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Relationship Between Thymidine Transport and Phosphorylation in Novikoff Rat Hepatoma Cells As Analyzed by a Rapid Sampling Technique

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Incorporation of thymidine into Novikoff rat hepatoma cells was analyzed with a rapid sampling technique which allowed collection of 12 time points in 20 sec. Transport was studied in the absence of metabolism by using either ATP-depleted cells or a thymidine kinase negative subline. Transport was a rapid, saturable, nonconcentrative process with a K_m of about 85 μM. The intracellular thymidine pool was also rapidly labeled in cells which phosphorylated thymidine, so that a group translocation process involving thymidine kinase can be ruled out. Under all conditions examined, phosphorylation, not the transport, of thymidine was the rate-determining step in its incorporation into the acid-soluble pool. Estimation of transport rates from total incorporation into cells which phosphorylate the substrate is invalid in this cell system and must be questioned in all instances.

Key words: **transport; incorporation; uptake; thymidine; nucleoside; Novikoff rat hepatoma cells; rapid sampling technique**

Transport rates of substances which are neither metabolized nor accumulated intracellularly (facilitated transport) are difficult to determine accurately. Not only is the total amount of substrate taken up small, but often the process has reached an equilibrium in a matter of seconds, thus making it very difficult to measure initial transport rates. Many investigators, therefore, have estimated the rates of facilitated transport by measuring rates of incorporation[1] of substrates that are rapidly metabolized, mainly phosphorylated, intracellularly, and thereby trapped (see Ref. 1). The phosphorylated products accumulate intracellularly and incorporation is generally linear with time for much longer periods of time (up to 10 min or longer) than in the absence of metabolism. It is obvious, however, that the rates thus obtained can only be considered transport rates if transport, and not metabolism, is the rate-limiting step in the overall process (1, 2). Often investigators have

[1] We define "incorporation" as the transfer of radioactivity from extracellular labeled substrate to total cell components which in general involves transport and phosphorylation of the substrate, and its subsequent incorporation into macromolecules. "Transport," on the other hand, refers strictly to the transfer of unaltered substrate by a saturable process from one side of the plasma membrane to the other.

A detailed description of the methodology employed here will be found in volume 20 of "Methods in Cell Biology" (D. Prescott, ed.)

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merely assumed that transport was the rate-limiting step, or that the error they introduced was small and unavoidable. In other cases indirect evidence was offered which suggested that indeed transport was the rate-limiting step, but the validity of these assumptions has been difficult to prove unequivocally (1) and has been questioned (2). Indeed, in recent experiments (3, 4) we have demonstrated that the kinetics of the facilitated transport of thymidine (dThd) as measured in ATP-depleted or dThd kinase-deficient cultured Novikoff rat hepatoma cells, in which dThd is not phosphorylated, differ markedly (both in K_m and V_{max}) from those for dThd incorporation by cells in which dThd is phosphorylated. Measurements of the kinetics of facilitated dThd transport in nonmetabolizing cells was only made possible by the application of a newly developed rapid sampling technique which allows us to obtain up to 12 time points within a 20-sec period (4). We have now applied this methodology to measuring the transport, phosphorylation, and incorporation of dThd into DNA in metabolizing Novikoff cells, and further document that in these cells the rate of dThd incorporation is governed by the rate of its phosphorylation rather than its rate of transport into the cell.

MATERIALS AND METHODS

Wild-type Novikoff rat hepatoma cells (subline N1S1-67) and a dThd kinase-deficient subline thereof (3) were propagated in Swim's medium 67 and enumerated as described previously (5, 6). Cells were collected from exponential phase cultures by centrifugation at 400 × g for about 2 min, and suspended in a basal medium, BM42B (7), to a concentration of 2–4 × 10^7 cells/ml for transport measurements or about 2 × 10^6 cells/ml for incorporation studies. Cells were depleted of ATP by incubation in glucose-free BM42B supplemented with 5 mM KCN and 5 mM iodoacetate (3).

In all incorporation and transport assays the influx of substrate against zero intracellular concentration was measured, i.e., with a "zero-trans" protocol (8). For incorporation measurements at time scales of minutes the cell suspension was supplemented with labeled dThd and incubated on a gyratory shaker at about 200 rpm. Duplicate 1-ml samples of suspension were analyzed for radioactivity in total cell material (9).

