPR, a concentration sufficient to completely occupy the transport inhibitory sites. Thus, the sites at which dephosphorylation of NBMPR occurs in HeLa cells are separate from and function independently of the high affinity sites which bind NBMPR.

The permeation of physiological nucleosides and nucleoside analogs through plasma membranes of erythrocytes [1–3] and of various types of cultured mammalian cells [4, 5] occurs mainly by a nucleoside-specific, facilitated diffusion process. NBMPR‡ is a potent inhibitor of the nucleoside transport process in a variety of cell types [5], but exceptions are known (see below). In cultured mammalian cells [6, 7] and in erythrocytes [8], inhibition of nucleoside transport by NBMPR is associated with high affinity, site-specific binding of NBMPR to cells, apparently to membrane sites [9]. In human erythrocytes, a proportionality has been shown between binding site occupancy by the inhibitor and fractional inhibition of uridine transport [8]. The latter observation and the absence of NBMPR binding sites on cell types genetically deficient in nucleoside transport [10–12] have indicated that the binding sites represent functional nucleoside transport elements in the cell membrane. Maximum values for the cellular binding of NBMPR (estimated from mass law analysis of equilibrium binding data) have been used to estimate the abundance of nucleoside transporter elements on cells [6, 7, 11, 12]. Earlier studies in this laboratory showed that HeLa cells and S49 mouse lymphoma cells possess about 10^5 high affinity sites per cell at which bound NBMPR had dissociation constants of 10^{-9} to 10^{-10} M [6, 11]. These high affinity sites, which are evidently located on nucleoside transport elements, are absent from cells of a nucleoside transport-deficient clone (AE_1) derived from a mutagenized population of S49 cells [11].

While the high affinity sites that bind NBMPR represent functional nucleoside transport sites in erythrocytes [8, 10] and in cultured S49 mouse lymphoma cells [11], nucleoside transport mechanisms of low sensitivity to NBMPR have been recognized in cultured cell lines of two rat neoplasms, the Novikoff hepatoma [13] and the Walker 256 carcinosarcoma [14, 15], and cell types with nucleoside permeation mechanisms having components of high and low sensitivity to NBMPR have been reported [14, 16].

NBMPR has been shown to protect cultured cells against a variety of cytotoxic nucleosides, apparently by reducing cellular uptake of the latter [7, 17]. The administration of NBMPR to mice together with toxic doses of the naturally occurring nucleosides, tubercidin, nebularine, or toyocamycin, markedly changed the toxicology of these agents in that mice were protected against otherwise lethal treatment protocols with these drugs [17]. Because of high aqueous solubility, NBMPR-P, the 5′-monophosphate ester of NBMPR [18], has been employed as a "prodrug" form of NBMPR in demonstrations of *in vivo* protection against toxic doses of nucleoside analogs and in chemotherapy experiments in which leukemic mice were treated with high, potentially lethal doses of nucleoside analogs [19, 20]. We have shown that administration of NBMPR-P together with tubercidin dramatically changes the toxicology of the latter agent in mice

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‡ Abbreviations: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBMPR-P, the 5′-monophosphate of NBMPR; NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; MEM, Eagle's minimal essential medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; and HPLC, high pressure liquid chromatography.

[21]. The present study indicates that (i) NBMPR-P *per se* does not interact with the high affinity transporter sites on erythrocytes and HeLa cells in that it does not inhibit NBMPR binding and is not transport inhibitory prior to dephosphorylation, and (ii) dephosphorylation of NBMPR-P by ecto 5′-nucleotidase proceeds independently of NBMPR binding.

5′-Nucleotidase is an intrinsic protein of the plasma membrane with its catalytic site located on the external aspect of the plasma membrane [22–26] in various mammalian cells. The presence of ecto 5′-nucleotidase activity has not been demonstrated in mammalian erythrocytes.

