THYMIDINE TRANSPORT BY CULTURED NOVIKOFF HEPATOMA CELLS AND UPTAKE BY SIMPLE DIFFUSION AND RELATIONSHIP TO INCORPORATION INTO DEOXYRIBONUCLEIC ACID

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ABSTRACT

The initial rate of thymidine-^{3}H incorporation into the acid-soluble pool by cultured Novikoff rat hepatoma cells was investigated as a function of the thymidine concentration in the medium. Below, but not above 2 μM, thymidine incorporation followed normal Michaelis-Menten kinetics at 22°, 27°, 32°, and 37°C with an apparent K_m of 0.5 μM, and the V_{max} values increased with an average Q_{10} of 1.8 with an increase in temperature. The intracellular acid-soluble ^{3}H was associated solely with thymine nucleotides (mainly deoxythymidine triphosphate [dTTP]). Between 2 and 200 μM, on the other hand, the initial rate of thymidine incorporation increased linearly with an increase in thymidine concentration in the medium and was about the same at all four temperatures. Pretreatment of the cells with 40 or 100 μM *p*-chloromercuribenzoate for 15 min or heat-shock (49.5°C, 5 min) markedly reduced the saturable component of uptake without affecting the unsaturable component or the phosphorylation of thymidine. The effect of *p*-chloromercuribenzoate was readily reversed by incubating the cells in the presence of dithiothreitol. Persantin and uridine competitively inhibited thymidine incorporation into the acid-soluble pool without inhibiting thymidine phosphorylation. At concentrations below 2 μM, thymidine incorporation into DNA also followed normal Michaelis-Menten kinetics and was inhibited in an apparently competitive manner by Persantin and uridine. The apparent K_m and K_i values were about the same as those for thymidine incorporation into the nucleotide pool. The over-all results indicate that uptake is the rate-limiting step in the incorporation of thymidine into the nucleotide pool as well as into DNA. The cells possess an excess of thymidine kinase, and thymidine is phosphorylated as rapidly as it enters the cells and is thereby trapped. At low concentrations, thymidine is taken up mainly by a transport reaction, whereas at concentrations above 2 μM simple diffusion becomes the principal mode of uptake. Evidence is presented that indicates that uridine and thymidine are transported by different systems. Upon inhibition of DNA synthesis, net thymidine incorporation into the acid-soluble pool ceased rapidly. Results from pulse-chase experiments indicate that a rapid turnover of dTTP to thymidine may be involved in limiting the level of thymine nucleotides in the cell.

INTRODUCTION

DNA synthesis by mammalian and other types of cells is most often measured by the incorporation of labeled thymidine into DNA or acid-insoluble cell material. However, it has frequently been ob-

served that changes in the rate of thymidine incorporation into DNA either during the cell cycle (1, 2) or as a result of various treatments (3–7) are preceded by or correlate with proportional changes in the rate of incorporation of thymidine from the medium into the nucleotide pool by the cells. It seems possible, therefore, that the rate of thymidine incorporation into DNA does not always accurately reflect the rate of DNA synthesis, and discrepancies between the two rates have been observed (8, 9). For instance, if the rate of thymidine incorporation into nucleic acids is limited by the rate of uptake from the medium, as has been demmonstrated for ribonucleosides (10, 11), changes in the rate of thymidine uptake could cause changes in the rate of thymidine incorporation into DNA unrelated to the rate of DNA synthesis. On the other hand, the level of thymine nucleotides in the cell or their rate of removal for DNA synthesis may have a regulatory effect on thymidine uptake and/or phosphorylation (2, 5, 12–15). The question is complicated by our ignorance with respect to the mode and regulation of thymidine uptake by mammalian cells and the fact that a change in the level of thymidine kinase activity usually occurs at about the same time as the change in thymidine incorporation (2, 5–7, 16, 17). During the cell cycle, however, the main increase in thymidine kinase activity occurs at the end of S phase and during G_2 phase (2, 18). It has been suggested that phosphorylation is part of the uptake reaction and represents the rate-limiting step in the incorporaof thymidine by hamster cells in culture (17, 19), but the isolation of mutants that fail to take up thymidine in spite of high levels of thymidine kinase activity (20) indicates that an additional protein(s) is involved in thymidine uptake.

We have studied the mode of uptake of thymidine and the relationship between uptake rates and rates of incorporation into DNA in Novikoff rat hepatoma cells growing randomly in suspension culture. Evidence is presented that thymidine, like ribonucleosides (10, 11), choline (21), or glucose (22), enters these cells by both a transport reaction and simple diffusion. At low concentrations in the medium, thymidine is mainly taken up by the transport system and at these concentrations uptake is the rate-limiting step in the incorporation of thymidine into DNA. We also studied the involvement of sulfhydryl groups in thymidine transport, the substrate specificity of the system, and the dynamics of the thymine nucleotide pool.

MATERIALS AND METHODS

Materials

Unlabeled thymidine, uridine, thymine nucleotides, and thymidine-6-^{3}H (10 Ci/mmole) were purchased from Schwarz/Mann, Orangeburg, N. Y. Solutions of thymidine-^{3}H with lower specific radioactivity were prepared by addition of unlabeled thymidine. About 5% of the radioactivity of the thymidine-^{3}H was associated with an unknown contaminant (23). Cytosine arabinoside (araC) was purchased from Sigma Chemical Co., St. Louis, Mo. and *p*-chloromercuribenzoate (PCMB) and dithiothreitol (DTT) from Nutritional Biochemicals Corporation, Cleveland, Ohio. Persantin [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*) pyrimidine] was a gift from Geigy Pharmaceuticals, Ardsley, N.Y. The compositions of basal medium 42 (BM42, reference 24) and balanced salt solution (BSS, reference 10) have been described previously.

Cell Culture

Novikoff rat hepatoma cells (subline N1S1-67) were propagated in suspension culture in Swim's medium 67 and enumerated by tabulation in a Coulter counter as described previously (24, 25). For experiments, cells were collected by centrifugation at 400 *g* for 1–2 min from cultures in the exponential phase of growth (2×10^6 to 2.8×10^6 cells/ml) and suspended to 2×10^6 cells/ml in BM42.

Incorporation of Thymidine-^{3}H

Suspensions of cells in BM42 were supplemented with thymidine-^{3}H as indicated in the appropriate experiments and incubated at 37°C or at 18°C where indicated on a gyratory shaker at about 200 rpm. At appropriate time intervals, replicate samples of suspension were analyzed for radioactivity associated with total cell material (acid soluble plus acid insoluble) or for radioactivity in cold perchloric acid-insoluble material as described previously (10, 23). The acid-soluble pools were extracted with cold perchloric acid from samples of 1×10^7 to 2×10^7 cells as described in reference 23 and analyzed chromatographically as described below. Culture fluid was obtained by sedimenting the cells at 400 *g* for 2 min and removing the supernatant fluid.

Assay of Thymidine Kinase Activity

Cell-free extracts were prepared from exponential-phase cells as described previously (26), except that 2-ml extracts were prepared from 2×10^8 rather than 4×10^7 cells. In routine assays for thymidine kinase activity, the reaction mixture contained in a

total of 0.2 ml: 5 mM $MgCl_2$, 10 mM adenosine triphosphate (ATP), 100 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol, 50 mM KCl, 0.2 mM thymidine-^{3}H (250 μCi/μmole), and cell-extract from 1 × 10^7 cells. Other substrate concentrations were employed as indicated in the appropriate experiment. The reaction mixtures were incubated at 37°C, and at appropriate time intervals 50-μl samples were removed and heated for 1 min in a bath of boiling water. The samples were clarified by centrifugation, and thymidine and the thymine nucleotides in the supernatant fluid were separated chromatographically as described below. The conversion of thymidine to total thymine nucleotides was linear with time until 30–40% of the thymidine had been phosphorylated. Thymidine kinase activity was estimated from the linear portion of a plot of the amount of total thymine nucleotides formed as a function of time of incubation.

