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Biochemical mechanisms in the Killmann experiment: critique of the deoxyuridine suppression test.

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Research Article

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Biochemical Mechanisms in the Killmann Experiment

CRITIQUE OF THE DEOXYURIDINE SUPPRESSION TEST

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ABSTRACT The degree of inhibition of [^{3}H]thymidine incorporation into DNA by exogenous deoxyuridine is assayed in a procedure known as the deoxyuridine suppression test. We report studies of the biochemical basis of this phenomenon in phytohemagglutinin-stimulated lymphocytes, which suggest that its mechanism has not been fully understood. Results show that inhibition by deoxyuridine is caused only in part by expansion of the intracellular pools of nonradioactive dTMP and dTTP, which dilutes the specific radioactivity of the [^{3}H]dTMP and [^{3}H]dTTP derived from [^{3}H]thymidine. Increased dTTP levels also inhibit thymidine kinase. In addition, thymidine kinase is competitively inhibited by intracellular deoxyuridine. Inhibition of thymidine kinase activity by both metabolites further decreases the specific radioactivity of [^{3}H]dTMP and [^{3}H]dTTP. Deoxyuridine also inhibits the incorporation of [^{3}H]deoxyadenosine and [^{3}H]deoxyguanosine into DNA in these cells.

Exogenous deoxyuridine still inhibits [^{3}H]thymidine incorporation in cells whose *de novo* thymidylate synthesis has been strongly inhibited by 5-fluorodeoxyuridine or methotrexate. In such drug-treated cells, exposure to high concentrations of exogenous deoxyuridine can partially overcome the inhibition of thymidylate synthetase with resulting increase in the severely depleted dTTP pools. This increase is associated with enhanced DNA synthesis, as measured by incorporation into DNA of labeled deoxyribonucleosides other than [^{3}H]thymidine.

We conclude that exogenous deoxyuridine has multiple effects on [^{3}H]thymidine incorporation, which must be considered in interpretations of deoxyuridine suppression test results.

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INTRODUCTION

Investigations of the role of cobalamins in DNA synthesis in animal cells have encountered problems of two kinds. First, studies in these cells disclosed no cobalamin-dependent enzymes other than 5′-deoxyadenosylcobalamin-dependent methylmalonyl CoA mutase (1–3) and methylcobalamin-dependent methyltetrahydrofolate (CH_3-H_4PteGlu)[1]: homocysteine methyltransferase (4), neither of which is directly on the pathway of DNA synthesis. Second, hypotheses seeking to establish indirect links between one or the other of these enzymes and DNA synthesis could be validated only by kinetic studies in intact tissues and for various reasons data from such studies have been either difficult to obtain or ambiguous.

In approaching this problem a number of workers have accumulated data that implicate cobalamin in DNA synthesis by giving it a role in the metabolic generation of H_4PteGlu from CH_3-H_4PteGlu. This concept, often termed the "methylfolate trap hypothesis," was first proposed in 1961 by Noronha and Silverman (5) to account for certain abnormalities of folate metabolism appearing to arise as sequelae of cobalamin deficiency in rats. The hypothesis, which was later extended to the situation in human cobalamin deficiency (6), suggested that accumulation of CH_3-H_4PteGlu would sequester folate in this form, thereby preventing its conversion to H_4-PteGlu, precursor of CH_2-H_4PteGlu, the coenzyme of thymidylate synthetase.

This hypothesis has occasioned much debate (7–11), some of it centering on the merits of an experiment by Killmann (12), which later workers made the basis of the deoxyuridine suppression test (13–17). With ra-

[1] *Abbreviations and nomenclature used in this paper*: CH_3-H_4PteGlu, N^5-methyltetrahydrofolate; CH_2-H_4PteGlu, N^5,N^{10}-methylenetrahydrofolate; d, deoxy(prefix); FdUrd, fluorodeoxyuridine; MTX, methotrexate; PBS, phosphate-buffered saline, pH 7.4; PCA, perchloric acid; PHA, phytohemagglutinin.

dioautographic methods Killmann showed that exogenous deoxyuridine strongly inhibits (or suppresses) the uptake of labeled thymidine into normal bone marrow cells, but does so by a smaller percentage in bone marrow cells from cobalamin-deficient subjects. Comparable results were subsequently reported in phytohemagglutinin (PHA)-stimulated lymphocytes (18–20).

