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METHOTREXATE DI-*n*-BUTYL ESTER EFFECTS ON THYMIDINE METABOLISM

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Abstract—The effects of methotrexate di-*n*-butyl ester (DBMTX) on thymidine (dThd) metabolism in cultured L1210 cells have been studied. In contrast to methotrexate, DBMTX markedly suppresses the incorporation of exogenous radiolabeled dThd into DNA. Uptake of dThd into the pools of thymidine nucleotides is decreased markedly. The DNA polymerases and thymidine kinase were unaffected by DBMTX *in vitro*. There was no net efflux of soluble dThd DNA precursors from cells. No specific block at any of the phosphorylation steps was found. dThd transport, however, was inhibited significantly, as were deoxyuridine (dUrd) and uridine (Urd) transport. These results suggest that DBMTX inhibits dThd incorporation by inhibiting initial transport of dThd across the cell membrane.

A major mechanism of methotrexate (MTX) resistance in experimental cell lines is decreased carrier-mediated active transport through which MTX uptake normally occurs [1]. Recently, lipid-soluble derivatives of MTX, which might circumvent this mechanism of resistance by penetrating cell membranes via passive diffusion, were synthesized in these laboratories [2]. The di-*n*-butyl ester of methotrexate (DBMTX) was found to be approximately as potent *in vitro* as MTX against MTX-sensitive cell lines, and in addition, was effective against cell lines resistant to MTX by virtue of decreased transport.* Several observations made in preliminary studies [3] strongly suggested that DBMTX affects cell metabolism differently than does MTX. DBMTX, in contrast to MTX, caused marked suppression of exogenous thymidine (dThd) incorporation into the DNA of L1210 and CCRF-CEM cells in culture. Also, DBMTX, while approximately equipotent with MTX as an inhibitor of cell growth, is about one to two orders of magnitude less effective as an inhibitor of dihydrofolate reductase [4]. These effects imply that DBMTX has either a different or an important additional mechanism of action when compared to MTX.

We have now studied in more detail the perturbations of dThd metabolism produced by DBMTX. The relevant pathways are shown in Fig. 1. The results of the present study show that the decrease in amount of exogenous dThd taken up into the acid-soluble dThd nucleotide pools is attributable to inhibition of dThd transport.

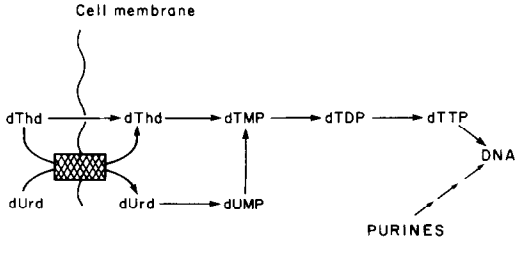

Fig. 1. Schematic representation of dThd and dUrd transport and intermediary metabolism

MATERIALS AND METHODS

Enzymes and chemicals. DBMTX was prepared as described previously [2]. [^{3}H]Thymidine ([^{3}H]dThd), 50 Ci/mmole, [^{14}C]uridine ([^{14}C]Urd), 35 mCi/mmole, and [^{3}H]deoxyuridine ([^{3}H]dUrd), 30 Ci/mmole, were obtained from New England Nuclear (Boston, MA). Authentic samples of dThd, dTMP, dTDP and dTTP were obtained from the Sigma Chemical Co. (St. Louis, MO). The lipid soluble antifols, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl-pyrimidine (DDMP) and 2,4,-diamino-5-(3′,4′-dichlorophenyl)-6-*n*-hexyl-pyrimidine (DDHexP), were gifts from Dr. C. A. Nichol, Burroughs Wellcome Research Laboratories (Research Triangle, NC). 1,3-Diamino-9-chlorobenzo-f-quinazoline (PY490) was prepared in our laboratories [5]. The alpha- and gamma-DNA polymerases were purchased from Worthington Biochemicals (Freehold, NJ). Rat liver beta-DNA polymerase was a gift from Dr. Earl Baril, Worcester Foundation for Experimental Biology, Worcester, MA. Terminal deoxynucleotidyl transferase (TdT) was prepared in our laboratories [6].