Chromatography

The various thymine nucleotides and thymidine in thymidine kinase reaction mixtures, acid-extracts from thymidine-^{3}H-labeled cells, or samples of culture fluid were separated by ascending paper chromatography with a solvent composed of 3 vol 1 M ammonium acetate (pH 5.0) and 7 vol 95% ethanol (solvent 28) as described previously (23).

Radioactivity Measurements

Radioactivity was measured by liquid scintillation counting as described previously (24).

RESULTS

Effect of Temperature on Thymidine Uptake as a Function of Thymidine Concentration in the Medium

Fig. 1 A illustrates the initial rates of thymidine uptake by NIS1-67 cells at 22°, 27°, 32°, and 37°C as a function of the thymidine concentration in the medium between 0.1 and 2 μM. Thymidine uptake was estimated by determining the rate of incorporation into total cell material (acid soluble plus acid insoluble) since the thymidine incorporated into DNA must first have passed through the intracellular acid-soluble pool. At these concentrations incorporation was approximately linear with time for about 5–10 min (see reference 23 and Figs. 2 and 4). Chromatographic analyses of acid-extracts prepared from these cells showed that practically all intracellular radioactivity was associated with thymine nucleotides (mainly deoxythymidine triphosphate [dTTP]; see reference 23), indicating that all thymidine was rapidly phosphorylated as it entered the cells. The Lineweaver-Burk plots of the initial rates of uptake in Fig. 1 C show that thymidine uptake followed normal Michaelis-Menten kinetics at the four temperatures tested. The apparent K_m values increased slightly (from 0.4 to 0.5 μM) with an increase in temperature from 22° to 37°C. The apparent V_{max} decreased from 10 pmoles/10^6 cells per min at 37°C to 4 pmoles/10^6 cells per min at 22°C with an average Q_{10} of 1.8. When the V_{max} values were analyzed in an Arrhenius plot a straight line was obtained from which an activation energy of about 11 kcal/mole was calculated (Fig. 1 D). Evidence will be presented later that phosphorylation was not the rate-limiting step in the incorporation of thymidine into the nucleotide pool by whole cells. Thus, the results indicate that the kinetics of thymidine incorporation into total cell material reflect those of a transport system and that at concentrations below 2 μM in the medium, thymidine enters the cells mainly via this transport system.

It will be noted from the data in Fig. 1 B, however, that the rate of thymidine uptake increased linearly with an increase in thymidine concentration in the medium above 2 μM. A similar linear phase has been observed with cultured hamster tumor cells with 0.1–1 mM thymidine in the medium (19). The linear increase in uptake rates was relatively unaffected by changes in the temperature of incubation (Fig. 1 B). When analyzed in a Lineweaver-Burk plot, the uptake rates above 2 μM deviated from the straight line in the direction of the origin (not shown). The deviation became apparent at progressively lower thymidine concentrations with a decrease in the temperature of incubation (see Fig. 1 C). These results are similar to those obtained with nucleosides (11, 27), choline (21), and deoxyglucose (22) and indicate that at concentrations above 2 μM, thymidine entered the cells at a significant rate by simple diffusion. The conclusion is further supported by the effect of PCBM treatment and heat shock on thymidine uptake (see below). We have estimated the initial rate of thymidine uptake due to simple diffusion as a function of thymidine concentration by drawing a broken line through the origin parallel to the solid line part of the uptake curves (Fig. 1 B). The results indicate that at a concentration of 200 μM in the medium, about 90% of the thymidine taken up by the cells during the first 5 min of incubation entered by simple diffusion and only 10%

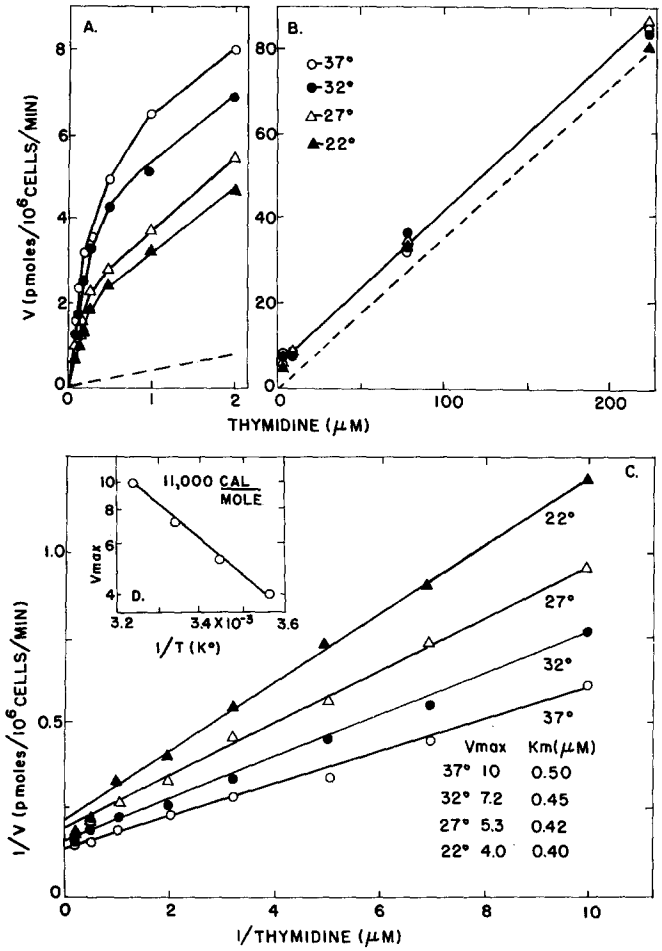

FIGURE 1 Effect of temperature on the initial rate of thymidine uptake as a function of thymidine concentration. Samples of cells were suspended to 2×10^6 cells/ml of BM42 that had been equilibrated at 22°, 27°, 32°, and 37°C. Samples of 10 ml of each suspension were supplemented (A) with 0.1, 0.15, 0.2, 0.3, 0.5, or 1 μM (1350 cpm/pmole) or 2 μM (83 cpm/pmole) thymidine-^{3}H, or (B) with 2 μM or 10 μM (83 cpm/pmole), 80 μM (17.5 cpm/pmole) or 220 μM (6.5 cpm/pmole) thymidine-^{3}H. After 5 and 10 min of incubation at the indicated temperatures, duplicate 1 ml samples were analyzed for radioactivity in total cell material as described in Materials and Methods. The initial rates of thymidine uptake were estimated from the duplicate 5-min values. For technical reasons the experiment was conducted in parts, one temperature at the time, but the whole experiment, except for the radioactivity analyses, was completed in 1 hr. (C) Lineweaver-Burk plot of the initial rates of thymidine uptake between 0.1 and 5 μM thymidine in A and B. (D) Arrhenius plot of the V_{max} values as estimated from the Lineweaver-Burk plots in (C).

by transport (Fig. 1 B), whereas simple diffusion contributed only little to the over-all rate of uptake at concentrations below 2 μM (Fig. 1 A).

Very little free labeled thymidine was present in acid-extracts prepared from cells incubated with thymidine concentrations up to 80–100 μM (see reference 23), but at 200 μM free thymidine accumulated intracellularly. For instance, after 2 hr of labeling with 200 μM thymidine-^{3}H (see Fig. 5 for time course) about half of the radioactivity in the acid-soluble pool was associated with thymine nucleotides (mostly dTTP) and the other half with free thymidine (see Table II). The intracellular amount of free thymidine was approximately 320 pmoles/10^6 cells, and, based on an average cell volume of 2–2.5 μl/10^6 cells (13, 23), was equivalent

to an over-all intracellular concentration of 130–160 μM free thymidine, which is close to the extracellular concentration, but did not exceed it. This is a minimum estimate because thymidine arising from turnover of *de novo* synthesized thymine nucleotides was not taken into account.

