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# Properties of the Thymidine Transport System of Chinese Hamster Ovary Cells as Probed by Nitrobenzylthioinosine

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*Summary.* The transport of thymidine into Chinese hamster ovary cells grown in suspension culture was measured under conditions in which thymidine was not metabolized, namely, when cells had been depleted of ATP. The system transporting thymidine was saturable ($K_m^{zt}=70$ μM), rapid (50% of transmembrane equilibrium level attained within 8 sec), and was apparently shared by other nucleosides, but not thymine or hypoxanthine. 6([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine, "nitrobenzylthioinosine", inhibited thymidine transport in a simple, noncompetitive fashion with an apparent $K_i=1.0$ nM (based on total concentration of inhibitor, which significantly overestimates that of free inhibitor). The rate of expression of inhibition was slow ($t_{1/2}=17$ sec) relative to the rate of association of thymidine with its transporter, and thymidine partially protected the transport system against inhibition by nitrobenzylthioinosine. The dissociation constant for the inhibitor-transporter complex was estimated at about 0.1 nM, and the number of binding sites per cell at about $6\times10^4$. HeLa, P388 murine leukemia, and mouse L cells were as sensitive to nitrobenzylthioinosine inhibition of thymidine transport as Chinese hamster ovary cells; Novikoff rat hepatoma cells were much less sensitive.

Various thioesters of 6-mercaptopurine ribosides have been identified as inhibitors of tumor growth (Montgomery *et al.*, 1961). A particularly potent member of this class is 6-([4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine, the *p*-nitrobenzylthio analog of inosine. Using the human erythrocyte as a model system, Paterson and his colleagues have demonstrated that this compound strongly inhibits the membrane transport system for nucleosides (Paterson & Oliver, 1971; Pickard *et al.*, 1973; Cass, Gaudette & Paterson, 1974). Nitrobenzylthioinosine binds tightly to the erythrocyte membrane (dissociation constant = 1 nM) but is not itself altered chemically. A causal relationship of transport inhibition to antitumor activity has not been demonstrated.

Nevertheless, this work has established nitrobenzylthioinosine as a selective and useful probe of nucleoside transport. Prospects for its use as a tool to characterize nucleoside transport systems, to titrate carrier

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molecules, and to affinity-label these carriers motivate its application to cells more complicated than the erythrocyte. Indeed, its inhibition of uridine uptake has been studied in transformed hamster fibroblasts (MCT line, Eilam & Cabantchik, 1976, 1977) and of uridine, thymidine and adenosine uptake in HeLa cells (Paterson, Naik & Çass, 1977*b*; Cass & Paterson, 1977; Paterson *et al.*, 1977*a*).

While these studies have rigorously identified the transport step as the level at which nitrobenzylthioinosine operates, they observe the effects of that operation on nucleoside uptake.[1] Uptake of a radiolabeled nucleoside is a multiphase process which often involves transport[2] of substrate across the cell membrane, phosphorylation by a nucleoside kinase, incorporation[3] into nucleoside triphosphates and eventually into nucleic acids. It has generally been assumed – and supported by indirect evidence – that transport limits the rate of this overall process (*see* review by Plagemann & Richey, 1974), and, consequently, that the kinetic characteristics of uptake are ascribable to the transport step. However, where the transport step has been isolated from the subsequent steps of uptake, by chemical, genetic, or kinetic means, its kinetic characteristics appear vastly different from those of the overall process. For example, the uptake of thymidine in wild-type Novikoff rat hepatoma cells proceeds with a $K_m$ of about 0.5 μM and a $V_{max}$ of about 0.1 pmol/μl cell water·sec, and is quite specific for thymidine; the transport of thymidine into cells of a thymidine kinase-deficient subline (like that into ATP-depleted, wild-type cells) proceeds with a $K_m$ of about 85 μM and a $V_{max}$ of 10 to 20 pmol/μl cell water·sec, and the system is shared by several nucleosides (Wohlhueter *et al.*, 1976; Marz, Wohlhueter & Plagemann, 1977*a*).

The implication of these data is that some step other than transport is rate limiting for the uptake process; recently the rate limiting step has been identified as intracellular phosphorylation in Novikoff cells (for thymidine uptake, Marz *et al.*, 1977*b*) and in 3T3 cells (for uridine uptake, Rozengurt, Stein & Wigglesworth, 1977). A correlary point is that the pattern and extent of inhibition by membrane-level inhibitors, as judged by their effect on uptake, is different from the pattern and extent of inhibition of transport *per se*. This point is illustrated by the inhibition of hexose transport by cytochalasin B (Plagemann, Graff & Wohlhueter, 1977).

1 By "uptake" we denote the total accumulation of radioactivity derived from a given substrate within the cell regardless of metabolic conversions.

2 "Transport" denotes passage of substrate across the plasma membrane.

3 "Incorporation" denotes appearance of radioactivity derived from a given substrate into a specified compound or class of compounds.

An adequate assessment of nucleoside transport (as opposed to uptake) in mammalian cells requires rapid sampling techniques (Berlin & Oliver, 1975; Wohlhueter *et al.*, 1978). In the present communication we probe the thymidine transport system of Chinese hamster ovary cells with such techniques.

## Materials and Methods

### *Cells and Media*

Novikoff rat hepatoma cells (subline N1S1-67) and mouse L cells were propagated in suspension culture in Swim's medium 67 and enumerated with a Coulter counter as described previously (Ward & Plagemann, 1969; Graff & Plagemann, 1976). P388 mouse leukemia, Chinese hamster ovary, and HeLa cells were propagated in the same manner, except that the growth medium was Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum and that for production of large batches (1–3 liters) the suspensions were agitated with a magnetic stirrer rather than by incubation on a gyrotory shaker. Cells to be used in experiments were harvested from late exponential phase cultures (between 0.5 and $2 \times 10^6$ cells/ml depending on the cell line) by centrifugation at $400 \times g$ for 1 min and resuspended in a basal medium (BM42B; Plagemann & Erbe, 1974) for transport assays.