MATERIALS AND METHODS

Cells. HeLa S3 cells were cultured as described previously [27]. Trypsinized monolayer cultures provided inocula for suspension cultures in MEM-S medium* kept under continuous agitation with vibrating mixers (Vibro-Mixer, model E1, Chemapec, Hoboken, NJ) and were kept in exponential growth by dilution. Such cultures were single cell suspensions. Cell culture materials were purchased from GIBCO Canada Ltd., Burlington, Ont.

Replicate monolayer cultures were employed in NBMPR binding studies unless otherwise specified. Cultures in 2- or 8-oz prescription bottles containing $1–2 \times 10^6$ cells or $3–4 \times 10^6$ cells, respectively, were used 24 hr after inoculation, at which time cells were proliferating exponentially.

Male hybrid mice (C57BL/10.A SgSnJ × DBA/2J F_1) were asphyxiated with carbon dioxide, and blood was collected into 3.8% sodium citrate solution by cardiac puncture. The blood was centrifuged, plasma and buffy coat were removed, and the red cells were washed four times with TES-buffered saline (140 mM NaCl, 1.4 mM $MgSO_4$ and 18 mM TES, at pH 7.4) before use in transport experiments.

NBMPR binding. Site-specific binding of radioactivity from labeled NBMPR or NBMPR-P was determined as the difference between the cellular content of isotope after incubation to binding equilibrium in the absence and the presence of 5 μM NBTGR. In the latter condition, the virtually total occupancy of the high affinity binding sites by NBTGR, a potent and tightly bound inhibitor of nucleoside transport [28, 29], provided a measure of non-specific binding of [^{3}H]NBMPR. In binding assays, growth medium of replicate monolayer cultures in 4-oz bottles was replaced with 4 ml of MEM-T medium containing [^{3}H]NBMPR and specified additives. MEM-T medium consisted of serum-free MEM medium containing 20 mM HEPES buffer (pH 7.4) [27]. After incubation at 22° for 30 min (virtual equilibrium between bound and free NBMPR was demonstrated under these conditions), monolayers were washed once with ice-cold 0.15 M NaCl solution (bottles were filled), drained, and solubilized in 2.0 ml of 0.5 M KOH. The latter solutions were rinsed into counting vials with a detergent-xylene scintillant [30] and assayed for ^{3}H by liquid scintillation counting. Assays were at least in duplicate.

Time courses of [^{3}H]NBMPR association with cellular binding sites were obtained by exposing replicate monolayer cultures to the labeled ligand for graded intervals. In determining time courses of dissociation of the [^{3}H]NBMPR–site complex, replicate monolayer cultures which had been labeled by equilibrium with [^{3}H]NBMPR were employed, and the ^{3}H-content of those monolayers remaining after intervals of exposure at 22° to MEM-T medium containing 0.5 to 2.5 μM NBMPR or NBTGR was determined. The latter ligands occupied the high affinity binding sites virtually completely under these conditions and prevented reassociation of [^{3}H]-NBMPR with the binding sites.

Uridine transport. Time courses of the zero-*trans* influx of [5-^{3}H]uridine into washed mouse erythrocytes were determined at 22°. During influx intervals, the suspensions contained 150 μM [^{3}H]uridine and 6×10^8 erythrocytes at a 5% hematocrit in TES-buffered saline. In assay suspensions for the determination of inhibitory effects of NBMPR and NBMPR-P, these substances were present at 5 μM concentrations. For some determinations, NBMPR-P was purified by HPLC on a Brownlee ODS RP-18 column (Brownlee Labs., Inc., Santa Clara, CA) eluted with 25% acetonitrile in 10 mM KH_2PO_4.

After specified time intervals of 2–10 sec, influx was terminated by the rapid addition of NBMPR to a final concentration of 10 μM. Zero-time points were determined by addition of NBMPR solution 5 sec before addition of permeant solution. The cells were immediately pelleted by centrifugation for 1 min at 12,800 *g*, and washed with 1 ml of ice-cold TES-buffered saline containing 20 μM NBMPR, before extraction of acid-soluble ^{3}H into cold 5% perchloric acid. Duplicate portions of each extract were assayed for ^{3}H by liquid scintillation methods.