Effect of PCMB Treatment and Heat Shock on Thymidine Transport

The results in Fig. 2 A and C demonstrate that preincubation of cells with various concentrations of PCMB at 37°C for 12 min or heating at elevated temperatures for 5 min, respectively, markedly reduced the capacity of the cells to take up thymidine when present at a concentration of 0.5 μM. An inhibition of thymidine incorporation by treatment with mercurials has been reported previously for cultured hamster cells (28). No significant morphological difference could be detected between untreated cells by light microscopy except after heating at 48.5 and 49.5°C. The proportion of trypan blue-stainable cells began to increase at about 10 min after heating at 48.5° or 49.5°C and reached about 10% and 20%, respectively, at the end of the experiment, whereas less then 2% of the cells were stainable in all other suspensions of treated and untreated cells.

The thymidine kinase activity of N1S1-67 cells as measured in cell-free preparations was reduced less than 10% by heating the cells at 49.5°C for 5 min or by incubation with 100 μM PCMB for 15 min. In agreement with this finding, chromatographic analysis of acid-extracts indicated that the thymidine taken up was rapidly phosphorylated by treated and untreated cells alike (Table I). This is indicated by the fact that significant amounts of free thymidine did not accumulate intracellularly. The data indicate that the decreased thymidine incorporation was due to an inactivation of the transport system rather than to a failure of the thymidine taken up to be phosphorylated. However, whereas PCMB treatment had no effect on the further phosphorylation of deoxythymidine monophosphate (dTMP) to dTTP, the formation of dTTP was progressively reduced with an increase in temperature of heating (Table I). Heating at 48.5° and 49.5°C also completely destroyed the capacity of the cells to incorporate thymidine into DNA (Fig. 2 D) and this effect was irreversible (not shown). In contrast, the incorporation of thymidine into acid-insoluble material was reduced by treatment with the various concentrations of PCMB to about the same extent as thymidine transport (compare Fig. 2 A and B), and the effect was reversible (see below).

As shown by the data in Fig. 3 A, only the saturable transport component of the uptake curve (below 2 μM) seemed to be inactivated by PCMB treatment or heat shock (left frame), whereas uptake of thymidine by simple diffusion remained unaffected (right frame).

Fig. 3 B illustrates Lineweaver-Burk plots of the initial rates of thymidine transport by untreated cells and cells whose transport system had been partially inactivated by PCMB treatment or heat

TABLE I

Chromatographic Analysis of Acid-Extracts from Thymidine-Labeled Untreated, PCMB-Treated, and Heat-Shocked Cells

Pretreatment		^{3}H/50 μl acid extract (cpm)				
		dTTP	dTDP	dTMP	Thymidine	Total
PCMB, 12 min,	0 μM	6,340	780	180	230	7,530
	40 μM	6,070	560	150	160	6,940
	100 μM	5,910	620	160	160	6,850
37°C, 5 min		10,840	760	180	180	11,960
47.5°C, 5 min		14,910	1,310	650	110	16,980
48.5°C, 5 min		8,290	3,440	3,450	140	15,320
49.5°C, 5 min		210	630	3,810	140	4,790

The details of the experiment are described in the legend to Fig. 2. After the indicated treatments, the cells were incubated with thymidine-^{3}H for 90 min. Acid-extracts were prepared from samples of 1.5×10^7 cells, and 50-μl samples of each acid-extract were chromatographed with solvent 28 as described in Materials and Methods.

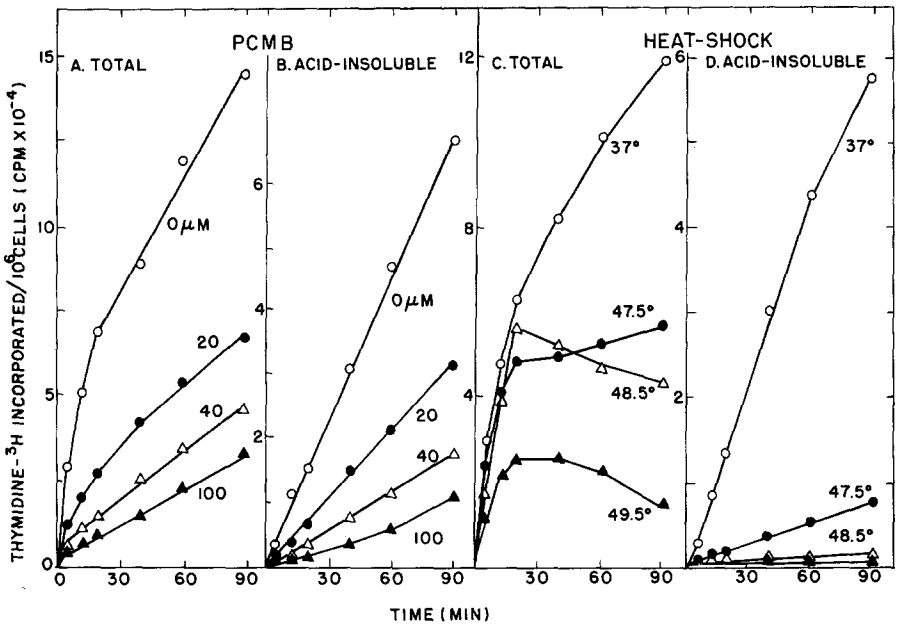

FIGURE 2 Effect of PCMB treatment (A–B) and heat shock (C–D) on the incorporation of thymidine-^{3}H into total cell material and acid-insoluble material. Samples of 4×10^7 cells were collected by centrifugation and further treated as follows: (A–B) The cells were suspended to 4×10^6 cells/ml in BM42 containing the indicated concentrations of PCMB. The suspensions were incubated on a gryratory shaker at 37°C for 12 min and the cells were again collected by centrifugation. (C–D) The cell pellets were incubated in water baths at the indicated temperatures for 5 min. Then the cell pellets from A–B and C–D were suspended (0 time) to 2×10^6 cells/ml of BM42 containing 0.5 μM thymidine-^{3}H (1200 cpm/pmole) and further incubated at 37°C. At the indicated times thereafter, duplicate 0.5 ml samples of each suspension were analyzed for radioactivity in total cell material (A and C) or acid-insoluble material (B and D). All points represent averages of the duplicate samples. At 90 min of incubation, about 20% of the cells heated at 49.5°C and about 10% of the cells heated at 48.5°C were stainable by trypan blue, whereas less then 2% of the cells in all other suspensions were stainable. At 90 min of incubation, acid-extracts were prepared from samples of 1.5×10^7 cells of each suspension and analyzed chromatographically (see Table I).

shock. The results indicate that PCMB treatment caused an increase in the apparent K_m without affecting the V_{max}, whereas heat shock caused a reduction in the V_{max} without significantly affecting the K_m. The results are consistent with the view that PCMB treatment caused a lowering of the affinity of the transport sites (or carrier protein) for substrate without affecting the number of sites, whereas heating completely inactivated a number of sites without changing the affinity of the remaining sites.

Reversal of PCMB Inactivation of Thymidine Transport System

A portion of a suspension of cells was incubated with PCMB for 15 min. Then the cells were collected by centrifugation and samples of the treated and untreated cells were monitored (0–1 hr) for thymidine incorporation into total cell material (Fig. 4) and into acid-insoluble material (not shown). Other samples of PCMB-treated and untreated cells were resuspended in BM42 with or without DTT. These suspensions were incubated at 37°C, and at 1.5 and 4 hr of incubation, samples of each suspension were monitored for thymidine-^{3}H incorporation. The results in Fig. 4 show that the cells incubated in BM42 plus DTT regained rapidly their capacity to incorporate thymidine into total cell material. After 4 hr of incubation with DTT, the initial rate of thymidine incorporation was close to that of the untreated control cells, whereas the reversal was minimal when the cells were incubated in BM42 alone (Fig.