This effect of exogenous nonradioactive deoxyuridine in normal cells has been attributed to a postulated increase in the size of the intracellular pool of nonradioactive dTMP and its product dTTP, which then (*a*) dilute the pools of [^{3}H]dTMP and [^{3}H]dTTP arising from [^{3}H]thymidine, and (*b*) inhibit thymidine kinase, the enzyme that catalyzes the conversion of [^{3}H]thymidine to [^{3}H]dTMP (13, 21). The result is substantial diminution in the labeling of DNA. The smaller percentage effect of exogenous deoxyuridine in cobalamin deficiency has been taken to mean that in that circumstance a smaller expansion of the dTMP pool arising from nonradioactive dUMP takes place. This result is the major item of evidence that conversion of dUMP to dTMP is in some way cobalamin-dependent.

This experiment in one or another modification has been widely used and has been recommended as a routine clinical test for cobalamin or folate deficiency (17, 22, 23). In view of the reliance placed on this methodology, it seemed appropriate to scrutinize it more closely. This paper reports such studies in PHA-stimulated lymphocytes in which thymidylate synthetase has been blocked by fluorodeoxyuridine (FdUrd) or methotrexate (MTX). The results show that the biochemical basis of deoxyuridine suppression has not heretofore been fully understood.

METHODS

Materials. The lymphocyte separation medium (density 1.077–1.080 g/ml) was supplied by Litton Bionetics, Kensington, Md.; the tissue culture medium RPMI 1640 with Hepes buffer (25 mM), glutamine (200 mM), and penicillin (10,000 U/ml)-streptomycin (10 mg/ml) by Grand Island Biological Co., Grand Island, N. Y.; the Bacto-phytohemagglutinin P by Difco Laboratories, Detroit, Mich.; preservative-free heparin by Fellows Medical Mfg. Co., Inc., Oak Park, Mich. MTX was obtained from Lederle Laboratories, Pearl River, N. Y.; and 5-fluorodeoxyuridine from Roche Diagnostics Div., Hoffman-LaRoche Inc., Nutley, N. J.

Thymidine and deoxyuridine were obtained from Sigma, St. Louis, Mo.; deoxyadenosine, deoxycytidine, and deoxyguanosine from Schwarz/Mann Div., Beckton, Dickinson & Co., Orangeburg, N. Y.; all nucleotides from Calbiochem-Behring Corp. American Hoechst Corp., San Diego, Calif. [*methyl*-^{3}H]Thymidine (2 or 50 Ci/mmol), [6-^{3}H]deoxyuridine (24.1 Ci/mmol), [5-^{3}H]deoxycytidine (22.6 Ci/mmol), [G-^{3}H]-dATP (13.6 Ci/mmol), and [*methyl*-^{14}C]thymidine (47.5 mCi/mmol) were from New England Nuclear Corp., Boston, Mass.; [8-^{3}H]deoxyadenosine (14.4 Ci/mmol), [8-^{3}H]deoxyguanosine (6.5 Ci/mmol), and [5-^{3}H]deoxyuridine (14 Ci/mmol) were from Schwarz/Mann Div. Poly-d(A-T) was supplied by Collaborative Research Inc., Waltham, Mass. and *Escherichia coli* DNA polymerase (5,000 U/mg) by Grand Island Biological Co. The DE 81 DEAE-filter paper disks (2.3 cm) and the GF/C microfiber glass filters (2.4 cm) were from Whatman Chemicals, Div. W & R Balston, Kent, England. Calf thymus DNA and dithiothreitol were supplied by Calbiochem-Behring Corp. Nucleoside solution added to lymphocyte cultures were sterilized by passage through a Millex filter unit (Millipore Corp., Bedford, Mass.), that was connected to a 12-ml disposable syringe.