Chemicals. NBMPR and NBTGR were prepared in this laboratory by Dr. Thomas P. Lynch by established methods [31, 32] using as starting materials 6-thioinosine and 6-thioguanosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. NBMPR-P was prepared by Dr. Lynch by a previously described method [18]. [5-^{3}H]Uridine, [G-^{3}H]NBMPR and [G-^{3}H]NBMPR-P were purchased from Moravek Biochemicals Inc., Brea, CA. We thank Dr. Josef Moravek for his cooperation in the tritiation of these compounds. [^{35}S]NBMPR-P was prepared by reaction of 4-nitrobenzyl bromide with [^{35}S]6-thioinosinate. The latter was labeled by exchange with [^{35}S]elemental sulfur by procedures previously employed to prepare [^{35}S]-NBMPR [6]. The product, [^{35}S]NBMPR-P, was purified by thin-layer chromatography with initial isolation on silica gel layers which were developed in 15% methanol in chloroform, and further purification on cellulose thin layers run in butanol–ethanol–water (4:1:2, by vol.). The procedures for ^{35}S-labeling of NBMPR-P were developed in this laboratory by Dr. P. M. Moore and Dr. T. P. Lynch.

* MEM-S medium is MEM without calcium salts and supplemented with 5% calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM HEPES (pH 7.4).

RESULTS AND DISCUSSION

The equilibrium binding of [^{3}H]NBMPR and [^{3}H]-NBMPR-P to HeLa cells were compared in the experiment of Fig. 1. In the presence of 5 μM NBTGR, the cellular content of ^{3}H from either compound was greatly reduced. This result was interpreted to mean that NBTGR occupancy of the high affinity sites precluded binding of ^{3}H from [^{3}H] NBMPR-P, and suggested that the cell-associated form of the latter might be the dephosphorylation product, NBMPR. Cellular ^{3}H acquired in the presence of NBTGR was considered to be nonspecifically associated with the cells.

The influence of graded extracellular concentrations of NBMPR-P on the equilibrium binding of [^{3}H]NBMPR was measured in the experiment of Fig. 2, which indicated that NBMPR binding to HeLa cells was competitively inhibited by NBMPR-P. Two possible explanations for this result were: (i) competition between NBMPR-P and the ^{3}H-ligand at the binding sites, or (ii) dilution of [^{3}H]NBMPR in the medium by dephosphorylation of nonisotopic NBMPR-P. Subsequent findings supported the latter possibility.

Results of the experiment summarized in Fig. 3 were consistent with the idea that extracellular NBMPR derived by dephosphorylation of NBMPR-P interacted with cellular binding sites. Figure 3 shows that the site-specific cellular association of ^{35}S which took place when HeLa cell monolayers were incubated with [^{35}S]NBMPR-P was abruptly reduced by the addition of nonisotopic NBMPR to the system. This result is interpreted to mean that the addition of nonisotopic NBMPR to the medium (at a concentration at least 100-fold higher than that of the isotopic free ligand, presumably [^{35}S]NBMPR)

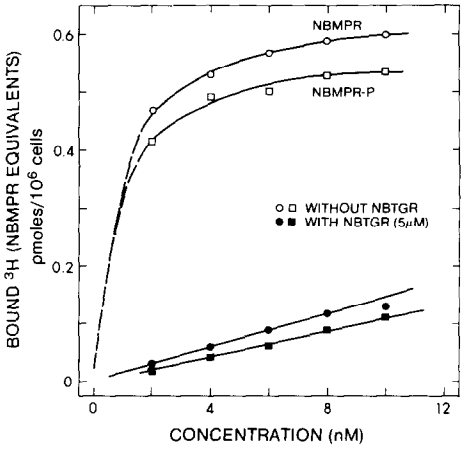

Fig. 1. Binding of NBMPR-P and NBMPR to HeLa cells. Replicate monolayer cultures were incubated for 30 min at 22° in MEM-T medium containing [G-^{3}H]NBMPR (○,●) or [G-^{3}H]NBMPR-P (□,■) at the indicated concentrations with (filled symbols) or without (open symbols) 5 μM NBTGR. Monolayers were washed once with ice-cold 0.15 M NaCl solution and assayed for ^{3}H content.