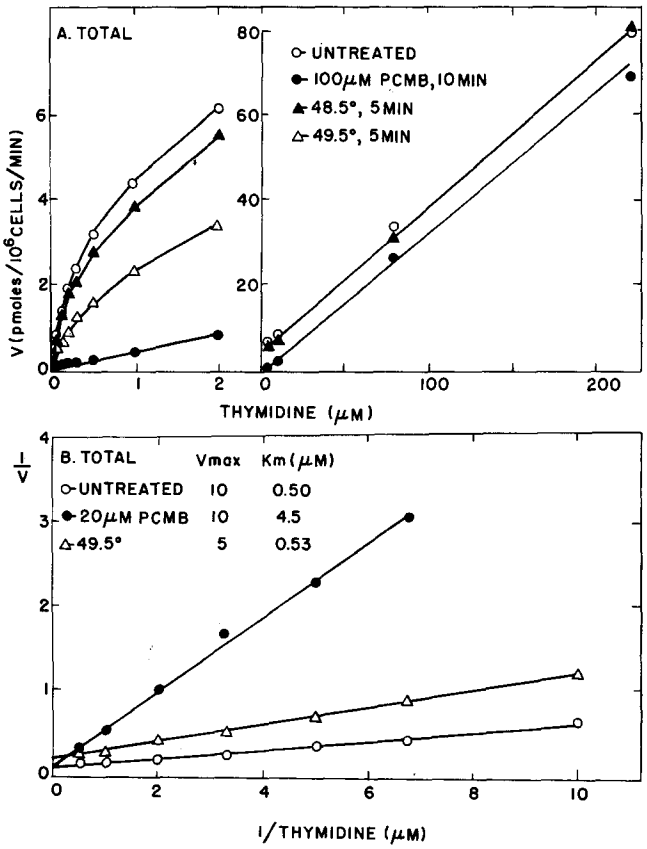

FIGURE 3 Initial rates of thymidine uptake by untreated, PCMB-treated, and heat-shocked cells as a function of thymidine concentration. (A) Samples of 2×10^8 cells were collected by centrifugation and treated as follows: (*a*) untreated (○——○); (*b*) The cells were suspended to 4×10^6 cells/ml in BM42 containing 100 μM PCMB. The suspension was incubated on a gyratory shaker at 37°C for 10 min and the cells again collected by centrifugation (●——●); (*c*) The cell pellets were heated at 48.5°C (▲——▲) or 49.5°C (△——△) for 5 min. Then the cells of all four samples were suspended in BM42 to 2×10^6 cells/ml and immediately thereafter samples of each suspension were supplemented with 0.1, 0.15, 0.2, 0.3, 0.5, or 1 μM (1650 cpm/pmole), or 2 or 10 μM (98 cpm/pmole), or 80 μM (21 cpm/pmole) or 220 μM (7.4 cpm/pmole) thymidine-^{3}H. The initial rates of uptake were estimated by analyzing duplicate 1 ml samples of suspension for radioactivity in total cell material after 5 min of incubation at 37°C. For technical reasons, the experiment was conducted in parts, one treatment at a time, but the whole experiment, except for the radioactivity analyses, was completed in 1 hr. (B) Lineweaver-Burk plots of the initial uptake rates between 0.1 and 2 μM thymidine.

4) or in BM42 that was supplemented with 1 mM glutathione or 5 mg/ml of bovine serum albumin (not shown). The presence of 10 mM unlabeled thymidine also did not increase the degree of reversal. The decrease in thymidine incorporation by the DTT-treated cells between 4.5 and 5 hr (Fig. 4) was probably a consequence of an inhibition of DNA synthesis by DTT, since DTT caused a marked decrease in thymidine incorporation into acid-insoluble material (not shown) and an inhibition of DNA synthesis by various antibiotics also resulted in a rapid cessation of thymidine incorporation into the nucleotide pool (see below). DTT, however, had no effect on the phosphorylation of thymidine to dTTP since about 85% of the radioactivity in acid-extracts from these cells was associated with dTTP (not shown).

Relation of Phosphorylation to Thymidine Uptake

Since the rate of thymidine incorporation into the acid-insoluble pool by various lines of hamster

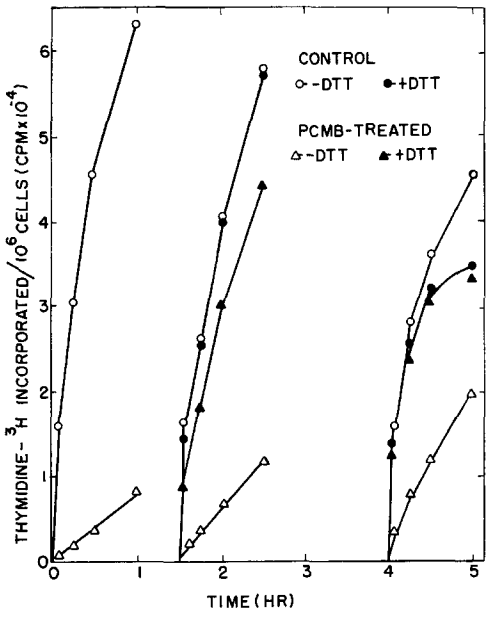

FIGURE 4 Reversal of the PCMB-inactivation of the thymidine transport system by incubation of the cells in BM42 with and without DTT. Suspensions of 4 × 10^6 cells/ml of BM42 without (○——○, ●——●) or with 50 μM PCMB (△——△, ▲——▲) were incubated on a gryratory shaker at 37°C for 15 min. Then the cells were collected by centrifugation and suspended (0 time) to 2 × 10^6 cells/ml in BM42 (○——○, △——△) or BM42 containing 1 mM DTT (●——●, ▲——▲). At 0, 1.5, and 4 hr of further incubation at 37°C, samples of the suspension were supplemented with 0.32 μM thymidine-^{3}H (1200 cpm/pmole) and monitored for the incorporation of radioactivity into total cell materials. All points represent averages of values from duplicate 0.5 ml samples of suspension. Other samples of cell suspension were analyzed for radioactivity in acid-insoluble material (not shown).

cells correlated approximately with the level of thymidine kinase activity in these cells, Hare and Schuster (17, 19) suggested that phosphorylation might act as a regulatory factor in thymidine movement across the cell membrane. In contrast, phosphorylation did not appear to be the rate-limiting step in thymidine uptake by N1S1-67 cells. At least at concentrations below 50 μM in the medium, the thymidine taken up by the cells was phosphorylated as rapidly as it entered, since significant amounts of free thymidine were not present intracellularly. This finding combined with the fact that PCMB treatment inactivated the transport system without significantly affecting the thymidine kinase suggested that transport is a reaction separate from phosphorylation and, at low thymidine concentrations in the medium, represents the rate-limiting step in the incorporation of thymidine into the nucleotide pool. These conclusions are supported by the finding that Persantin markedly inhibited thymidine incorporation into total cell material (Fig. 5 A) without affecting the distribution of the intracellular label among the thymine nucleotides (Table II). It also had no effect on the phosphorylation of thymidine by cell-free preparations, even at a relatively high concentration of 1 mM (not shown). Further, the incorporation of thymidine into acid-insoluble material was reduced to about the same extent as its incorporation into the nucleotide pool (compare Fig. 5 A and B). Persantin previously has been shown to be a competitive inhibitor of the transport of ribonucleosides (10, 11, 29, 30) and monosaccharides (22, 31). The inhibition of thymidine transport was also of the simple competitive type (Fig. 6 A). On the other hand, at concentrations of thymidine in the medium at which it is taken up mainly by simple diffusion (200 μM), Persantin, even at a higher concentration of 200 μM, had relatively little effect on thymidine uptake or its incorporation into acid-insoluble material (Fig. 5 C and D, Table II).

The data summarized in Table III show that the V_{max} for the phosphorylation of thymidine by cell-free preparations was about 20 times higher than that for thymidine uptake by whole cells, whereas the apparent K_m for the thymidine kinase

TABLE II

Chromatographic Analysis of Acid-Extracts from Cells Labeled with Thymidine-^{3}H in the Presence of Various Concentrations of Persantin

Thymidine-^{3}H (μM)	Persantin (μM)	^{3}H/50 μl acid-extract: dTTP + dTDP	dTMP	Thymidine	Total
0.3	0	2700	80	120	2900
	20	2500	50	120	2670
200	0	700	90	840	1630
	200	560	60	720	1340

The details of the experiment are described in the legend to Fig. 5. After labeling with thymidine-^{3}H in the presence of the indicated concentrations of Persantin for 120 min, acid-extracts were prepared from 1 × 10^7 cells, and 50-μl samples of each acid-extract were chromatographed with solvent 28 as described under Materials and Methods.