Lymphocyte cultures. The method of Böyum (24) was followed except that normal heparinized blood (20 U/ml) was diluted 1:1 with tissue culture medium instead of saline. Harvested lymphocytes were washed, suspended in fresh medium, and counted in a Coulter Counter Model S (Coulter Electronics Inc., Hialeah, Fla.). Wright's-stained smears showed that over 90% of the nucleated cells were mononuclear forms. Cells were cultured in RPMI 1640 with 25 mM Hepes buffer (a medium that contains 1 mg folic acid/liter 5 μg vitamin B_{12}/liter, and no thymine or thymidine) with the following additions (final concentrations in parenthesis): glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml); heparin (20 U/ml); and an autologous diluted plasma (30% vol/vol) collected aseptically from the top of the gradient tubes into which diluted blood had been introduced. Hence, approximate actual plasma concentration in the medium was 10%. Cells were cultured with PHA (7.7 μg/ml) at a density of 5×10^5 cells/ml in a 1-ml vol in tightly capped 12 × 75 mm Falcon polystyrene tubes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 37°C. Cells were harvested at 72 h. After the culture period cells were counted in hemocytometers according to Wilson and Thomson (25).

Incorporation of radioactive precursors. Sterilized nucleoside solutions were added to replicate cultures 2 h before harvesting where indicated and radioactive nucleosides ([^{3}H]-thymidine 1 μCi/ml, 2 Ci/mmol, others 5 μCi/ml unless otherwise stated) 1 h before harvesting. Cells were harvested by adding 2 ml of cold phosphate-buffered saline (PBS), centrifuging at 1,000 *g* at 4°C for 10 min, and washing once with PBS. The pellet was suspended in water, 0.1 mg of carrier DNA, and 1:2 vol 0.6 N PCA were added (26). After centrifugation the supernatant fraction was removed (PCA-soluble fraction) and the precipitate was washed twice with 0.2 N perchloric acid (PCA). Except in incubations in which the labeled precursor was [^{3}H]thymidine, precipitates were treated overnight with 0.3 N KOH at 37°C to hydrolyze RNA. After the addition of 1:3 vol 2 N PCA, the resulting DNA-containing precipitate was washed twice with 0.2 N PCA and extracted twice with 1 N PCA, 20 min at 80°C. Tritium in the PCA-soluble fraction and in the resulting DNA hydrolysate was assayed in Aquasol (American Cyanamid Co., Bound Brook, N. J.) in a Beckman LS 150 scintillation counter of 30% efficiency (Beckman Instruments Inc., Fullerton, Calif.).

Thymidine kinase activity. Pellets of cells harvested from cultures by centrifugation at 1,000 *g* at 4°C for 10 min were resuspended in a buffer containing 0.02 M potassium phosphate, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, and 20% wt/vol glycerol (27). Cells were disrupted with an MSE ultrasonic disintegrator (Measuring & Scientific Equipment Ltd., London, England) at 20 kHz. Sonic extracts were centrifuged for 30 min at 27,000 *g* at 4°C.

Thymidine kinase was assayed by measurement of the phosphorylation of radioactive thymidine to thymidine phosphates, which are then bound to DEAE-cellulose paper (28, 29). The incubation mixture contained 100 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 5 mM ATP, 50 μM [*methyl*-^{14}C]thymidine (0.2 μCi/ml), and crude lymphocyte extract corresponding to $1–5 \times 10^6$ cells in a final volume of 0.5 ml. The mixture was incubated

in 12 × 75 mm disposable plastic tubes at 37°C. At intervals, 50-μl aliquots of the reaction mixture were applied to DEAE-cellulose paper disks, which were promptly immersed into 1 mM ammonium formate (20 ml/disk); residual thymidine was eluted from disks by washing twice for 10 min in 1 mM ammonium formate, once in water, and once in 95% ethanol for 5 min, respectively. Disks were dried for 10 min in counting vials by infrared lamp, cooled and assayed for radioactivity in 10 ml of Aquasol.

The above assay conditions were found to be optimal for thymidine kinase extracted from 72 h lymphocyte cultures and showed that the reaction rate was linear for at least 40 min over the range of crude extract volumes used in these assays. The enzyme activity is expressed as nanomoles of thymidine phosphorylated per minute per 10^7 cells.

Assay of dTTP pools. Cells were collected from cultures by centrifugation at 1,000 *g* at 4°C for 10 min. Nucleotides were extracted by suspension of the pellet in 1 ml of 60% methanol at −20°C (30). After 20 h at that temperature the supernatant fraction obtained after centrifugation at 1,000 *g* at 4°C for 10 min was collected and evaporated to dryness under vacuum at room temperature. Dried extracts were stored at −20°C and dissolved in sterile water immediately before assay.