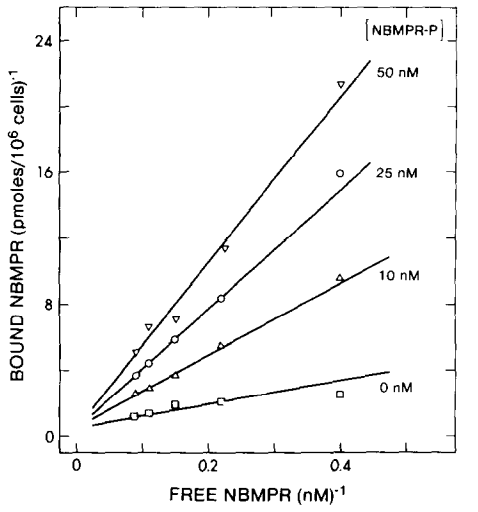

Fig. 2. Influence of NBMPR-P on NBMPR binding to HeLa cells. Replicate monolayer cultures were incubated at 22° for 20 min in MEM-T medium containing various concentrations of [G-^{3}H]NBMPR and nonisotopic NBMPR-P. The monolayers were washed once with ice-cold 0.15 M NaCl solution and then assayed for cellular ^{3}H-content.

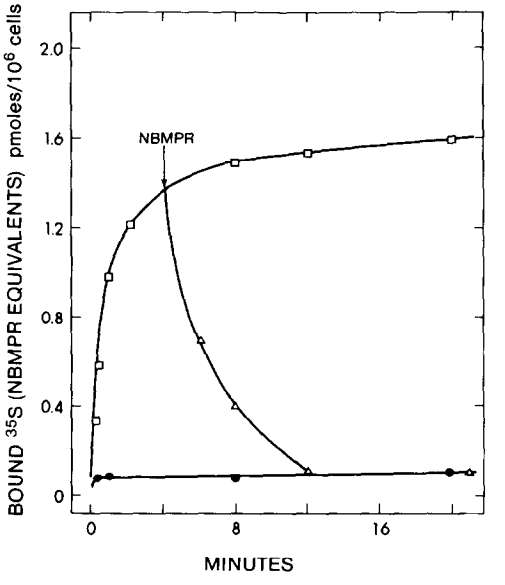

Fig. 3. Dissociation of ^{35}S from HeLa cells after incubation with [^{35}S]NBMPR-P. Replicate samples of HeLa cells in suspension were incubated at 22° with MEM-T medium containing 5 nM [^{35}S]NBMPR-P in the absence (□) and presence (●) of 5 μM NBTGR to determine site specific binding. Nonisotopic NBMPR (△) (final concentration 0.5 μM) was added to the medium after 5 min of incubation (arrow). After incubation for the periods indicated, cells were recovered by centrifugation from the replicate assay mixtures and were washed once with ice-cold 0.15 M NaCl solution. The cellular content of ^{35}S was determined after KOH solubilization.

drastically altered the cell content of the latter because, during the equilibration between free and site-bound ^{35}S-ligand, binding sites freed by dissociation of the [^{35}S]NBMPR–site complex became occupied by the nonisotopic ligand. Thus, the time course shown for the changes in cellular ^{35}S-content following the addition of nonisotopic NBMPR would appear to represent dissociation of the NBMPR-binding site complex without the occurrence of significant reassociation of the free isotopic ligand, that is, as if dissociation into an infinitely large volume had occurred. Reversible binding of the ^{35}S-ligand is clearly implied by the results of Fig. 3.