In the case of the Novikoff and L cell lines, we have available thymidine kinase deficient mutants, which transport thymidine, but are unable to metabolize it. For the other cell lines, where it was necessary to preclude complications due to metabolism of transported substrate, the cells were depleted of ATP by incubation at 37° for 15 min in a glucose-free variant of basal medium (BM42A), containing 5 mM KCN and 5 mM iodoacetate (Plagemann, Marz & Erbe, 1976). Such ATP-depleted cells are not impaired with respect to thymidine transport, but are unable to phosphorylate the nucleoside (nor otherwise metabolize it), so that isotopic thymidine is accumulated within the cell only to the extent of equilibrium between extra- and intracellular water (Wohlhueter *et al.*, 1978).

### *Transport Measurements*

The methodology employed to assess initial rates of transport is described in detail elsewhere (Wohlhueter *et al.*, 1978). Cell suspension and radioactive substrate were mixed in constant proportion (448:61 μl) at intervals of as little as 1.5 sec means of a dual-syringe device. The emergent mixtures were dispensed into twelve tubes loaded in the rotor of an Eppendorf microcentrifuge, and containing 100 μl of a silicone oil mixture of density 1.034 g/ml. After the final tube was dispensed, the centrifuge was started, whereby cells were centrifugally removed from the medium within 2 sec. Subsequently the medium was aspirated, the upper part of the tubes rinsed, and the radioactivity present in the cell pellet quantitated by scintillation spectrometry.

Two different experimental protocols were employed: (i) "Zero-trans" (zt), in which radioactive substrate is introduced exogenously, and enters the cell against an internal concentration assumed initially to be zero; and (ii) "Equilibrium exchange" (ee), in which ATP-depleted (nonmetabolizing) cells are preloaded with a given concentration of nonradioactive substrate and then mixed by means of the dual-syringe with radiolabeled substrate at the same concentration. For exchange experiments and for zero-trans experiments in

nonmetabolizing cells, sampling times were adjusted to encompass the full time course of the approach to extra-/intracellular equilibrium. An integrated rate equation

$$R_i = R_{i,\infty}(1 - e^{-k't}) \tag{1}$$

was fit to the data; $R_i$ = radioactivity in the intracellular space (after correction for radioactivity in the extracellular space of the pellet) at time $t$; $R_{i,\infty}$ = asymptotic value of $R_i$; and $k'$ = a pseudo-first-order rate constant, which, actually, is a function of exogenous substrate concentration (*see* Wohlhueter *et al.*, 1978). The slope of this curve at zero time is given by $k'R_{i,\infty}$ and may be converted to appropriate dimensions, given the specific radioactivity of the substrate and the intracellular space in the cell pellet. For zero-trans experiments with cells capable of phosphorylating thymidine, Eq. (1) does not apply, because radioactivity is accumulated within the cells as thymine nucleotides far above the expected equilibrium levels. In such experiments sampling times were adjusted to span an initial period where uptake was linear with time.

Intra- and extracellular volumes contained in the cell pellets were analyzed with $^3H_2O$ and [$^{14}$C] carboxylinulin, respectively (Wohlhueter *et al.*, 1978). All experiments were conducted at 23 to 25°.

### *Data Processing*

Theoretical equations (e.g., Eqs. (1) and (4) and the Michaelis-Menten equation) were fit to data, where indicated, by means of a generalized, nonlinear regression program (Dietrich & Rothmann, 1975). Values of parameters cited are those giving a minimal sum of squared deviations, and are reported ± the standard error of the parameter estimate, i.e., the square-root of the variance of the given parameter.

### *Chemicals*

6-([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine ("nitrobenzylthioinosine") was made available to us through the generosity of Drs. Carol Cass and Alan Paterson of the University of Edmonton. 2 amino-6([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine (NSC 49813, "*p*-nitrobenzylthioguanosine") and 2 amino-6-([2-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine (NSC 49814, "o-nitrobenzylthioguanosine") were obtained from the National Cancer Institute, Bethesda, Md., under Contract NOI-CM-43784. [Methyl-$^3$H]thymine (50 Ci/mmol) was a product of Schwarz-Mann (Orangeburg, N.Y.) and [G-$^3$H]hypoxanthine (1.8 Ci/mmol) of Amersham (Arlington Heights, Ill.). [Methyl-$^3$H]thymidine (10 Ci/mmol) was purchased from ICN (Irvine, Calif.); [carboxyl-$^{14}$C] carboxylinulin (2.6 Ci/g) and $^3H_2O$ (1 mCi/g) were purchased from New England Nuclear (Boston, Mass.). Silicone oil no. 550 was from Dow Chemical Corporation (Midland, Mich.). Other chemicals were reagent grade from standard suppliers.

## Results and Discussion

### *Thymidine Transporters of Various Cell Lines Differ in Sensitivity to Nitrobenzylthioinosine*

We have kinetically characterized the nucleoside transport system of Novikoff rat hepatoma cells (R. Marz, R.M. Wohlhueter & P.G.W.