Cell culture and isotopic labeling. L1210 murine leukemia cells were maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) medium supplemented with fetal bovine serum to 10% (v/v), glutamine (2 mM), and 2-mercaptoethanol (50 μM). For dThd uptake experiments, cells in early log phase growth, at a density of approximately 5×10^5 cells/ml, were collected by centrifugation at 800 *g* for 5 min and resuspended in RPMI 1640 medium with glutamine and 2-mercaptoethanol but without serum at a final cell density of 2×10^6 cells/ml. Five μl of a solution of DBMTX or MTX in dimethylsulfoxide (DMSO) such that the final drug concentration was 10^{-5} M were

* H. Lazarus, personal communication.

added to triplicate tubes containing 5 ml of the cell suspension. An equivalent amount of DMSO was added to control tubes. The final DMSO concentration in the medium was 0.1% (v/v). The cells were incubated at 37° in a 5% CO_2 atmosphere for 30 min. [^{3}H]dThd (50 μCi/ml, final concentration 0.2 M) was added to all tubes and incubation continued. At intervals, triplicate sample and control tubes were removed and [^{3}H]dThd uptake was halted by rapid cooling in an ice–water bath and dilution with ice-cold Earle's solution without glucose (EBSS). The cells were collected by centrifugation at 800 *g* and washed three times with EBSS. After the last washing, the cell pellet was resuspended in 1 ml of EBSS and the cells were lysed by adding 10 μl of 5% sodium dodecyl sulfate (SDS) solution. Macromolecular species were precipitated by the addition of 10 μl yeast RNA (1 mg/ml) as carrier, followed by 3 ml of 10% trichloroacetic acid (TCA); the mixture was allowed to stand for 30 min at 0°. The precipitate was removed by membrane filtration and counted, using a Searle Mark III scintillation counter. For measurement of [^{3}H]dThd uptake into the total acid-soluble fraction, 0.5-ml aliquots of the filtrate were removed and counted. The effect of 0.1% DMSO was tested in an experiment in which equivalent amounts of water were added to control tubes. There was no difference in the amount of thymidine uptake or incorporation in DMSO-treated versus control cells.

dThd and dThd nucleotide pools. For measurement of uptake into the individual dThd nucleotide pools, 10^7 cells, prepared as above, were exposed to 0.1 μM [^{3}H]dThd (50 Ci/mmole) for 30 min. Macromolecular species were precipitated and the acid-soluble fraction was obtained as above. The TCA was removed by 3-fold extraction with ether. The aqueous phase was concentrated to a volume of 0.5 ml by lyophilization and reconstitution with distilled water. dThd and dThd nucleotides were separated by one-dimensional thin-layer chromatography on PEI-cellulose coated sheets (F1440 PEI-LS254 Schleicher & Schull, Dassel, West Germany) developed with 1.0 M lithium chloride [7]. A mixture of authentic dThd, dTMP, dTDP and dTTP was co-chromatographed with the sample. The spots were located by ultraviolet light, cut out, eluted with 28% aqueous ammonia, and counted. The recovered radioactivity amounted to 95 per cent of the total counts applied to the plate. The number of cpm in each spot was always more than twenty times background.

For measurements of uptake of [^{14}C]Urd and [^{3}H]dUrd, a double-labeling experiment was carried out. Cells were preincubated with drug (DBMTX or MTX) for 45 min, following which 50 μl each of [^{14}C]Urd (0.01 μCi/μl), final concentration 0.32 μM) and [^{3}H]dUrd (1.0 μCi/μl, final concentration 0.32 μM) were added as above to triplicate sample and control tubes. Incubation was continued for an additional 30 min. The cells were washed lysed, and the acid-soluble and acid-precipitable fractions were isolated and counted as above.