The results shown in Figs. 1–3 indicated that the isotopic, site-bound molecular species on cells exposed to isotopically labeled NBMPR-P could be NBMPR. This interpretation was tested directly in experiments which showed that the isotopic material extracted from HeLa cells after incubation with [G-^{3}H]NBMPR-P behaved chromatographically like NBMPR. Preliminary experiments (data not shown) showed that cell-bound radioactivity could be extracted with methanol virtually quantitatively (95–97%) from HeLa cells exposed to NBMPR-P labeled with ^{3}H or ^{35}S. Figure 4 shows that the isotopic material in extracts from cells exposed to [G-^{3}H]-NBMPR-P comigrated with NBMPR on cellulose thin layers* and was the principal isotopic product in the extracts regardless of the duration of cell exposure to [^{3}H]NBMPR-P.

The preceding evidence indicated that NBMPR was the principal, if not the exclusive, derivative of NBMPR-P found in association with HeLa cells. Our previous demonstration that NBMPR-P was dephosphorylated by 5′-nucleotidase [18], together with the well recognized existence of ecto 5′-nucleotidase as an intrinsic constituent of the plasma membrane in various cells [22–26], including HeLa cells [33], suggested that the observed formation of cell-bound NBMPR from extracellular NBMPR-P proceeded via cellular ecto 5′-nucleotidase activity. The latter idea was tested, as shown in Figs. 5 and 6, by measuring (i) cell-mediated conversion of extracellular NBMPR-P to extracellular NBMPR, and (ii) inhibition of NBMPR-P dephosphorylation by AMP, a recognized substrate of ecto 5′-nucleotidase. In evaluating cellular dephosphorylation of NBMPR-P, washed monolayers and serum-free medium were employed.

The experiment of Fig. 5 showed that the dephosphorylation of NBMPR-P was progressive and proceeded with the formation of NBMPR in the medium under conditions that assured saturation of cellular NBMPR binding sites, that is, in the presence of 5 μM nonisotopic NBMPR. Dephosphorylation rates were similar in the absence of NBMPR (data not shown). The formation of extracellular NBMPR from NBMPR-P was not due to the release of cellular phosphorolytic enzymes into the incubation medium because cell-free medium following incubation with HeLa cell monolayers under the conditions specified in Fig. 5 did not dephosphorylate NBMPR-P (data not shown).

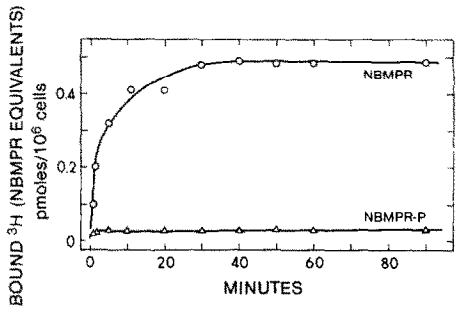

Fig. 4. Formation of bound NBMPR in HeLa cells incubated with [G-^{3}H]NBMPR-P. Replicate monolayer cultures in 8-oz bottles were washed twice with MEM-T medium and then incubated at 22° in MEM-T medium containing 10 nM [G-^{3}H]NBMPR-P for the indicated intervals. To end incubation intervals, the monolayers were washed once with ice-cold 0.15 M NaCl solution and extracted several times with methanol. Chromatographic carriers, nonisotopic NBMPR and NBMPR-P were added to the pooled methanol extracts which were dried under a nitrogen stream and redissolved in 100 μl of methanol. Measured volumes of the methanol-extracted material were chromatographed on cellulose thin layers in Solvent III. Carrier-containing areas scraped from the chromatogram plates were combusted in a Packard model 306 Sample Oxidizer for determination of ^{3}H-content, expressed in NBMPR equivalents that accompanied NBMPR (○) and NBMPR-P (△) carriers.