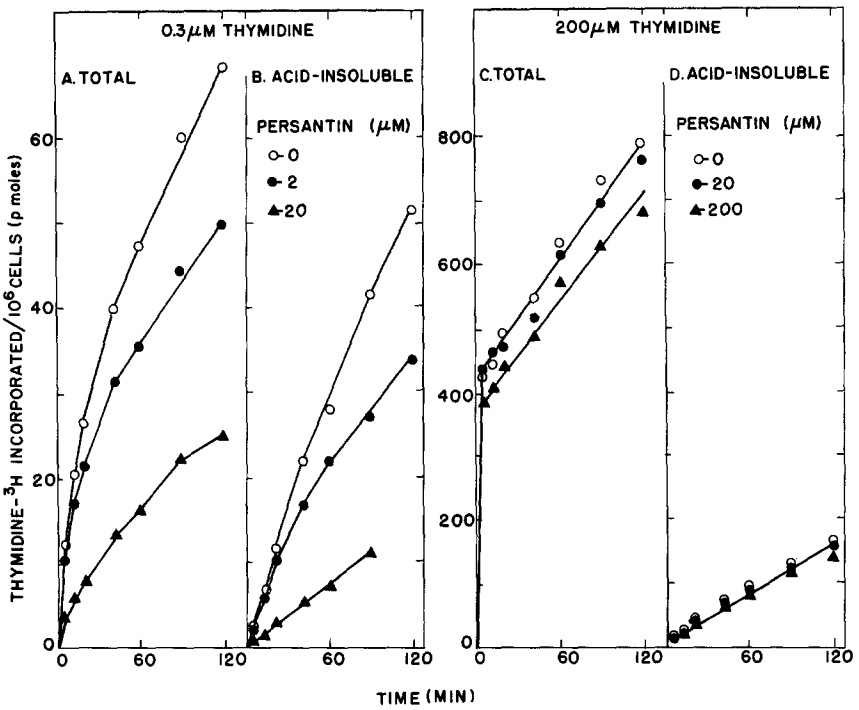

FIGURE 5 Effect of Persantin on thymidine incorporation into total cell material and acid-insoluble material at 0.3 μM (A–B) and 200 μM thymidine (C–D). Samples of a suspension of 2×10^6 cells/ml of BM42 were supplemented with the indicated concentrations of Persantin and immediately thereafter with 0.3 μM (1170 cpm/pmole) or 200 μM (7.75 cpm/pmole) thymidine-^{3}H. After various times of incubation at 37°C, duplicate 0.5 ml samples of each suspension were analyzed for radioactivity in total cell material (A and C) or acid-insoluble material (B and D). All points represent averages of the duplicate samples. At 120 min of incubation, acid-extracts were prepared from 1.6×10^7 cells of each suspension and analyzed chromatographically (see Table II).

was about 400 times higher than that for thymidine transport. Only at concentrations above 50–100 μM thymidine in the medium did the rate of uptake apparently exceed the capacity of the cells to phosphorylate it and free thymidine accumulate intracellularly (Table II). It is of interest that the ratio of the V_{max} for the thymidine kinase activity over that for thymidine transport by an equivalent number of cells (about 20) was about the same as the corresponding ratio for uridine kinase activity and uridine transport (Table III), though the apparent V_{max} values were about 10-fold higher for uridine than for thymidine.

Specificity of the Thymidine Transport System

On the basis of results from exchange diffusion studies Oliver and Paterson (32) concluded that human erythrocytes possess a single system for the transport of uridine and thymidine and other ribo- and deoxyribonucleosides. In contrast, our previous results indicated that N1S1-67 cells possess at least three systems for the transport of ribonucleosides and that thymidine is transported by a different system (11). This was also suggested by the data of Steck et al. (33) from the studies with chick embryo cell cultures, and the conclusion is further supported by the data summarized in Table III. As pointed out already, the V_{max} and K_m values for uridine and thymidine transport differ by about one order of magnitude. Although uridine transport is competitively inhibited by thymidine (11) and thymidine transport by uridine (Fig. 6 A), the extent of inhibition was low in either case. As indicated by the low K_m/K_i ratios (Table III), the transport systems had much lower affinities for the respective inhibitors than for their substrates. Similarly, Hare (17) reported that uridine had little, if any, effect on the uptake of thymidine by cul-

TABLE III

Characteristics of Uridine and Thymidine Transport Systems and Uridine and Thymidine Kinase Activities

Substrate	Reaction	V_{max}*	K_m (μM)	Competitive inhibition			Inactivation		
				Inhibitor	K_i (μM)	K_m/K_i	47.5°C	49.5°C	PCMB
Thymidine	Transport by whole cells	8–12	0.4–0.5	Uridine	500	0.001	10	40–50	50–60
	Cell-free kinase	200	200						
Uridine	Transport by whole cells	100–150	13–16	Thymidine	700	0.020	60–70		20–30
	Cell-free kinase	2800	500						

The kinetic constants for thymidine transport and its inhibition by uridine were estimated from the Lineweaver-Burk plots in Figs. 2 C, 4 C, and 7 and those from other similar experiments. The degree of inactivation of the thymidine and uridine transport systems by treatment with 20 μM PCMB or heat shock was estimated from the data in Fig. 2 and data presented previously (11), respectively. The kinetic constants for the phosphorylation of thymidine by a cell-free extract were estimated from a Lineweaver-Burk plot of initial velocities which were determined as described under Materials and Methods, except that the reaction mixtures contained 0.1, 0.14, 0.2, 0.3, and 0.5 mM thymidine-^{3}H. The corresponding values for uridine transport and uridine kinase activity have been estimated previously (10).

* pmoles/10^6 cells per min.

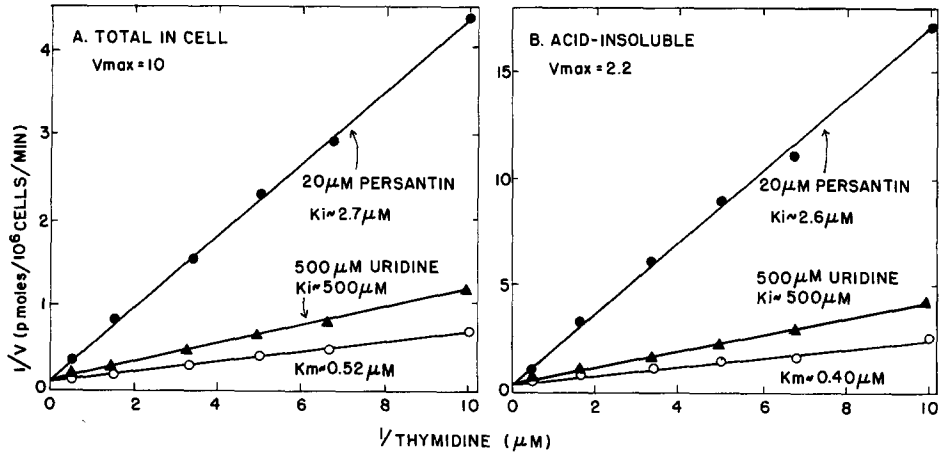

FIGURE 6 Competitive inhibiton of thymidine transport (A) and incorporation into acid-insoluble material (B) by Persantin and uridine. Samples of a suspension of 2 × 10^6 cells/ml of BM42 were supplemented with 20 μM Persantin or 500 μM uridine. Then samples of an untreated suspension and of the treated suspensions were supplemented with 0.1, 0.15, 0.2, 0.3, 0.6, or 2 μM thymidine-^{3}H (1200 cpm/pmole). The initial rates of thymidine uptake (A) or incorporation into acid-soluble material (B) were estimated by analyzing duplicate 1 ml samples for radioactivity in total cell material after 5 min of incubation and other samples for radioactivity in acid-insoluble material after 10 min, respectively. For technical reasons the experiment was conducted in parts, one treatment at a time, but the whole experiment, except for the radioactivity analyses, was completed in 45 min.

tured hamster cells. Further, the uridine transport system was markedly more heat labile than the thymidine transport system, whereas the reverse was true for the sensitivity to PCMB inactivation (Table III).