Table 1. Inhibition of thymidine transport in various cell types by nitrobenzylthioinosine[a]

| Cell line | Cell density $\times 10^{-7}$ (cells/ml) | Concentration of nitrobenzyl thioinosine (nM) | Inhibition (%) |
|---|---|---|---|
| Chinese hamster ovary | 2.18 | 10 | 83 (5.2[b]) |
| | | 100 | 90 |
| HeLa | 1.12 | 10 | 68 (1.8[b]) |
| | | 100 | 77 |
| P388 leukemia | 1.22 | 10 | 84 (7.6[b]) |
| | | 100 | 86 |
| L ($TK^-$) | 2.07 | 10 | 86 (10.3[b]) |
| | | 100 | 88 |
| N1S1-67 ($TK^-$) hepatoma | 1.23 | 100 | 4 (1.2[b]) |
| | 2.17 | 2000 | 37 (9.9[b]) |
| | | 8000 | 53 |

[a] Initial rates of (methyl-$^3$H) thymidine transport (zero-trans) were measured at 23 to 25° in suspensions of various cell types, either depleted of ATP or deficient in thymidine kinase ($TK^-$), as described in Methods. Exogenous thymidine concentration was 10 μM (N1S1-67 at $1.23 \times 10^7$ cells/ml), 120 μM (P388 cells), or 160 μM (all others), and cells were preincubated for at least 2 min with the indicated concentrations of nitrobenzylthioinosine.

[b] Values in parenthesis are transport velocities in control cells expressed as pmol/μl cell water · sec.

Plagemann, *in preparation*) and sought, as a complement to those studies, to probe the nucleoside carrier of these cells with nitrobenzylthionucleosides. Novikoff hepatoma cells proved, however, to be singularly insensitive to nitrobenzylthioinosine (*see* Table 1). Ortho- and para-nitrobenzylthioguanosine were also only weak inhibitors of thymidine transport in Novikoff cells (data not shown), whereas in HeLa cells (Paterson *et al.*, 1977*b*) the para compound is a potent inhibitor of uridine uptake. We suspect that the nitrobenzylthionucleosides may be simply alternate substrates for the nucleoside transporter of Novikoff cells. Both inosine and nitrobenzylthioinosine exhibited competitive inhibition of thymidine transport in experiments analogous to that shown in Fig. 4. The $K_i$ for inosine was 31 μM (Marz *et al.*, *in preparation*); that for nitrobenzylthioinosine was 2.2 μM, as can be estimated also from the data of Table 1.

Like human erythrocytes (Cass & Paterson, 1977) and MCT hamster fibroblasts (Eilam & Cabantchik, 1976, 1977), on the other hand, Chinese hamster ovary (CHO), HeLa, mouse L, and P388 murine leukemia cells were very sensitive to inhibition by nitrobenzylthioinosine. The dissocia-

tion constant for nitrobenzylthioinosine with the nucleoside carrier in these cells is on the order of 0.1 nM (*see below*).

Studies similar to these, but employing higher concentrations of inhibitor, confirmed a feature evident in Table 1, i.e., that inhibition of thymidine transport never exceeded about 90%. We conclude that about 10% of thymidine entering (nitrobenzylthioinosine-sensitive) cells does so by a route distinct from the main nucleoside transporter. This residual entry component is comparable to the rate of nonmediated permeation of cytosine into Novikoff hepatoma cells (ca. 0.5 pmol/μl cell water·sec at 160 μM; *see* Graff, Wohlhueter & Plagemann, 1977), and may represent nonmediated entry of thymidine. However, preliminary experiments indicate that this component which is insensitive to nitrobenzylthioinosine, is partially inhibited by hypoxanthine. Transport of hypoxanthine in Chinese hamster ovary cells was saturable (data not shown), so that the possibility that thymidine enters to a minor extent via the hypoxanthine transporter cannot be excluded.

The present methodology – sampling at short intervals and the use of nonphosphorylating cells – assures that the inhibition observed pertains to the nucleoside transport system. The uptake of thymidine, as measured in phosphorylating cells over longer periods of time (i.e., the appearance of cell-associated radioactivity comprising thymidine and metabolic derivatives of it), is also depressed by nitrobenzylthioinosine (Cass & Paterson, 1977). But the behavior of the overall process (transport plus metabolism) is more complex than that of transport alone, and the interpretations, accordingly, less straightforward.

### *Nitrobenzylthioinosine Inhibits Transport of Thymidine, but not Hypoxanthine or Thymine*

Initial rates of entry into CHO cells of radiolabeled thymine, hypoxanthine, and thymidine are plotted in Fig. 1 against the concentration of nitrobenzylthioinosine with which cell suspensions had been pretreated. The entry of the nucleic acid bases was apparently unaffected by concentrations of nitrobenzylthioinosine which curtailed entry of thymidine by as much as 90%. We conclude that the main route of entry of the bases is different from that of the nucleoside.

Now the transport of thymidine in CHO cells is nonconcentrative, but saturable ($K_m^{zt} \cong 70$; *cf.* Figs. 2 and 4). Fig. 2 shows that uridine is