The data of Fig. 6 demonstrate that dephosphorylation of NBMPR-P was inhibited by AMP and that the inhibition was competitive. A slope replot (Fig. 6

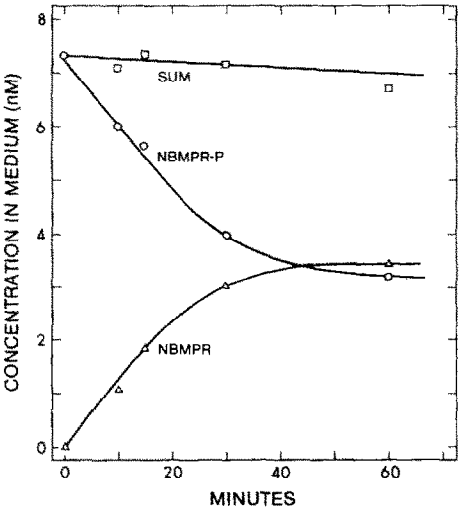

Fig. 5. Dephosphorylation of NBMPR-P by HeLa cell monolayers in the presence of 5 μM NBMPR. Monolayer cultures in 8-oz prescription bottles were washed twice with MEM-T medium and preincubated with 5 μM NBMPR in MEM-T medium for 20 min at 22°. [G-^{3}H]NBMPR-P (final concentration 10 nM) was added to the medium and incubation at 22° was continued for the indicated intervals. Samples (100 μl) of the medium from each incubation mixture were chromatographed with NBMPR and NBMPR-P carriers on cellulose thin layers with solvent I, and the ^{3}H accompanying the carriers was determined by the combustion method.

* This was confirmed in three chromatographic solvents: I, *n*-butanol–95% ethanol–water (4:1:2, v/v); II, isoamyl alcohol saturated with 5% Na_2HPO_4; III, 95% ethanol–1 M ammonium acetate (7:3, v/v).

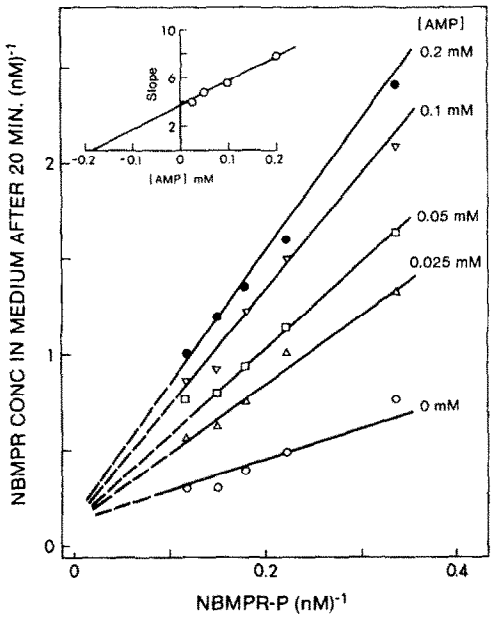

Fig. 6. Influence of AMP on the rate of NBMPR-P dephosphorylation by HeLa cell monolayers. Monolayer cultures in 8-oz prescription bottles were washed twice with MEM-T medium and preincubated with 5 μM NBMPR in MEM-T medium for 20 min at 22°. [G-^{3}H]NBMPR-P (final concentration 10 nM) was then added to the medium in the absence and in the presence of various concentrations of AMP, and incubation at 22° was continued for 20 min. Samples of assay medium (100 μl) from each incubation mixture were chromatographed with NBMPR and NBMPR-P carriers on cellulose thin layers using solvent I, and the ^{3}H accompanying the carriers was determined by the combustion method. The replot (inset) indicates that the K_i for the AMP inhibition of NBMPR-P dephosphorylation was 0.19 mM.

inset) indicated that the K_i value for AMP in this inhibition was 0.19 mM. The apparent competitive inhibition by AMP of NBMPR-P dephosphorylation indicates that the latter reaction was mediated by the HeLa cell ecto 5′-nucleotidase.