Relationship between Thymidine Transport and Its Incorporation into DNA

The incorporation of thymidine into acid-insoluble material by N1S1-67 cells attains a relatively

constant and maximum rate within a few minutes after the addition of thymidine to the medium, regardless of the concentration of thymidine added (reference 23; see also Figs. 2 B and D, and 5 B and D). This finding indicates that the thymine nucleotide pool of N1S1-67 used for DNA synthesis is very small and equilibrates rapidly with extracellular thymidine. This conclusion was previously indicated by the data of Gentry et al. (13), and similar results have been reported for human leukocytes (15). Further, the rate of thymidine incorporation into DNA and the initial rate of transport in N1S1-67 cells varied in the same manner as a function of thymidine concentration. This is indicated by the fact that thymidine incorporation into DNA followed normal Michaelis-Menten kinetics with an apparent K_m which was about the same as that for thymidine transport (compare Fig. 6 A and B). Combined, these results are consistent with the conclusion that the rate-limiting step in the incorporation of thymidine into DNA, like its incorporation into the nucleotide pool, is its transport into the cell. In agreement with this conclusion is the finding that the competitive inhibition of thymidine transport by Persantin or uridine resulted in an apparent competitive inhibition of thymidine incorporation into acid-insoluble material and that the K_i values were about the same for both inhibitors (Fig. 6). Similarly, as pointed out already, inactivation of the thymidine transport system by PCMB resulted in a proportional reduction in the incorporation of thymidine into acid-insoluble material (Fig. 2 A and B).

Effect of Inhibitors of DNA Synthesis and Thymidine Incorporation into Nucleotide Pool and Turnover of Thymine Nucleotides

The inhibition of DNA synthesis by cytosine arabinoside, puromycin, or Acti-dione had little effect on the initial rate of thymidine incorpo-

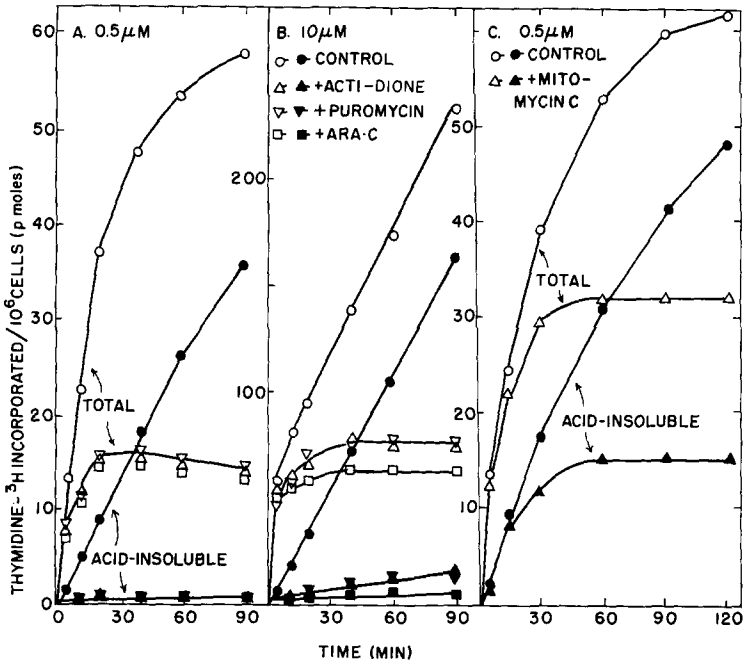

FIGURE 7 Effect of Acti-dione, puromycin, araC, and mitomycin C on thymidine incorporation into total cell material and acid-insoluble material. Suspensions of 2×10^6 cells/ml of BM42 were supplemented with 100 μg/ml (A) or 80 μg/ml (B) of Acti-dione, or 25 μg/ml (A) or 20 μg/ml (B) of puromycin, or 50 μM araC (A and B), or 100 μg/ml of mitomycin C and immediately thereafter (0 time) with (A and C) 0.25 μM thymidine-^{3}H (1200 cpm/pmole) or (B) 10 μM thymidine-^{3}H (70 cpm/pmole). Cell suspensions without antibiotics were supplemented with thymidine-^{3}H in the same manner. At the indicated times of incubation, duplicate 0.5 ml samples of each suspension were analyzed for radioactivity in total cell material (open symbols) or acid-insoluble material (closed symbols). All points represent averages of the duplicate samples.

ration into the nucleotide pool, although subsequently it became progressively reduced and incorporation ceased altogether after 20–30 min (Fig. 7 A and B). The reason for the rapid cessation of thymidine incorporation into the nucleotide pool in the absence of DNA synthesis is not entirely clear. In contrast, the incorporation of ribonucleosides into the nucleotide pool by N1S1-67 cells continues at an undiminished rate for hours in the absence of RNA synthesis, resulting in a marked expansion of the ribonucleotide pools (23, 26). Chromatographic analysis of the acid-soluble pools showed that as in untreated cells the thymidine incorporated by cells treated with the various inhibitors of DNA synthesis accumulated intracellularly mainly as dTTP (not shown). This finding coupled with the observation that Acti-dione, puromycin, and araC had an identical effect on thymidine incorporation into total cell material (Fig. 7 A and B) suggests that the latter effect was not due to a direct inhibition of the transport or phosphorylation of thymidine but rather was related to the inhibition of DNA synthesis. Mitomycin C affected DNA synthesis by N1S1-67 cells only slowly, but the inhibition of thymidine incorporation into acid-insoluble material was also accompanied by a cessation of thymidine incorporation into the nucleotide pool (Fig. 7 C).

The inhibition of thymidine incorporation into the nucleotide pool could be a consequence of feedback inhibition of thymidine kinase by the dTTP (12) accumulating as a result of the inhibition of DNA synthesis (2, 14). Also, as previously suggested (2, 13), feedback inhibition by dTTP could account for the rapid decrease in the initial rate of thymidine incorporation after 5–10 min of incubation with higher concentrations of thymidine (reference 23; see also Figs. 5 C and 7 B). The data in Fig. 8, on the other hand, raise the possibility that the cessation of the net incorporation of thymidine in the absence of DNA synthesis may represent an equilibrium between the rate of dTTP synthesis and turnover. In this experiment, one set of replicate cultures was labeled with 0.25 μM thymidine-^{3}H in the presence and absence of Acti-dione or araC. Another set of cultures was incubated with these inhibitors plus 10 μM unlabeled thymidine for 70 min. Then the cells of the latter cultures were collected by centrifugation, suspended in fresh medium containing the drugs and 0.25 μM thymidine-^{3}H, and the incorporation of radioactivity into total cell material and acid-insoluble material was measured. As indicated by the data, thymidine-^{3}H was readily incorporated into the nucleotide pool by the latter cells in spite of the fact that their dTTP pool should have been completely saturated during incubation with the inhibitors plus an excess of unlabeled thymidine. The time course of thymidine incorporation by these cells was about the same as that by the cells that had not been preincubated with unlabeled thymidine, considering that the incorporation by the untreated control cells after 70 min of incubation in BM42 was also somewhat lower than that by those labeled immediately after suspension in BM42 (Fig. 8). That a