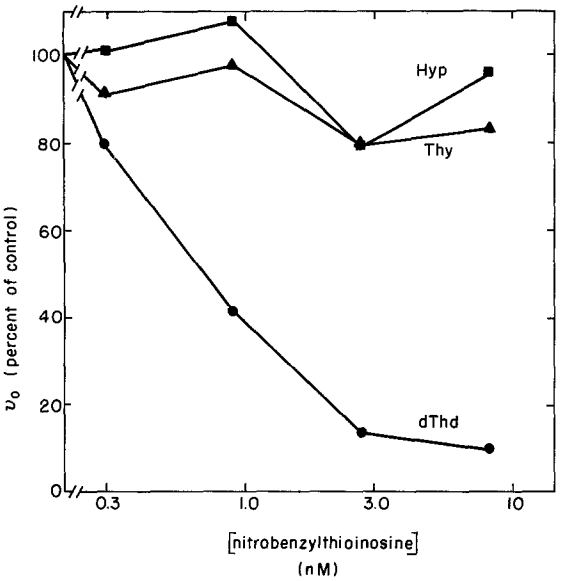


Fig. 1. Transport of thymidine, thymine, and hypoxanthine as a function of concentration of nitrobenzylthioinosine. A suspension of Chinese hamster ovary cells ($1.1 \times 10^7$ cells/ml basal medium) was depleted of ATP by treatment with 5 mM KCN and 5 mM iodoacetate at 37°. The suspension was then divided into five flasks containing nitrobenzylthioinosine at a final concentration of 0, 0.3, 0.9, 2.7 or 8.1 nM, incubated $\geq 5$ min at 24°, and assayed for zero-trans influx of isotopic thymidine (●–●; 10 μM, 816 cpm/μl), thymine (▲–▲; 10 μM, 559 cpm/μl) or hypoxanthine (■–■; 10 μM, 972 cpm/μl) by the rapid sampling technique described in *Methods*. During the incubation times employed, thymidine and thymine attained transmembrane equilibrium, and the corresponding initial velocities were estimated (*see* text) from the time course of approach to equilibrium; hypoxanthine did not attain equilibrium, and its influx was estimated from initial linear slopes. Control influxes were 0.74, 0.16, and 0.044 pmol/μl cell water·sec, respectively, for thymidine, thymine, and hypoxanthine. Neither extra- nor intracellular space was affected by nitrobenzylthioinosine: 1.2 and 7.0 μl/cell pellet, respectively

a competitive inhibitor of thymidine transport ($K_i = 87$ μM), which we interpret as evidence that both nucleosides are transported by the same carrier. Inosine also inhibits thymidine transport, though we have not investigated the kinetics of this inhibition. Thus it appears that the thymidine transport system inhibited by nitrobenzylthioinosine is in reality a general nucleoside transporter, which functions as a facilitated diffusion system in CHO cells as in erythrocytes (Oliver & Paterson, 1971), leukocytes (Taube & Berlin, 1972), and Novikoff rat hepatoma cells (Plagemann *et al.*, 1976; Marz *et al.*, 1977a).

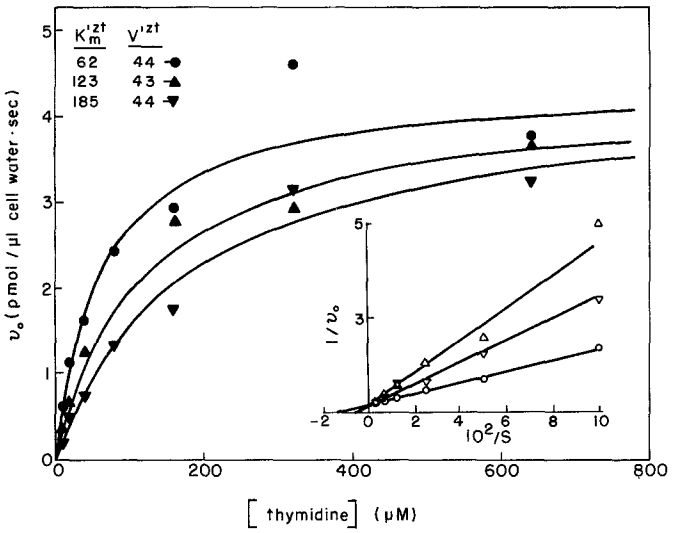


Fig. 2. Competitive inhibition of thymidine transport by uridine. Initial velocities of thymidine transport at 23° were measured in suspended, ATP-depleted, Chinese hamster ovary cells ($1.1 \times 10^7$ cells/ml) at thymidine concentrations of 10, 20, 40, 80, 160, 320 and 640 µM (700 cpm/µl, irrespective of concentration), and uridine concentrations of 0 (●–●), 90 (▲–▲), and 180 (▼–▼) µM. Extra- and intracellular volumes in the cell pellets were 1.4 and 6.4 µl, respectively. The values given for the Michaelis-Menten parameters, and the corresponding curves, are those best-fitting the set of velocity data at each inhibitor concentration. The inset shows the same data and parameters in a Lineweaver-Burk plot. Nonlinear regression of the pooled data on the rate equation for simple, competitive inhibition (*cf.* Segel, 1975) estimated $V^{zt} = 4.4 \pm 2.0$ pmol/µl cell water·sec, $K_m^{zt} = 61 \pm 11$ µM, and $K_i = 87 \pm 25$ µM ($r_{y,\hat{y}} = 0.978$). The equation for mixed inhibition did not fit the data substantially better ($r_{y,\hat{y}} = 0.978$)

### *Nitrobenzylthioinosine Binds Slowly Relative to Natural Nucleosides*

The transport of nucleosides into mammalian cells is a fast process. For example, the first-order rate constant for thymidine entry in CHO cells (as calculated from control group in Fig. 1) equals 0.09 $sec^{-1}$; accordingly, a 20% deviation from initial rate of entry obtains already at 2.5 sec. It is reasonable to assume that membrane permeation is the rate-limiting step in the entry process, and that substrate-carrier association occurs on a millisecond time scale.