Efflux of [^{3}H]dThd from intact cells was studied by first exposing cells to [^{3}H]dThd as above for 15 min prior to drug addition. Under these conditions, about 50 per cent of the total cell-associated label was contained within the acid-soluble fraction. The medium was replaced by fresh medium without dThd but with 10^{-5} M DBMTX. Cell-associated label was measured by removing aliquots of cells at appropriate time intervals, washing three times with ice-cold EBSS, and counting the final cell pellet.

Transport studies. For transport studies, the method

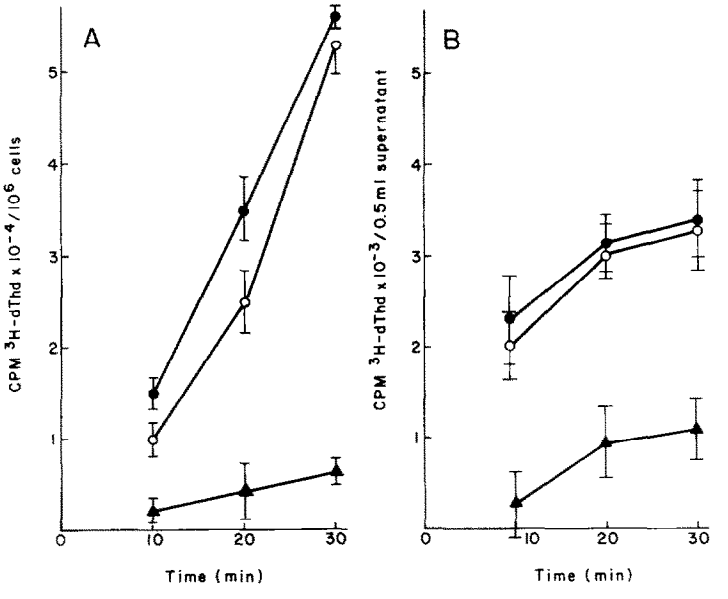

Fig. 2. Effects of 10^{-5} M DBMTX (▲——▲) and 10^{-5} M MTX (●——●) on uptake and incorporation of [^{3}H]dThd into L1210 cells. Untreated controls are shown as (○——○). Drug was added to early log phase cultures of 10^6 L1210 cells 30 min prior to time = 0. [^{3}H]dThd was then added and the (^{3}H) in 0.5-ml aliquots of the acid-soluble and the total acid-precipitable fractions was subsequently assayed at the indicated times. Data shown are from three experiments. Bars indicate ± 1 standard deviation. Panel A shows incorporation of [^{3}H]dThd into the acid-precipitable fraction. Panel B shows uptake of [^{3}H]dThd into the acid-soluble fraction.

of Plagemann *et al.* [8] was applied to monolayers of mouse 3T3 fibroblasts. The monolayers were preincubated for 10 min at 37° with 5 mM KCN and 5 mM iodoacetate in glucose-free medium which contained, in addition, 10^{-5} M DBMTX and 0.1% ethanol (drug-treated) or 0.1% ethanol alone (controls). This medium was then aspirated and replaced with the same medium containing either [^{3}H]dThd (2×10^3 cpm/pmole, 1.0 μM), [^{3}H]dUrd (2×10^3 cpm/pmole, 1.0 μM), or [^{3}H]Urd (2×10^3 cpm/pmole, 10 μM) as indicated. At various time periods, the medium was quickly aspirated and the monolayer was rapidly rinsed three times with ice-cold EBSS containing 50 μM persantin. After drying, the monolayers were scraped off the plate, transferred into scintillation vials, and their radioactivity was determined.

Enzyme studies. DNA polymerases were assayed according to established methods [9]. For inhibition studies, various concentrations of DBMTX in DMSO were incubated with each of the polymerases for 5 min at 37° prior to addition of other reaction components. Control tubes contained equivalent amounts of DMSO.

Thymidine kinase was obtained from the cytoplasmic fraction of L1210 cells [10], and from normal human liver [11]. Assays of enzyme activity were carried out by established methods [12].