For transport and incorporation studies at time scales in seconds, the rapid sampling technique, which has been described in detail elsewhere (4), was employed. Briefly, fixed aliquots of a suspension of cells were rapidly mixed with a solution of radioactive dThd at short time intervals by means of a hand-operated, dual syringe apparatus. Samples emerging from the mixing chamber were dispensed into 12 tubes mounted in an Eppendorf microcentrifuge which contained an oil mixture (density = 1.034 g/ml). Dispensing of samples into sequential centrifuge tubes was paced with a metronome and could be accomplished, comfortably, at 1.5-sec intervals. After the last sample had been mixed, the centrifuge was started, and within an estimated 2 sec the cells had entered the oil phase thus terminating transport. When more than 12 samples were required, cells and substrate were mixed in the same proportions as provided by the mixing apparatus and samples were removed at appropriate times for centrifugation through oil.

After centrifugation the supernatant medium was aspirated. The upper part of the tube was washed once with 1 ml of water which was subsequently removed together with most of the oil. Then 0.2 ml of 0.5 N TCA was added to the tube, and immediately vortexed to disperse the pellet. After 30 min of incubation at 70°C the entire tube and its contents were transferred to a vial containing 8 ml of a modified Bray's solution (5) and analyzed for radioactivity in a liquid scintillation spectrometer.

In order to stop substrate metabolism when cells capable of phosphorylating dThd were used, the centrifuge tubes were set up with 3 phases: the cell substrate mixture (density ~ 1.0 g/ml), above oil (density = 1.034 g/ml), above 0.5 N trichloroacetic acid in 10% (wt/vol) sucrose (density = 1.04 g/ml). The acid phase was separated into 3 fractions: a fraction which was not precipitated by $LaCl_3$ at neutral pH which contained free dThd, an acid-soluble fraction precipitated by $LaCl_3$ at neutral pH, which contained dThd nucleotides, and an acid-insoluble pellet containing DNA.

Total water space and extracellular space in cell pellets obtained by centrifugation through oil were determined in parallel runs in which substrate was replaced by [^{14}C]-carboxylinulin in [^{3}H]H_2O. All data were corrected for substrate radioactivity in the extracellular space (generally about 12% of total water space) and normalized to rates per μl cell H_2O.

Initial velocities of transport were computed as zero-time slopes of an integrated rate equation to which data were fit by the method of least squares (4); Michaelis-Menten parameters were computed according to Wilkinson (10).

Chemicals

Radiochemicals were obtained from the following sources: [methyl-^{3}H]dThd (10 Ci/mmole) from ICN (Irvine, California); [carboxyl-^{14}C]carboxyl-inulin (2.6 Ci/g) and [^{3}H]-H_2O (1 mCi/g) from New England Nuclear (Boston, Massachusetts). Thymidine was obtained from Sigma Chemical Co. (St. Louis, Missouri), $LaCl_3$ from Gallard-Schlesinger (Carle Place, New York). Other chemicals were reagent grade from standard suppliers.

RESULTS

The incorporation of dThd (0.2 and 2 μM) into total cell material by untreated wild-type Novikoff cells was approximately linear with time for several minutes both at 25 and 37°C (Fig. 1A, B). Chromatographic analysis of acid extracts of 6-min labeled cells showed that over 95% of pool radioactivity was associated with thymine nucleotides (data not shown). Initial rates of incorporation, as estimated from the 3-min points, followed normal Michaelis-Menten kinetics (Fig. 2A). The results are similar to those reported previously for Novikoff cells and other cell lines (1).

Quite different results were obtained, when dThd transport per se was measured in ATP-depleted cells. A steady-state intracellular concentration of free dThd, which was about equal to the extracellular concentration, was attained within 20 sec of incubation both at 25 and 37°C (Fig. 1C). The approach to equilibrium was fit to an integrated form of the first-order rate equation: $S_{i,t} = S_0 (1 - e^{-kt})$, where S_i is the intracellular concentration of substrate at time t, S_0 is the extracellular concentration of substrate, and k is a pseudo-first-order rate constant. The rationale for these fits has been described (4). Initial velocities (v_o) were calculated as the slope at zero time. At a dThd concentration of 2μM the v_o of transport was significantly higher than the dThd incorporation rates exhibited by the metabolizing cells (Fig. 1). All values reported in Fig. 1 are directly comparable, since they were obtained in experiments conducted on the same day with the same cell population.