The rate at which nitrobenzylthioinosine exerts its inhibitory effect is relatively slow. In an equilibrium exchange assay (Table 2), maximal inhibition was manifest after preincubation for only 0.8 min – the shortest time in which we were able to complete a transport assay after mixing inhibitor and cell suspension. Yet, when inhibitor was first presented

Table 2. Rate of expression of transport inhibition by nitrobenzylthioinosine[a]

| Time from addition of inhibitor to completion of transport assay | % Inhibition |
|---|---|
| Simultaneous | 25 |
| 0.8 min | 58 |
| 5.3 min | 65 |
| 15 min | 63 |
| Resuspended in medium without inhibitor | |
| + 1 min | 43 |
| + 5 min | 38 |
| +15 min | 51 |

[a] Equilibrium exchange of isotopic thymidine was measured at 24° by first equilibrating ATP-depleted Chinese hamster ovary cells ($1.3 \times 10^7$/ml) with 83 μM thymidine, then incubating for various times with 1.6 nM nitrobenzylthioinosine, and finally introducing isotopic thymidine (8.0 cpm/pmol) at the same concentration by means of a dual syringe device. "Simultaneous" indicates that the nitrobenzylthioinosine was first introduced along with substrate. The cells remaining after 15 min incubation with nitrobenzylthioinosine were separated from the medium by centrifugation, resuspended in the same volume of fresh medium without inhibitor, and assayed again 1, 5, and 15 min later for isotope exchange. The rate constants for exchange were estimated from the time courses for the attainment of isotopic equilibrium, and amounted to 0.040 $sec^{-1}$ in control assays. The actual time courses of exchange in control cells and those exposed simultaneously to thymidine and inhibitor are presented in Fig. 3.

to cells simultaneously with substrate, a progressive inhibition of the isotope exchange was evident during the 40-sec duration of the equilibrium exchange experiment (*see* Fig. 3). This deviation was used to estimate the rate of the inhibitory reaction as follows. We assume that the rate constant for exchange [$k'$ of Eq. (1)], is not constant here, but a decreasing function of time of exposure to nitrobenzylthioinosine. In Fig. 3 we allow $k'$ to decrease exponentially with time from the initial value (0.040 $sec^{-1}$) found for controls to the final value (0.015 $sec^{-1}$) found in this experiment for longer ($>1$ min) pretreatment with nitrobenzylthioinosine. Empirically this model accommodated the data quite well, and assigned a half-time of 17 sec for the expression of inhibition. Resuspension of cells in fresh medium without inhibitor effected only a partial alleviation of inhibition (Table 2). Such reversal as did occur, however, was completed also within about 1 min.

A similarly rapid expression of inhibition was observed with zero-trans influx of thymidine (10 μM) into normally phosphorylating CHO cells (data not shown). Fifty-two percent inhibition was apparent after

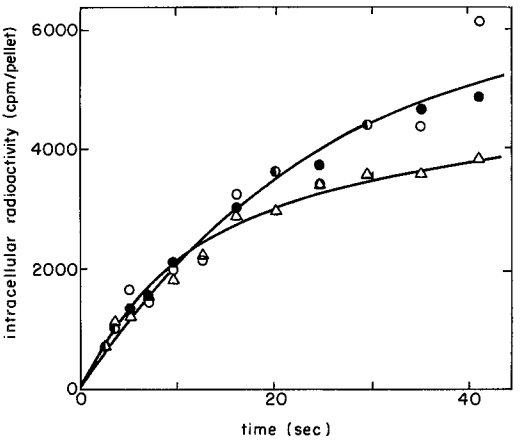


Fig. 3. Rate of expression of transport inhibition by nitrobenzylthioinosine. Experimental details for this experiment are presented in the legend to Table 2. Appearance of radioactivity in the cell pellet, after correction for radioactivity in the extracellular space (3.0 µl of a total pellet space of 12.3 µl), is plotted against time for duplicate assays without nitrobenzylthioinosine (●, ○), and a single assay in which 1.5 nM nitrobenzylthioinosine was added simultaneously with [$^3$H]thymidine (△). Equation (1) was fit to the pooled data from uninhibited cells; the curve shown is for a first-order rate constant of exchange, $k' = 0.040\ \text{sec}^{-1}$ (SE 0.006) and a final intracellular radioactivity of 6400 cpm/cell pellet (SE 560). For cells in the presence of nitrobenzylthioinosine, $k'$ of Eq. (1) was replaced by a time function, $k'_t = k'_o e^{-it} + k'_\infty$, where $k'_o$ was set equal to $0.04\ \text{sec}^{-1}$ and $k'_\infty$ to $0.015\ \text{sec}^{-1}$ (the rate constant measured after long preincubation with inhibitor). The best-fitting value for $i$ was $0.042\ \text{sec}^{-1}$ (SE 0.002) corresponding to a half-time of inactivation of 16.5 sec

1.4 min of preincubation with nitrobenzylthioinosine and persisted until 120 min, the last time of assay.

These data suggest that the inhibitory interaction of nitrobenzylthioinosine with the thymidine carrier of CHO cells is probably more involved than a simple substrate: carrier association. The nature of that interaction cannot be deduced from the present data, though the studies of Pickard *et al.* (1973) with radiolabeled nitrobenzyl nucleosides indicate that cell-bound inhibitor remains structurally intact.

### *Thymidine Interferes with the Interaction between Nitrobenzylthioinosine and the Nucleoside Transporter*

An equilibrium exchange experiment lends itself to an investigation of the question whether the presence of nucleoside interferes with nitro-

Table 3. Protection by thymidine against inactivation of thymidine transport by nitrobenzylthioinosine[a]

| Equilibration medium | Preincubation with nitrobenzylthioinosine | Rate constant of [$^3$H] dThd Exchange $\times 10^3$ ($sec^{-1}$) | Inhibition (%) |
|---|---|---|---|
| 10 μM dThd | − | 125 | − |
| | + | 44 | 65 |
| 1000 μM dThd | − | 13.9 | − |
| | + | 8.5 | 39 |
| 10 μM dThd + 1000 μM Thy | − | 94.9 | − |
| | + | 29.7 | 69 |