Cell viability. Cell viability after DBMTX treatment for up to 1 hr was greater than 90 per cent as judged by Trypan blue exclusion. Furthermore, cells incubated in medium containing 10^{-5} M DBMTX for 30 min and returned to growth medium at a density of 10^5 cells/ml grew over the subsequent 48 hr to the same cell number and cell density as untreated cells.

RESULTS

As expected from previous studies [3], DBMTX produced a marked inhibition of [^{3}H]dThd incorporation into DNA of L1210 cells, as measured by acid-precipitable counts (Fig. 2A). In short-term pulse labeling experiments, cells treated with MTX have been shown to incorporate [^{3}H]dThd as well as or better than control cultures [3, 13], and this was confirmed in the present experiment. In contrast, treatment with 10^{-5} M DBMTX resulted in a 90 per cent or greater decrease in [^{3}H]dThd incorporation into DNA.

One explanation for the above observation is that DBMTX might inhibit DNA synthesis by acting directly as an inhibitor at the DNA polymerase level. However, at concentrations up to 10^{-5} M, DBMTX had no significant inhibitory effect on alpha, beta and gamma polymerases or TdT as isolated enzymes *in vitro*.

After preincubation with 10^{-5} M DBMTX for 30 min, cells were exposed to [^{3}H]dThd, and the amount of label found in the total intracellular acid-soluble fraction was determined. A pronounced decrease in the amount of [^{3}H]dThd taken up into this intracellular fraction was produced by 10^{-5} M DBMTX as shown in Fig. 2B. After 30 min, the acid-soluble fraction of DBMTX-treated cells contained only about 20 per cent of the amount of label in MTX-treated or untreated controls.

The marked suppression of dThd uptake into the combined thymidine phosphate pools which include dThd, dTMP, dTDP and dTTP could result from specific inhibition of one or more of the phosphorylation steps that convert dThd into dTTP. This possibility was eliminated when the uptake of [^{3}H]dThd into individual dThd nucleotide pools was examined. The results, presented in Table 1, are ratios of the amounts of tritium found in the various pools. If DBMTX produced blockage at one of the phosphorylation steps, the effect would be to decrease the ratio of tritium in pools distal to the block relative to the ratio of tritium in pools proximal to

Table 1. Uptake of [^{3}H]dThd into nucleotide and nucleoside pools in early log phase L1210 cells

Ratio*	Experiment 1		Experiment	
	Control	DBMTX	Control	DBMTX
$\frac{\text{dTTP}}{\text{dTDP}}$	3.16	3.04	2.98	3.19
$\frac{\text{dTDP}}{\text{dTMP}}$	3.48	2.93	3.18	3.26
$\frac{\text{dTMP}}{\text{dThd}}$	4.20	6.22	5.01	5.03
$\frac{\text{dTTP}}{\text{dThd}}$	41.2	55.6	47.6	52.4

* Ratios are the quotients of cpm ^{3}H associated with the chromatographic spots of the indicated dThd derivatives from DBMTX-treated (10^{-5}M) L1210 cells and untreated controls (see Materials and Methods). The total cpm ^{3}H recovered amounted to 95 per cent of the amount initially applied (~ 10,000 cpm/plate).

Table 2. Effect of MTX and DBMTX on the uptake and incorporation of [^{3}H]dUrd and [^{14}C]Urd into acid-soluble and -insoluble fractions of L1210 cells*

Drug	Concn. (M)	Uptake into acid-soluble fraction (% control)		Incorporation into acid-precipitable fraction (% control)	
		[^{3}H]dUrd	[^{14}C]Urd	[^{3}H]dUrd	[^{14}C]Urd
MTX	1.0×10^{-5}	4.8 ± 1.8	65.7 ± 10.7	2.8 ± 1.2	60.0 ± 13.6
	2.0 ± 10^{-5}	3.4 ± 1.3	39.0 ± 2.4	1.0 ± 0.6	40.3 ± 12.9
DBMTX	1.0 ± 10^{-5}	5.6 ± 2.1	51.6 ± 9.6	1.7 ± 0.72	31.2 ± 6.3
	2.0×10^{-5}	4.5 ± 0.2	24.9 ± 1.6	0.52 ± 1.6	10.7 ± 2.9

* Cells were exposed to drug at the indicated concentration for 30 min, following which [^{3}H]dUrd and [^{14}C]Urd were added. After 30 min, the acid-soluble and -insoluble fractions were assayed for ^{3}H and ^{14}C. Data shown are means ± S. D. for three experiments.

the block. These ratios are essentially unchanged by 10^{-5} M DBMTX compared to untreated controls.