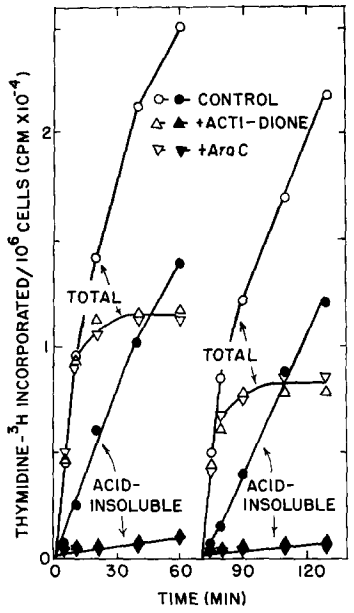

FIGURE 8 Thymidine-^{3}H incorporation in the presence of Acti-dione or araC after preincubation of the cells with the drugs plus an excess of unlabeled thymidine. Duplicate 20-ml samples of a suspension of 2 × 10^6 cells/ml of BM42 were supplemented with 10 μM unlabeled thymidine and as indicated with 100 μg/ml of Acti-dione (△——△, ▲——▲) or 50 μM araC (▽——▽, ▼——▼). Immediately thereafter, the cells from one set of samples were collected by centrifugation and suspended to the original cell density in BM42 containing 0.25 μM thymidine-^{3}H (1000 cpm/pmole) and Acti-dione or araC where indicated. The second set of suspensions was first incubated at 37°C for 70 min. Then the cells were collected and suspended in fresh medium containing thymidine-^{3}H and the drugs as described for the first set. At the indicated times of incubation, duplicate 0.5 ml samples of the suspensions were analyzed for radioactivity in total cell material (open symbols) or acid-insoluble material (closed symbols). All points represent averages of the duplicate samples.

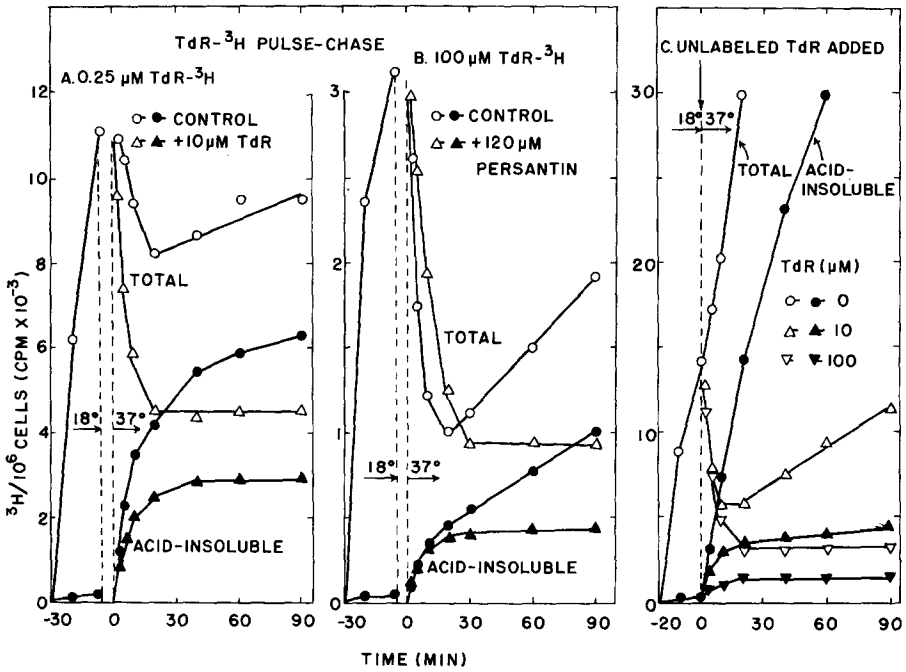

FIGURE 9 Pulse-labeling of cells with thymidine-^{3}H at 18°C and chase at 37°C after removal of thymidine-^{3}H (A and B) or direct addition of an excess of unlabeled thymidine (C). Suspensions of 2×10^6 cells/ml of BM42 at 18°C were supplemented with (A and C) 0.25 μM thymidine-^{3}H (1500 cpm/pmole) or (B) 100 μM thymidine-^{3}H (7.2 cpm/pmole) and incubated at 18°C. (A and B) After 25 min of incubation at 18°C, the cells of replicate samples were collected by centrifugation, washed once in cold (18°C) BM42, and then suspended to the original cell density in warm (37°C) BM42 containing where indicated 10 μM unlabeled thymidine (A) or 120 μM Persantin (B). The suspensions were further incubated at 37°C. (C) After 20 min of incubation at 18°C, samples of the suspension were supplemented as indicated with unlabeled thymidine. The flasks were transferred to a gyratory water bath shaker maintained at 37°C and further incubated at 37°C. Temperature equilibration occurred within 2 min. At the indicated times during the pulse and the chase periods, duplicate 0.5 ml samples of the various suspensions were analyzed for radioactivity in total cell material (open symbols) or acid-insoluble material (closed symbols). All points represent averages of the duplicate samples.

rapid turnover of dTTP occurs in mammalian cells was suggested by previous results that indicated that in pulse-chase experiments with thymidine-^{3}H, a substantial proportion of the dTTP that is synthesized during the pulse, is rapidly lost during the chase (15, 35). In contrast, such nucleotide loss was not observed in similar pulse-chase experiments with various ribonucleosides (35), except when the cells were treated with phenethyl alcohol during the chase (36). Additional results illustrating a rapid turnover of dTTP are presented in Fig. 9. The cells were incubated with 0.25 μM (Fig. 9 A) or 100 μM (Fig. 9 B) thymidine-^{3}H at 18°C for 25 min. At this temperature, thymidine was not incorporated into DNA and accumulated intracellularly mainly as dTTP (Table IV). Then the cells were freed of extracellular thymidine-^{3}H and suspended in fresh medium, incubated at 37°C, and monitored for radioactivity in total cell material and acid-insoluble material. As indicated by the data in Fig. 9 A, about 55% of the acid-soluble thymine nucleotides accumulated during labeling at 18°C was chased into acid-insoluble material during the 90 min of incubation at 37°C. About 20% of the label was lost from the cells during the first 20 min of the chase, and then the amount of label in total cell material began to increase slowly and progressively. A similar but more pronounced initial loss of labeled acid-soluble thymine nucleotides during the chase was observed when the cells were pulse-labeled with 100μM thymidine-^{3}H (Fig. 9 B). Chromatographic analyses of acid-extracts prepared from samples of these cells at various times during the chase indicate that the loss of la-

TABLE IV

Chromatographic Analysis of Acid-Extracts Prepared from Thymidine-^{3}H-Labeled Cells at Various Times during a Chase with and without Unlabeled Thymidine

Exp.	Chase		Acid-extract, ^{3}H/50 μl (cpm)				Culture fluid, ^{3}H/50 μl (cpm): Thymidine
	Thymidine added (μM)	Time (min)	dTTP	dTDP	dTMP	Thymidine	
A	0	0	4460	920	340	130	50
		20	1360	300	300	110	360
		90	660	200	200	100	150
	10	20	180	60	100	150	650
		90	140	70	100	130	650
C	0	0	4970	1180	310	260	
	10	10	1030	190	40	250	
		90	550	100	10	150	
	100	10	850	140	70	370	
		90	140	10	10	250	

The details of the experiment are described in the legend to Fig. 9 A and C. Cells were prelabeled with 0.25 μM thymidine-^{3}H at 18°C. Samples of these cells were collected by centrifugation, washed, and further incubated at 37°C in fresh medium containing the indicated concentrations of unlabeled thymidine (A), or samples of the suspension were directly supplemented with the indicated concentrations of unlabeled thymidine and further incubated at 37°C (C). At the indicated times during the chase, acid-extracts were prepared from samples of 1×10^7 cells, and 50-μl samples of each acid-extract were chromatographed with solvent 28 as described in Materials and Methods. In A, 50-μl samples of culture fluid were also chromatographed with solvent 28.