[a] Samples of a suspension of ATP-depleted, Chinese hamster ovary cells ($1.1 \times 10^7$ cells/ml) were mixed with nonradioactive thymidine and/or thymine at the concentrations indicated, and incubated at 24° sufficiently long (between 8 and 30 min) to equilibrate these components across the cell membrane. The equilibrated mixtures were divided, and one part was treated with 2 nM nitrobenzylthioinosine for an additional 8 to 12 min. Isotopic exchange was initiated by addition of [$^3$H] thymidine (675 cpm/μl final solution) to the extracellular space, and followed from 2.5 to $\geqq 90$ sec by means of the rapid sampling technique described in Methods. Rate constants for the attainment of isotopic equilibrium were computed by nonlinear regression techniques. Values are the means of duplicate runs, with an average deviation of ±5%.

benzylthionucleoside binding, since this experimental design requires preincubation of cells with nonradioactive substrate. Table 3 reports the results of such an experiment. CHO cells equilibrated with 10 μM thymidine ($K_m^{zt} \cong 70$ μM) and subsequently exposed to 2 nM nitrobenzylthioinosine were inhibited 65% in isotopic exchange. Cells exposed to nitrobenzylthioinosine in the presence of 1000 μM thymidine were inhibited only 39%. In contrast, 1000 μM thymine, which is not a substrate for the nucleoside transporter and whose transport is not inhibited by nitrobenzylthioinosine (*see above*), did not protect against inhibition (69%). Thymine itself, however, weakly inhibited thymidine transport.

The protective effect of thymidine evident in this equilibrium exchange experiment is most appreciated in contrast to the results of zero-trans experiments, in which exposure to nitrobenzylthioinosine necessarily precedes (or accompanies) exposure to substrate thymidine. From the kinetic constants given in the legend to Fig. 4 (and discussed below), we calculate that the zero-trans influx of thymidine at 10 μM would be inhibited 68% by 2 nM nitrobenzylthioinosine – equivalent to the percent inhibition measured in equilibrium exchange (Table 3). At 1000 μM thymidine we expect

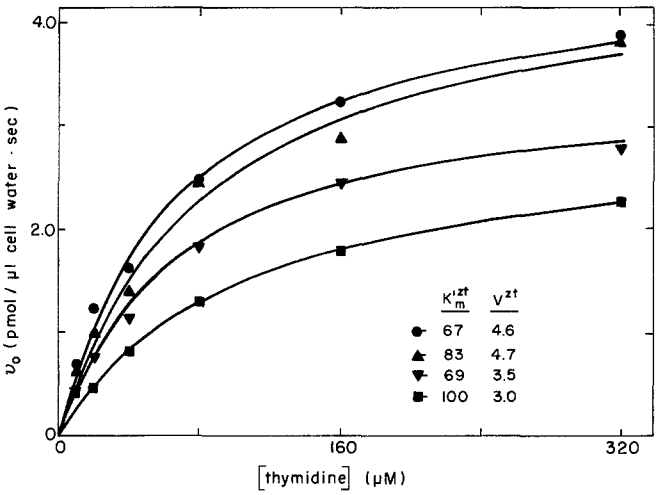


Fig. 4. Noncompetitive inhibition of thymidine transport by nitrobenzylthioinosine. Initial velocities of thymidine transport at 23° were measured in suspended, ATP-depleted, Chinese hamster ovary cells ($1.7 \times 10^7$ cells/ml; 6.8 µl/$10^7$ cells) at thymidine concentrations of 10, 20, 40, 80, 160 and 320 µM (770 cpm/µl, irrespective of concentration), and nitrobenzylthioinosine concentrations of 0 (●–●), 0.04 (▲–▲), 0.2 (▼–▼), and 0.9 (■–■) nM. Cells were preincubated with inhibitor for ≧2 min before introduction of substrate [$^3$H]thymidine. The curves shown are those for the Michaelis-Menten parameters best-fitting the set of velocity data at each inhibitor concentration. Nonlinear regression of the pooled data on the rate equation for simple, noncompetitive inhibition (*cf.* Segel, 1975) estimated $V^{zt} = 4.7 \pm 0.2$ pmol/µl cell water·sec, $K_m^{zt} = 76 \pm 7$ µM, and $K_i = 1.0 \pm 0.1$ nM ($r_{y,\hat{y}} = 0.991$). The equation for mixed inhibition did not fit the data substantially better ($r_{y,\hat{y}} = 0.992$)

67% inhibition in zero-trans, but measure only 39% with equilibrium exchange. Thus the apparent pattern and extent of inhibition by nitrobenzylthioinosine depends on the order of addition of substrate and inhibitor.

We interpret thymidine protection to mean that at least one stage of the interaction between nitrobenzylthioinosine and the thymidine carrier involves recognition of inhibitor by the nucleoside binding site. It is in this sense an affinity label for the nucleoside carrier. Whether, in the final stage of interaction it still occupies the nucleoside binding site (and thus renders the carrier nonfunctional) is not established. Such a model is, however, consistent with all our data, and is favored by us.

### *Inhibition by Nitrobenzylthioinosine Affects $V^{zt}$, not $K_m^{zt}$*

The data summarized in Fig. 4 conform to the pattern of simple, noncompetitive inhibition, as defined for enzyme systems (Cleland, 1963):

nitrobenzylthioinosine diminishes the maximum velocity of thymidine transport, but not the effective $K_m^{zt}$. At first glance this may seem contradictory to our previous conclusion that nitrobenzylthioinosine binds at the substrate binding site. The contradiction is resolved when one considers that the inhibitor binds virtually irreversibly, i.e., that the rates at which its ultimate, inhibitory effect is attained or reversed are much slower, and its affinity much greater, than the rates of thymidine interaction with and affinity for the nucleoside carrier.