Thymidine kinase from human liver [11], as well as from the cytoplasmic fraction of L1210 cells [10], was studied *in vitro*. DBMTX over the range of 10^{-9} to 10^{-5} M did not inhibit thymidine kinase from either source. In addition, 30 min of pretreatment of L1210 cells with 10^{-5} M DBMTX did not affect the amount of thymidine kinase activity extractable from the cells.

DBMTX might impair the ability of cells to retain dThd or phosphorylated dThd derivatives. To test this possibility, cells prelabeled with [^{3}H]dThd were exposed to 10^{-5} M DBMTX and the total amount of cell-associated tritium was followed over time. No significant loss of label occurred from either control or drug-treated cells.

The effect of DBMTX on the uptake and incorporation of other pyrimidine nucleosides was also studied. Table 2 shows the effects of DBMTX and MTX on the amount of [^{3}H]dUrd and [^{14}C]Urd found in acid-soluble and -insoluble fractions of L1210 cells. Both drugs caused diminutions in the labeling of total intracellular acid-soluble material, which includes the nucleosides and their phosphorylated derivatives. The effect was most pronounced in the case of [^{3}H]dUrd, where both DBMTX and MTX at 10^{-5} M produced reductions in uptake to approximately 5 per cent of control values.

DBMTX and MTX also caused significant decreases in the amount of [^{14}C]Urd taken up into the acid-soluble fraction, although these effects were less dramatic than for [^{3}H]dThd. Both drugs also produced diminutions in the incorporation of [^{3}H]dUrd and [^{14}C]Urd into the acid-precipitable fraction, as expected (Table 2).

The effect of DBMTX on transport of dThd, dUrd

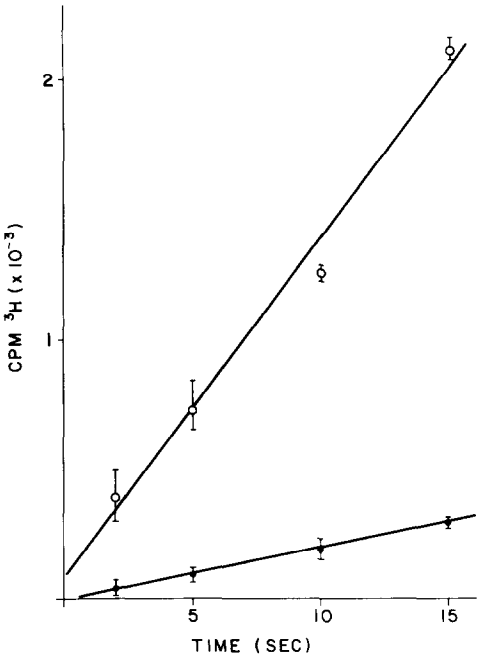

Fig. 3. Effect of DBMTX on dThd transport in 3T3 cells. 3T3 cells were grown as monolayers on 50 mm Petri dishes (10^6 cells/dish) and were treated with cyanide and iodoacetate as described under Materials and Methods. The monolayers were then exposed to [^{3}H]dThd (2×10^3 cpm/pmole, 1.0 μM) for the indicated time periods, rinsed rapidly as described in Materials and Methods, and transferred to scintillation vials and counted. Bars show range of values obtained for triplicate plates of each time point. Key: (○——○) controls; and (●——●) 10^{-5} M DBMTX.