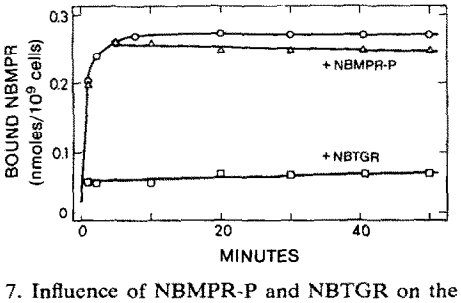

Fig. 7. Influence of NBMPR-P and NBTGR on the time course of NBMPR binding to mouse erythrocytes. Washed erythrocytes were prepared as a set of replicate centrifuge pellets containing 2.0×10^9 cells each. Each cell pellet was suspended at 22° in a 2.0-ml portion of MEM-T medium containing 0.5 nM [G-^{3}H]NBMPR without (○) and with 0.1 μM NBMPR-P (△) or 0.1 μM NBTGR (□). Incubation intervals began with the suspension of cells and were ended by centrifugation. The cellular ^{3}H-content was determined before and after incubation. The values shown are averages of duplicates.

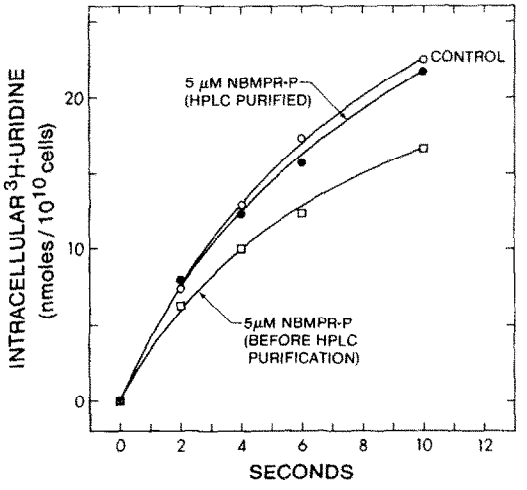

Fig. 8. Uridine transport in mouse erythrocytes in the presence of NBMPR-P before and after its purification by HPLC. Zero-*trans* influx of 150 μM [^{3}H]uridine at 22° was stopped at the specified times by addition of NBMPR at a final concentration of 10 μM. Zero-time values, representing ^{3}H nonspecifically trapped in the cell pellets, were subtracted from values measured at each time point. Each point represents results from duplicate assay suspensions. The suspensions contained: (control) no NBMPR-P (○), 5 μM NBMPR-P purified by HPLC (●), or 5 μM NBMPR-P *before HPLC purification* (□).

In other experiments (Fig. 7), we have observed that (a) high affinity, site-specific binding of [G-^{3}H] NBMPR at equilibrium by mouse erythrocytes was reduced by only 8–10% by a 200-fold excess of NBMPR-P. The small reduction that did occur in that experiment was probably due to NBMPR present in the NBMPR-P preparation (see Fig. 8).

The experiment summarized in Fig. 8 demonstrated that uridine influx in mouse erythrocytes was not affected by the presence of highly purified NBMPR-P at 5 μM concentrations. The time course data for control assays and those containing purified NBMPR-P were the same within experimental error. Other experimental data (not shown) demonstrated that 5 μM concentrations of NBMPR were sufficient to reduce uridine influx to less than 3% of control values under these conditions. Influx inhibitions resulting from graded NBMPR concentrations between 10 and 100 nM ranged from 5 to 43% and were proportional to NBMPR concentration. Uridine influx was inhibited 17–18% in assays containing 5 μM concentrations of NBMPR-P which had not been purified by HPLC and which was shown by HPLC analysis to contain 0.4 to 0.6% free NBMPR. This extent of inhibition is consistent with that which would be expected in an assay containing 25 nM NBMPR.

These results show that NBMPR-P itself is not transport inhibitory and are consistent with an apparent absence of ecto 5′-nucleotidase activity in erythrocytes. Thus, high affinity sites which bind NBMPR are present on HeLa cells and erythrocytes, but HeLa cells differ from erythrocytes in manifesting ecto 5′-nucleotidase activity. HeLa cells are known to possess plasma membrane-bound 5′-nucleotidase activity [33].

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