These results are just opposite of those reported by Eilam and Cabantchik (1977) and by Paterson and coworkers (Cass & Paterson, 1977; Paterson *et al.*, 1977*a*; Paterson *et al.*, 1977*b*), all of whom observe competitive inhibition of uridine, thymidine, and adenosine uptake by nitrobenzylthioinosine. The difference in kinetic patterns of inhibition, we believe, stem directly from the difference between uptake and transport. When we measure uptake of thymidine into CHO, HeLa, Novikoff rat hepatoma (Plagemann *et al.*, 1975), or mouse L cells (Plagemann *et al.*, 1976), we observe uptake $K_m$'s of 0.5 to 2.0 μM, comparable to that (0.5 μM) reported by Cass and Paterson for HeLa cells (1977). When, instead, we measure transport in these cells (Wohlhueter *et al.*, 1976; and Figs. 2 and 4), we observe zero-trans $K_m$'s of about 70 μM. Furthermore, we have demonstrated for thymidine in Novikoff cells (Marz *et al.*, 1977*b*) that transport is much more rapid than intracellular phosphorylation, so that the kinetic behavior of uptake must reflect predominantly phosphorylation. For this situation, consider the consequences of treatment with an inhibitor which acts only by diminishing the rate of transport. If substrate concentration is varied only within the first-order range with respect to transport (i.e., [dThd] $\leqq$ ca. 30 μM), the influence of an inhibitor-caused decrease in the effective first-order rate constant of transport on overall uptake can be overcome by increasing the substrate concentration. That is tantamount to competitive inhibition of uptake, although the inhibitor has altered only the rate of transport, not the affinity of the transport carrier for its substrate, Figure 5 presents a computer simulation of this situation.

### *Thymidine Carriers may be Titrated with Nitrobenzylthioinosine*

During the course of these experiments we had observed that, at low nitrobenzylthioinosine concentrations, the extent of transport inhibition depended upon cell density as well as nitrobenzylthioinosine concen-

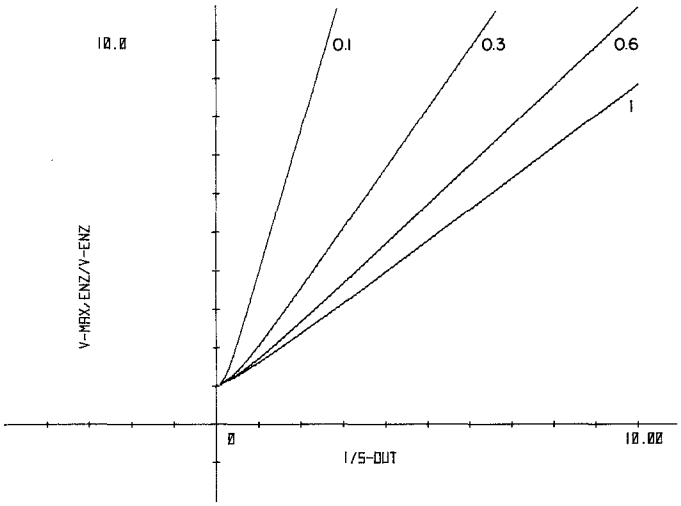


Fig. 5. Computer simulation of transport and phosphorylation operating in tandem. As a simple model we take

$$S_{out} \overset{t}{\rightleftharpoons} S_{in} \overset{e}{\rightarrow} S - P_i$$

in which reaction "$t$" is a symmetrical, facilitated transport system, operating bi-directionally ($K_{eq}=1$) with Michaelis-Menten parameters $K_m^t$ and $V^t$. Reaction "$e$" is an irreversible kinase, operating with $K_m^e$ and $V^e$, and whose product $S-P_i$ is trapped within the intracellular compartment. Solution for the steady-state concentration $S_{in}$ in terms of these kinetic parameters gives

$$aS_{in}^2 + bS_{in} + c = 0$$

where

$$a = -V^t K_m^t - V^e K_m^t - V^e S_{out}$$
$$b = V^t K_m^t S_{out} - V^t K_m^e K_m^t - K_m^{t2} V^e - V^e K_m^t S_{out}$$

and

$$c = V^t K_m^t K_m^e S_{out}.$$

In this Lineweaver-Burk simulation the reciprocal of the velocity of the enzymatic reaction normalized to the maximal enzyme velocity ($V^e/v^e$) is plotted against the reciprocal of the *exogenous* substrate concentration ($1/S_{out}$) for $0.1 \leqq S_{out} \leqq 10$. $K_m^t$ is fixed at 80 (comparable, in μM, to $K_m$ of thymidine transport); $K_m^e$ is fixed at 0.5 (comparable to $K_m$ of thymidine uptake); and $V^e$ is fixed arbitrarily at 0.004. The four curves represent decreasing velocities of transport ($V^t=1, 0.6, 0.3, 0.1$) at constant $K_m^t$, simulating addition of a transport inhibitor which affects only transport velocity, not transport $K_m$. Data falling on these curves might be construed, fallaciously, to represent competitive inhibition of a transport system operating with $K_m=1$, if transport were thought to be limiting, and if measurements were made over a time interval in which the rate of accumulation of $S-P_i$ was equivalent to the rate of uptake, i.e., where $S_{in}$ was in steady-state

tration. This observation suggested that the concentration of binding sites and inhibitor were comparable, and, accordingly, that Scatchard analysis might be feasible. *In lieu* of radiolabeled ligand, we measure binding site saturation indirectly by assuming that fractional saturation

is equal to fractional inhibition of transport velocity, i.e., that the fractional inhibition ($i$) equals concentration of bound ligand ($L_b$) divided by concentration of binding sites ($B$), $i = L_b/B$. Substituting this relationship into the Scatchard equation (Segel, 1975),

$$\frac{L_b}{L_f} = -\frac{L_b}{K_d} + \frac{B}{K_d} \tag{2}$$

and noting that $B$ = cell density ($D$, in cells per liter) times the number of binding sites per cell ($N_b$) divided by Avogadro's number ($N_A$) and that total concentration of ligand, $L_t = L_b + L_f$, allows one to write the following equation

$$0 = (DN_b/N_A)i^2 + (-L_t - DN_b/N_A - K_d)i + L_t. \tag{3}$$

The solution of Eq. (3) by means of the quadratic equation gives $i$ as a function of total ligand concentration, cell density, and the binding parameters $K_d$ and $N_b$,

$$i = \frac{(L_t + DN_b/N_A + K_d) \pm [(L_t + DN_b/N_A + K_d)^2 - 4(L_t DN_b/N_A)]^{1/2}}{2DN_b/N_A}. \tag{4}$$