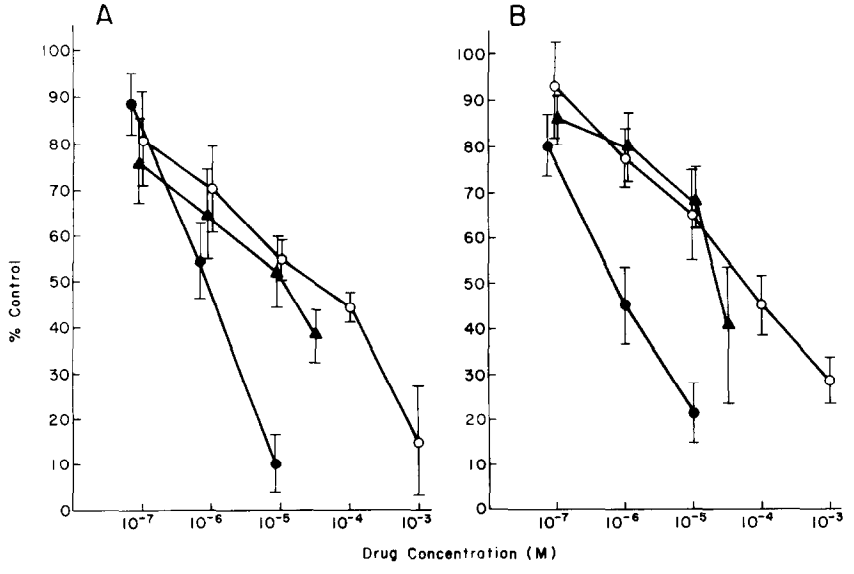

Fig. 4. Effects of DBMTX (●——●), DDMP (○——○), and PY490 (▲——▲) on uptake and incorporation of [^{3}H]dThd into L1210 cells, plotted as percent control cpm vs increasing drug concentration. Data shown are averages from at least three experiments; bars show ± 1 standard deviation. Panel A shows incorporation of [^{3}H]dThd into the acid-precipitable fraction. Panel B shows uptake of [^{3}H]dThd into the acid-soluble fraction.

and Urd was studied using short exposure times in 3T3 monolayer cells. Further metabolism of the nucleosides was inhibited by cyanide and iodoacetate pretreatment. Under these conditions, uptake of the tritiated nucleoside reflects membrane transport [8]. As shown in Fig. 3, DBMTX markedly inhibited the uptake of [^{3}H]dThd. Similar results were obtained for [^{3}H]Urd and [^{3}H]dUrd.

Several other lipid-soluble antifols which are not derivatives of MTX were also studied as inhibitors of dThd uptake and incorporation. Figure 4 shows the effects of DBMTX, DDMP and PY490 on acid-soluble and acid-precipitable [^{3}H]dThd in L1210 cells. Both of these drugs resemble DBMTX in that they produce decreases in [^{3}H]dThd uptake into soluble precursor pools as well as decreased incorporation into DNA. However, they are far less potent than DBMTX in this regard. Similar results were found with DDHexP (data not shown).

DISCUSSION

The present data demonstrate that DBMTX inhibits uptake of exogenous dThd into the total intracellular acid-soluble fraction of L1210 cells. Results obtained in the 3T3 fibroblast monolayer system suggest that this inhibition takes place at the level of dThd transport.

Outward leakage of phosphorylated derivatives does not occur to any significant extent. The absence of any effect of DBMTX on any of the DNA polymerases *in vitro* makes it highly unlikely that they are a target of DBMTX action *in vivo*. The decreased uptake of exogenous dThd into the total acid-soluble fraction is not due to a specific block at any one of the phosphorylation steps. This is supported by the constancy of the tritium ratios found in the various pools (Table 1), as well as the lack of any effect on thymidine kinase activity *in vitro*, or on the amount of thymidine kinase activity extractable from these cells.