Transport of dThd was a saturable process (Fig. 2B and C) and the "zero-trans" K_m and V_{max} were about 100 times higher than the corresponding values for incorporation into metabolizing cells. The dThd transport K_m at 37°C was significantly higher than that at 25°C. This finding does not imply that the affinity of the carrier for dThd differed

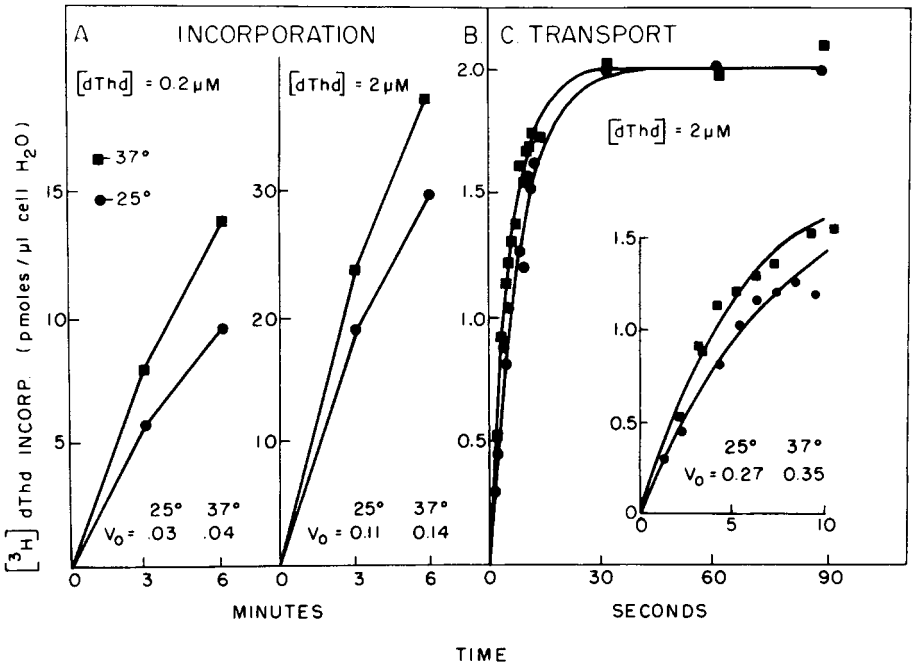

Fig. 1. dThd incorporation into total cell material by untreated N1S1-67 cells (A, B) and dThd transport into ATP-depleted cells (C). The cells were harvested from a single exponential phase culture and suspended to 2×10^6 cells/ml in BM42B (A, B) or to 2×10^7 cells/ml in glucose-free BM42B containing 5 mM KCN and 5 mM iodoacetate (C). The suspensions were incubated at 37°C for about 10 min and then one half of each suspension was equilibrated at 25°. The suspensions were supplemented with 0.2 μM (A) or 2 μM (B) [^{3}H]dThd (680 cpm/pmole) and incubated at the appropriate temperatures. Duplicate 1-ml samples of suspension were analyzed for radioactivity in total cell material (acid-soluble plus acid-insoluble). In (C) cell suspension and substrate solution were mixed in rapid succession (total 509 μl/mixture; 2 μM dThd final concentration, 500 cpm/ pmole) centrifuged through an oil layer and the cell pellet was analyzed for total radioactivity as described in Materials and Methods. The values were corrected for nonspecific trapping. The maximum level of intracellular dThd was assumed to represent equilibrium with the extracellular dThd concentration (2 μM). This assumption agreed in repeated experiments within 15% with results from independent intracellular [^{3}H]H_2O space determinations. The insert in (C) is a replot of some of the data at an expanded time scale. Initial velocities (v_0) of incorporation (A, B) and of transport (C) were estimated from these data (see text) and are expressed in pmoles/μl cell H_2O·sec.

correspondingly. According to the kinetic equations developed by Eilam and Stein (8) to describe a simple carrier model, a differential effect of temperature on the mobility of the loaded and unloaded carrier would give the same results.