beled acid-soluble material was mainly due to the loss of dTTP (Table IV), and chromatography of the culture fluid demonstrated the concomitant appearance of labeled thymidine in the medium (Table IV). Extracellular label was found to be associated exclusively with thymidine. The increase in radioactivity in total cell material during the latter part of the chase period (Fig. 9 A and B) was apparently due to reincorporation of the thymidine-^{3}H that was released from the degradation of dTTP-^{3}H, since it was prevented by the presence of Persantin during the chase (Fig. 9 B). Persantin also prevented the second phase of incorporation of label into acid-insoluble material which coincided with the increase in radioactivity in total cell material and thus reflected the reincorporation from the medium of the released thymidine. The presence of an excess of unlabeled thymidine during the chase, on the other hand, markedly increased the loss of labeled thymine nucleotides (Fig. 9 A). The cells lost about 95% of their dTTP during the first 20 min of the chase (Table IV). Only about 25% of the dTTP lost was incorporated into DNA (Fig. 9 A). The remainder was converted to thymidine and was released into the medium (Table IV). We interpret these data to indicate that in the absence of an excess of unlabeled thymidine in the medium, most of the thymidine released from the turnover of dTTP during the chase was rapidly rephosphorylated, whereas it was immediately diluted if an excess of unlabeled thymidine was present in the medium and was thus prevented from being reincorporated. The results in Fig. 9 C illustrate that the loss of dTTP during the chase was not an artifact resulting from the centrifugation and washing of the cells. The simple addition of an excess of unlabeled thymidine at the time of temperature shift from 18° to 37°C caused a rapid loss of most of the intracellular labeled dTTP, and little radioactivity was transferred to acid-insoluble material (Fig. 9 C, Table IV). Incubation of the cells at 18°C had little effect on the capacity of the cells to synthesize DNA since the control cells began to incorporate label into acid-insoluble material immediately upon the temperature shift to 37°C (Fig. 9 C).

DISCUSSION

The present results indicate that, in addition to being taken up by a transport reaction, thymidine

also enters N1S1-67 cells by simple diffusion. This conclusion is indicated by the fact that at concentrations above 5 μM, the initial rate of thymidine incorporation into total cell material is directly proportional to the thymidine concentration in the medium and that the rate of incorporation is relatively unaffected by the temperature of incubation, by inactivation of the transport system by PCMB treatment or heat shock, or by the presence of Persantin which competitively inhibits thymidine transport. Results with cultured hamster cells (19) support this conclusion. Thymidine, however, enters the cells by simple diffusion only at a significant rate if its concentration in the medium is relatively high, whereas at low concentrations transport is the main mode of entry of thymidine into the cells. Similar observations have been made for the uptake by N1S1-67 cells of various ribonucleosides (23, 27), choline (21), or glucose (22). The K_m values for the various transport reactions, however, differ markedly. The K_m for thymidine transport (0.4–0.5 μM) is more than one order of magnitude lower than the K_m's for ribonucleoside (7–24 μM) or choline transport (4–7 μM) and at least 2000-fold lower than the K_m for glucose transport (1–2 mM). These differences in K_m values for the different transport systems could have a physiological significance and reflect differences in the concentrations of the various substrates in serum or other body fluid. That the K_m values for the transport systems of N1S1-67 cells may still be similar to those of other body cells, in spite of the fact that the cells have been propagated continuously in culture for the last 15 yr (24), is indicated by the fact that the uridine transport system of N1S1-67 cells exhibits about the same K_m as that of primary cultures of chick embryo cells (30, 33, 37, 38; Plagemann, unpublished data) and mouse 3T3 cells (38).

From the present data, however, it cannot be decided whether the thymidine transport reaction represents active transport or facilitated diffusion. The cells possess an excess of thymidine kinase and the thymidine taken up by the cells thus becomes phosphorylated as rapidly as it enters the cells, except at concentrations of 100 μM or higher in the medium. At the latter concentrations the thymidine kinase appears to be saturated by the thymidine taken up, and free thymidine accumulates intracellularly. The finding that under these conditions the intracellular concentration of thymidine does not exceed the extracellular concentration, however, cannot be taken as evidence that uptake occurs by facilitated diffusion since at these concentrations thymidine enters the cells mainly by simple diffusion. The energy requirements for the transport reactions are difficult to evaluate in N1S1-67 cells since the cells possess large pools of ATP (34) and energy is derived from both oxidative and glycolytic reactions (22). Nevertheless, our data clearly indicate that the phosphorylation of thymidine is a reaction distinct from transport. This is indicated by the fact that PCMB and heat treatment or the presence of Persantin inhibit the incorporation of thymidine by whole cells without affecting the phosphorylation of thymidine by cell-free preparations. The PCMB-induced inhibition of thymidine uptake by hamster cells also occurred in the absence of any loss of thymidine kinase activity (28). The finding that the K_m and V_{max} values for thymidine incorporation by whole cells differ from the corresponding values for the in vitro phosphorylation of thymidine by factors of 10 and 400, respectively, is also consistent with this conclusion. In addition, Breslow and Goldsby (20) have isolated mutants of Chinese hamster fibroblasts that fail to incorporate thymidine in spite of the fact that their specific thymidine kinase activity is still about 50% of that of the parent cells.

The mechanism of transport of small molecular weight substances by mammalian cells is not understood. The relative sensitivity of the transport systems of N1S1-67 cells for thymidine, ribonucleosides (11), or glucose (22) to inactivation by PCMB suggests that sulfhydryl groups are involved. This conclusion is supported by the finding that the PCMB inactivation of thymidine transport is readily reversed by incubating the cells with DTT. The finding that the partial inactivation of the transport system by PCMB causes an increase in the K_m without changing the V_{max} is consistent with the view that blocking of the sulfhydryl groups with PCMB reduces the affinity of the transport sites for substrate without altering the number of sites and that this effect is overcome by high concentrations of substrate. Since, however, excess concentrations of unlabeled thymidine fail to reverse the effect of PCMB on the subsequent uptake of trace amounts of thymidine-^{3}H, high concentrations of thymidine must overcome the PCMB-induced lowered affinity for substrate without displacing the bound PCMB. The mechanism by which this could occur is not clear. In contrast, heat shock seems to result in the loss of the number of transport sites without affecting the affinity of the remaining sites for substrate.

There is also little question that thymidine and

uridine are transported by different systems. The results summarized in Table III clearly illustrate the marked differences in the kinetic properties of the two systems and their sensitivities to PCMB and heat inactivation. Further, thymidine transport and uridine transport are relatively little inhibited by each other.

The mechanism of turnover of dTTP observed during pulse-chase experiments or its physiological significance is not known. This loss of dTTP seems to be unique for dTTP since ribonucleotides (26), phosphorylcholine (39), or glucosamine-6-phosphate (40) seem to be stable in similar pulse-chase experiments with N1S1-67 cells. In this connection, it is of interest that the density-dependent inhibition of mouse 3T3 cells is accompanied by a 90% decrease in pool sizes of the deoxyribonucleoside triphosphates, whereas the levels of ribonucleotides remain relatively unaffected (41). It also cannot be decided at present whether the cessation of thymidine incorporation after inhibition of DNA synthesis reflects an equilibrium between dTTP synthesis and turnover or is due to feedback inhibition of thymidine kinase by dTTP. Similarly, it is not clear whether the limited incorporation of thymidine into the nucleotide pool by cells during various stages of the cell cycle other than the S period (1, 2) is due to an inhibition of thymidine transport or phosphorylation or simply reflects a rapid turnover of dTTP. Further work is in progress with synchronized N1S1-67 cells to distinguish between these possibilities. The cultures of randomly growing cells used in the present study probably consist largely of cells in the S period since the latter generally takes up 60–70% of the cell cycle in cells with a generation of about 12 hr (42, 43). Thymidine uptake could be solely due to the cells in the S period if it can be ascertained that N1S1-67 cells fail to either transport or phosphorylate thymidine during other periods of the cell cycle.

The reason for the rapid inhibition of DNA synthesis by Acti-dione and puromycin observed previously in other cell systems (44, 45) is also not clear. Since both antibiotics have an identical effect, it seems likely that the effect is a consequence of the inhibition of protein synthesis. The present results show that the inhibition of thymidine incorporation by either antibiotic is not due to failure of the thymidine to become phosphorylated to the triphosphate level.

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