DBMTX apparently produces decreased dThd incorporation into DNA by limiting the initial transport across the cell membrane. This process ordinarily occurs via facilitated diffusion, a saturable, nonconcentrative system that can be dissociated from subsequent metabolic steps (phosphorylation). The process is extremely rapid, equilibrium between extracellular and intracellular dThd being attained within seconds [14]. There is wide substrate specificity and, in fact, all nucleosides are probably transported by the same system [15], but with different efficiencies [8]. DBMTX might inhibit this process either by a fortuitous interaction with the presumed protein carrier molecule within the membrane or by deranging the lipid matrix–carrier protein interaction which is probably necessary for transport. Very little is known about the molecular nature of the nucleoside transport system. The nucleosides are mutually competitive inhibitors of each others' transport [8], while a number of structurally diverse compounds, including nitrobenzylthioinosine [16], papaverine [8], and persantin [8], are inhibitors of nucleoside transport which display more complex kinetics.

The effects of DBMTX on metabolic processes must also be considered. As mentioned above, our results imply that DBMTX does not produce a metabolic block at any of the phosphorylation steps. However, a generalized decrease in phosphorylation, such as might occur with ATP depletion [17], cannot be ruled out. Another possibility is that DBMTX may cause an increase in the levels of feedback inhibitors of thymidine phosphorylation, e.g. nucleoside triphosphates.

Both DBMTX and MTX produce dramatic decreases in the amount of [^{3}H]dUrd taken up into the acid-soluble fraction. Part of this decrease is probably due to a block in metabolism, subsequent to the nucleoside transport step, at the dUMP to dTMP conversion as a result of dihydrofolate reductase inhibition. DBMTX as well as its potential metabolites, MTX and MTX monobutyl esters, are inhibitors of this enzyme [4]. Since 80–90 per cent of the total intracellular pyrimidine nucleotide pool in growing cells consists of nucleoside triphosphates [18], metabolic blockade proximal to these is likely to lead to a decrease in total uptake. It is interesting to note that DBMTX and MTX are approximately equipotent in inhibiting [^{3}H]dUrd uptake and incorporation, although in comparison to MTX, DBMTX is a much less effective inhibitor of dihydrofolate reductase *in vitro* [4]. The relative extent to which membrane transport and metabolic effects of DBMTX contribute to the observed diminution of [^{3}H]dUrd uptake remains open.

Both MTX and DBMTX produce significant decreases in the amount of [^{14}C]Urd taken up into the acid-soluble fraction, with corresponding decreases in the amount incorporated into the acid-precipitable fraction. The same effect has also been found recently in MTX-treated 3T6 cells.* This observation is not explained by a metabolic block at the dUMP to dTMP conversion as described above, because only a small fraction of Urd is ordinarily reduced to dUrd and subsequently cycled through the thymidylate synthetase pathway. MTX has been shown previously to produce a decrease in Urd incorporation into acid-precipitable species; presumably this represents decreased RNA synthesis resulting from limitation of purine precursors [19]. Lack of purines would not be expected to decrease the pools of soluble pyrimidine ribonucleotide precursors however, and the origin of the effect remains unclear. As we have shown, DBMTX produces some inhibition of [^{14}C]Urd uptake through limitation of transport. The extent and nature of subsequent metabolic contributions to the effect are not known.

The other drugs studied also showed effects on thymidine uptake. The qualitative similarities among DBMTX, DDMP, DDHexP and PY490 in causing suppression of [^{3}H]dThd uptake and incorporation, despite quantitative differences, suggests a common mode of action.

The effect of DBMTX on dThd uptake is seen at concentrations which are readily attainable clinically with MTX. Whereas inability to take up exogenous thymidine *per se* would not be cytotoxic to cells, a concomitant inability to produce endogenous thymidylate could lead to "thymineless death". DBMTX has the potential to produce both of these effects through inhibition of dihydrofolate reductase and dThd transport. Inability to take up exogenous dThd would also have implications for use of dThd "rescue" after DBMTX treatment [20].

Finally, our studies demonstrate that a number of

* L. F. Johnson, personal communication.

antifols can affect the uptake of nucleosides into acid-soluble material and point out the importance of examining the components of this fraction in detail.

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