Kinetic parameters similar to those in Fig. 2 (25°C) were obtained in studies with the dThd kinase-deficient subline of Novikoff cells whether or not the cells were depleted of ATP (data not shown). In 9 experiments with these types of cells the K_m at 25°C ranged from 71 to 118 μM (mean = 88 μM) and the V_{max} values ranged from 11 to 28 pmoles/μl cell H_2O·sec, with no obvious connection between the kinetic parameters and the growth stage of cells or the type of cell employed. The observed variability, therefore, may reflect limitations in the methodology, although more detailed studies are needed to determine whether real differences in the cell populations studied may also have been a contributory factor.

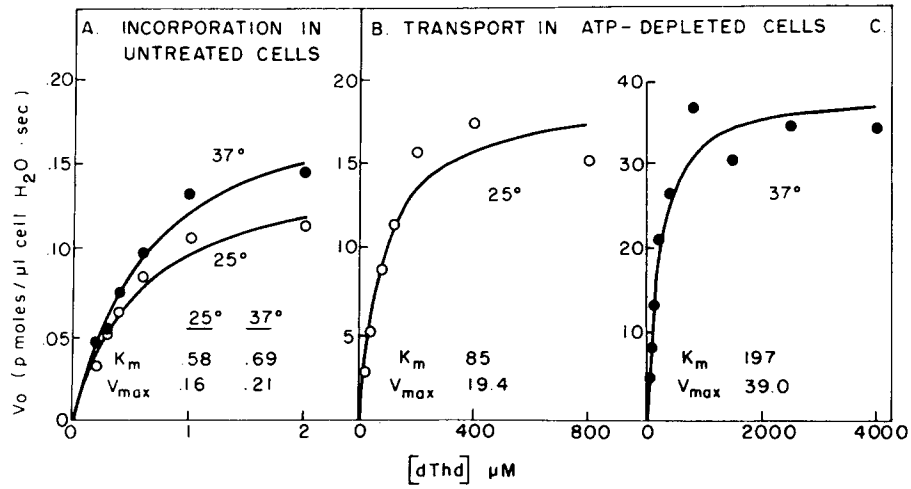

Fig. 2. Initial velocities of dThd incorporation by untreated N1S1-67 cells (A) and of dThd transport into ATP-depleted cells (B, C) as a function of dThd concentration. The experiment was conducted as described in the legend to Fig. 1, except that additional substrate concentrations were employed. Michaelis-Menten parameters were computed using the method of Wilkinson (10). A) Samples of the suspension of 2 X 10^6 cells/ml were supplemented with 0.2, 0.3, 0.4, 0.7, 1, and 2 µM [^{3}H]dThd (680 cpm/pmole) and the initial rate of incorporation was estimated from duplicate 1-ml samples of suspension analyzed for total cell-associated radioactivity after 3 min of incubation. B) Initial velocities were estimated from complete time courses of intracellular [^{3}H]dThd accumulation (see Fig. 1C). Samples of the suspension of 2 X 10^7 ATP-depleted cells/ml were mixed with [^{3}H]dThd to a final concentration of 500,000 cpm/sample and unlabeled dThd to final concentrations of 20, 40, 80, 120, 250, 400, and 800 µM. C) As described for B) except that dThd concentrations of 1.5, 2.5, and 4.0 µM were also used.

The discrepancy between the kinetic parameters of transport and incorporation was explored by using the rapid kinetic technique to measure the incorporation of 0.25, 20, and 320 µM dThd into 3 metabolite classes of wild-type cells: free dThd, dThd nucleotides, and DNA.

An intracellular steady-state level of free dThd was achieved in less than 60 sec, at the lower 2 concentrations, and by 200 sec at 320 µM dThd (Fig. 3). These steady states represent the balance between rates of influx, efflux, and phosphorylation of dThd and are determined by, among other things, the exogenous concentration of dThd.

At the lowest dThd concentration, 0.25 µM, the quantity of isotopic dThd incorporated into nucleotides exceeded that of intracellular free dThd after 2 min (Fig. 3A). At this substrate concentration, the incorporation of dThd into the nucleotide pool continued in a linear fashion for a few minutes. At the higher dThd concentrations, net incorporation into nucleotides practically ceased after 1–2 min of incubation (Fig. 3B and C), probably because of feedback inhibition of dThd kinase by TTP and a limited expandibility of the size of the dThd nucleotide pool (11). The amounts of dThd incorporated into DNA during the experimental periods were very small and have thus not been included in Fig. 3; at 23°C macromolecular synthesis in Novikoff cells nearly ceases.