The negative square root term proves to correspond to physically meaningful $i$'s. Figure 6 represents the results of an experiment in which fractional inhibition was measured at various cell densities ($D$ varied between 0.8 and $3.7 \times 10^{10}$ cells/liter at constant $L_t = 1 \times 10^{-9}$ M nitrobenzylthioinosine) or at various inhibitor concentrations ($L_t$ varied between 0.27 and $1.3 \times 10^{-9}$ M at constant $D = 1.13 \times 10^{10}$ cells/liter). Equation (4) was fit to these data by a nonlinear regression program, which converged at the values $K_d = 8.2 \times 10^{-11}(\pm 6.5 \times 10^{-11})$ $M$ and $N_b = 6.3 \times 10^4(\pm 0.9 \times 10^4)$binding sites per cell.

We construe this number, $6.3 \times 10^4$ sites/cell, as the number of broadly specific nucleoside transporters in the CHO cell membrane. It closely approximates the number of nitrobenzylthioinosine binding sites attributed by Eilam and Cabanchik (1977) to hamster fibroblasts (about $8 \times 10^4$ per cell), on the basis of inhibition of uridine uptake. Pickard *et al.* (1973) estimated that about $10^4$ sites on the erythrocyte membrane bind radiolabeled nitrobenzylthioinosine. Our estimate of the dissociation constant $K_d = 0.1$ nM is about one tenth that by Pickard *et al.* (1973) and Eilam and Cabanchik (1977).

The apparent inconsistency between our estimate of $K_d$ and of $K_i$ for noncompetitive inhibition (*see* Fig. 4) may be rationalized as follows.

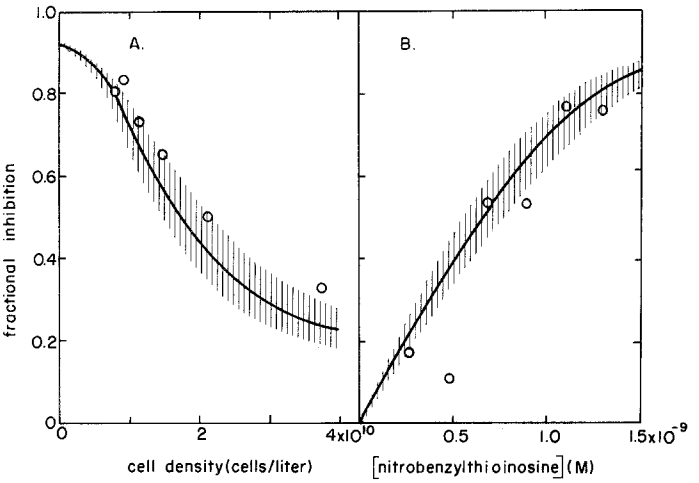


Fig. 6. Titration of nucleoside carrier sites with nitrobenzylthioinosine. A suspension of ATP-depleted, Chinese hamster ovary cells was divided and diluted so that one series of six tubes contained 1 nM nitrobenzylthioinosine and from 3.74 to $0.79 \times 10^{10}$ cells/liter (data in panel *A*), and another series of eight tubes contained $1.13 \times 10^{10}$ cells/liter and from 0 to 1.31 nM nitrobenzylthioinosine (data in panel *B*). Transport of [$^3$H]thymidine (10 μM, 71 cpm/pmol) was measured into cells from each tube, and initial velocities computed from the rate of approach to equilibrium. Initial transport velocity in duplicate control tubes ($1.13 \times 10^{10}$ cells/liter and no inhibitor) were 0.84 and 0.82 pmol/μl cell water·sec. The lines drawn are for the binding Eq. (4) (given in the text), where $K_d = 8.2 \times 10^{-11}$ M and $N_b = 6.3 \times 10^4$ binding sites per cell. The shaded areas correspond to $\pm 0.9 \times 10^4$ binding sites per cell, the SE or estimate of $N_b$

The derivation of rate equations for enzyme inhibition assume that the concentration of free inhibitor is not significantly different from that of total inhibitor. This is manifestly not the case for the experiment shown in Fig. 4. Using the dissociation constant and number of binding sites estimated here, we can compute the concentrations of *free* nitrobenzylthioinosine present in the experiment of Fig. 4 corresponding to the total concentrations employed and the cell density. For total concentrations 0.04, 0.2, and 0.9 nM, the computed free concentrations are $2.05 \times 10^{-3}$, $1.14 \times 10^{-2}$, and $9.03 \times 10^{-2}$ nM, respectively. Fitting the equation for simple, noncompetitive inhibition to the data of Fig. 4 using these concentrations of free inhibitor yields a $K_i = 0.11$ nM. Thus corrected, our values of $K_d$ and $K_i$ become virtually identical.

Taking a maximal velocity of thymidine transport into CHO cells at 4.7 pmol/μl cell water·sec (*see* Fig. 4) and a cellular volume of $7 \times 10^{-7}$ μl/cell, the absolute flux per carrier under substrate-saturating zero-trans conditions is about 31 thymidine molecules/carrier·sec.

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