The initial rates of dThd transport and phosphorylation calculated from the data in Fig. 3 are summarized in Table I. The initial rate of transport was calculated by summation of the initial rates of appearance of radioactivity in all intracellular components:

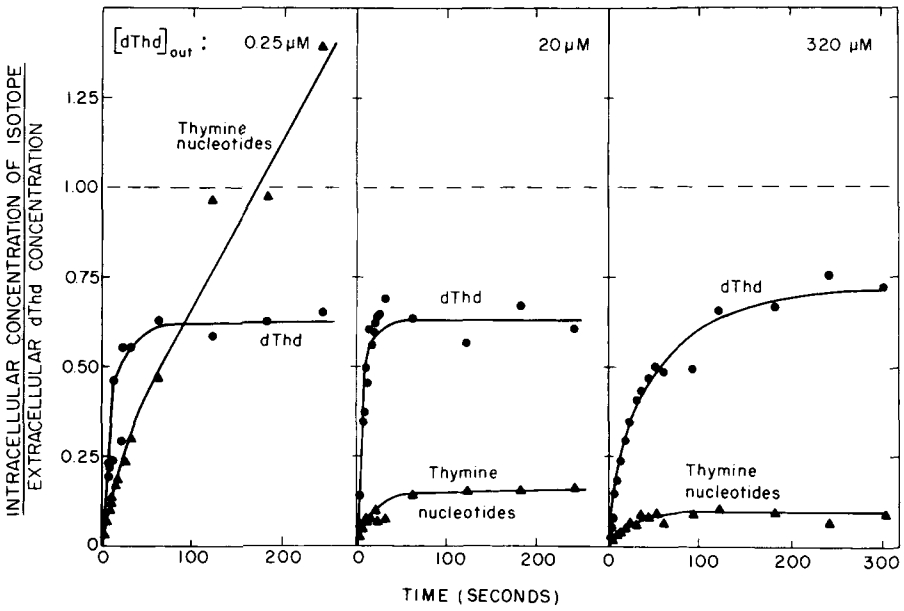

Fig. 3. Accumulation of labeled dThd and thymine nucleotides as a function of time of incubation with various concentrations of [^{3}H]dThd in the medium. Samples of a suspension of 4×10^7 untreated N1S1-67 cells/ml of BM42B were mixed at short intervals with solutions of [^{3}H]dThd to final concentrations of 0.25, 20, and 320 μM and 3,030 cpm/μl. The mixtures were centrifuged through an oil layer into a sucrose layer containing 0.2 N trichloroacetic acid, which was later fractionated into free dThd (●—●), thymine nucleotides (▲—▲), and DNA (not shown) as described in Materials and Methods. The data were normalized by dividing the intracellular concentration of dThd equivalents by the extracellular dThd concentration. The experiment was carried out at ambient temperature (23°C).

TABLE I. Initial Velocities of Thymidine Transport and In Situ Phosphorylation*

External thymidine concentration (μM)	Initial rates (pmoles/μl cell H_2O·sec)	
	Transport	Phosphorylation
0.25	0.0107	0.0025
20	1.61	0.126
320	8.13	1.82

*The experiment was conducted as described in the legend to Fig. 3. The initial rate of transport was calculated by summation of the initial rates of appearance of radioactivity in dThd, dThd nucleotides, and DNA; the initial rate of phosphorylation was similarly the sum of the rates of isotope appearance in dThd nucleotides and DNA.

dThd, dThd nucleotides, and DNA; the initial rate of phosphorylation was similarly the sum of the rates of isotope appearance in dThd nucleotides and DNA. Due to the complexity of the experimental procedure the rates thus obtained must be regarded as estimates. It is obvious, nevertheless, that at all concentrations of dThd tested, the rate of transport was significantly higher than the rate of phosphorylation.

DISCUSSION

Our results clearly show that at all dThd concentrations examined, the rate of dThd transport into the cells occurs at appreciably higher rates than its subsequent intracellular phosphorylation. This conclusion is based on comparisons of the rates of dThd incorporation into total cell material by metabolizing cells with the rates of dThd transport measured directly with cells in which the substrate is not phosphorylated because of lack of ATP or dThd kinase (Figs. 1 and 2), as well as on results from experiments in which both the intracellular accumulation of free dThd and its conversion into nucleotides was analyzed in metabolizing cells (Fig. 3; Table I). When cells are exposed to labeled dThd an intracellular steady-state level of free dThd is attained within 30–200 sec, depending on the extracellular dThd concentration. The fact that even at low extracellular dThd concentrations sizable amounts of free dThd are rapidly accumulated intracellularly seems to exclude a direct involvement of dThd kinase in transport.

At relatively low dThd concentrations (2 μM and below), where the intracellular steady state of dThd is attained quickly, the overall rate of dThd incorporation into cell material (measured at relatively long time scales) must reflect the rate of phosphorylation. Within this range of dThd concentrations, incorporation appears saturable (cf. Fig. 2A; apparent K_m = 0.6 μM). Yet it will be noted in Fig. 3 that, at much higher dThd concentrations, the rate of phosphorylation continues to rise, suggesting that the kinetic behavior of the phosphorylation reaction in situ is more complicated. We do not yet know whether the complication is due to multiple kinases or to an increase in apparent K_m as a function of concentration of the kinase effector dTTP.

In any case, the "zero-trans" K_m for transport in nonmetabolizing cells is about 100 times higher than the K_m apparent in Fig. 2A. Other experiments (12) have shown that the transport system exhibits a broad substrate specificity, and thus resembles the nucleoside transport systems described by Oliver and Patterson, for human erythrocytes (13) and by Taube and Berlin for rabbit polymorphonuclear leukocytes (14). Uridine and dThd are not metabolized in human erythrocytes and initial rates of uptake were estimated from the linear disappearance of substrate from the culture fluid over a 30-sec period. A "zero-trans" K_m for uridine transport of 710 μM was obtained, a value significantly higher than that for dThd transport in Novikoff cells. Uptake data for dThd did not yield a straight line in a Lineweaver-Burk plot, but it was concluded that dThd is transported by the same system as uridine, since it accelerates the efflux of uridine from the cells (13).

A "zero-trans" K_m for dThd transport by polymorphonuclear leukocytes of 50 μM (14) was based on measuring dThd incorporation into metabolizing cells. After 45 sec of incubation with 40 μM [^{3}H] dThd only 17% of the intracellular radioactivity was associated with free dThd, but dThd incorporation was linear for 45 sec; thus the authors assumed that the 45-sec time points used to estimate initial rates were short enough to be valid estimates of the initial rate of dThd transport. It is obvious from our studies (Fig. 3), that in Novikoff cells at least, intracellular dThd concentrations close to the steady-state level

are attained in about this time period and thus a 45-sec time point cannot be used to estimate initial rates. The magnitude of the error will depend on the intracellular level of unmetabolized substrate; at concentrations approaching those found in the medium, transport rates will be seriously underestimated because of the high rate of efflux (2).

Our results emphasize that true initial rates of dThd transport are very difficult to obtain in metabolizing cells. In cells incapable of metabolizing dThd, time courses of substrate accumulation in the cells may be fit to an equation describing the approach to intra-extracellular equilibrium and thus time points at which a sizable amount of dThd has accumulated intracellularly still yield information from which initial velocities can be computed. Intracellular accumulation of substrate is linear for an impractically short time because of backtransport of the substrate.

We are not yet equipped with equations to describe the time course of dThd accumulation and phosphorylation in metabolizing cells. Such equations must allow for the kinetics of dThd kinase in situ. From our preliminary data (Fig. 3) it seems that the in situ rates of phosphorylation do not bear a simple hyperbolic relation to dThd concentration. These data also show that at higher concentrations of dThd in the medium (20 μM and above) the dTTP pool becomes maximally labeled within a very short period of time, probably due to feedback inhibition of the dThd kinase and the limited expandability of the dTTP pool (11). Under these conditions the eventual rate of dThd incorporation by the cells will reflect the rate of dTTP incorporation into DNA. Thus our data in Fig. 3 and Table I do not as yet allow calculation of a K_m of the dThd transport process in metabolizing cells. Nevertheless, the time courses of accumulation of dThd in these cells are generally consistent with a value of about 85 μM observed in ATP-depleted or dThd kinase-deficient cells.

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