Determination of dTTP pools was based on the method of Lindberg and Skoog (31). The reaction mixture contained in a final volume of 300 μl: 20 μmol Tris-HCl buffer, pH 8.0, 2 μmol $MgCl_2$, 0.5 μmol dithiothreitol, 3 pmol poly d(A-T), ~80 pmol [^{3}H]dATP (1–5 Ci/mmol), 0.1 U *E. coli* DNA polymerase, and either 0–10 pmol of dTTP for a standard curve or lymphocyte extract corresponding to 1–2 × 10^5 control cells or 1–2 × 10^6 drug-treated cells. Polymerase was added immediately before incubation at 37°C for 30 min. After incubation, acid-insoluble polymer was precipitated along with 0.05 mg of carrier DNA with 10% trichloracetic acid in 1% sodium pyrophosphate. The precipitate was collected with vacuum filtration on GF/C microfiber glass filters, washed with 9 ml 0.01 M HCl, and 95% ethanol. The filters were dried in scintillation vials under an infrared lamp, cooled, and assayed for radioactivity in 10 ml Aquasol. The standard curve was linear at least to 10 pmol of dTTP. Recovery of known amounts of dTTP added to the methanol extracts was 100% from control cell extracts and ~50% from drug-treated cell extracts at the amount of extract used in these studies. Final results, corrected for recovery losses, are expressed as picomoles per 10^6 cells.

Assay of dTMP pools. The dTMP pool was measured by an original method, which will be described in detail elsewhere.[2] In brief, the method employs a crude hemolysate that contains a kinase that catalyzes a substantial conversion of dTMP to dTTP (32) and that is approximately eight times more active with dTMP than with dUMP as substrate. Cells are extracted with 60% methanol as described above and assays are performed on fractions eluted with 0.3 M triethylammonium bicarbonate (33) from small Dowex-1 (Dow Corning Corp., Midland, Mich.) chromatograms of cell extracts. A reaction mixture containing the following ingredients in a final volume of 200 μl is incubated 30 min at 37°C: 1.7 μmol $MgCl_2$, 0.2 μmol ATP, 1.7 μmol 3-phosphoglycerate, 100 μl hemoyzate prepared by three freeze-thaw cycles of a 1:4 suspension of normal erythrocytes in PBS and 50 μl aqueous solution of an evaporated Dowex-1 eluate containing dTMP, but no dTDP or dTTP. After incubation the tube is immersed in boiling water for 2 min, chilled, and centrifuged for 20 min at 1,000 *g*. A 10-μl aliquot of the supernatant fraction is assayed for dTTP as described above. Standard curves are practicably useful functions of dTMP content from 0 to 3 nmol per incubation and duplicate determinations are in good agreement.

Although dUMP elutes from Dowex-1 in the same position as dTMP, the results of assays performed on extracts from control cells (incubated without deoxyuridine) were taken to represent dTMP alone, because the erythrocyte kinase used in the assay has a low affinity for dUMP and levels of dUMP in control cells are presumably low. However, intracellular dUMP pools rise in cells incubated with deoxyuridine and dUMP accumulating in this unusual circumstance interferes with the dTMP assay, which lacks absolute specificity for dTMP. To control this problem, the size of the dUMP pool was determined in these cells by incubating them with [5-^{3}H]-deoxyuridine of known specific radioactivity, which had been rigorously freed of trace contamination with [^{3}H]thymidine by thin-layer chromatography. Because [5-^{3}H]dUMP is stripped of its tritium on conversion to dTMP (34), assay of radioactivity in a Dowex-1 fraction chromatographically freed of deoxyuridine and containing only dUMP and dTMP provides a measure of dUMP in the fraction. The assumption that exogenous [5-^{3}H]deoxyuridine has the same specific radioactivity as the dUMP pool is supported by experiments showing direct proportionality between the concentration of exogenous deoxyuridine and the size of the dUMP pool. By reference to standard curves for dUMP in the DNA polymerase assay it is possible to assign to dUMP a fraction of final assay results and to obtain dTMP levels by difference. Experiments with known mixtures of dUMP and dTMP validate this procedure.

Estimation of intracellular deoxyuridine levels. Intracellular deoxyuridine pools were estimated in cells exposed to 1 mM [6-^{3}H]deoxyuridine by determining the amount of radioactivity in the PCA-soluble fraction and its distribution between deoxyuridine and its phosphorylated derivatives. Cells were incubated with 1 mM [6-^{3}H]deoxyuridine (30 Ci/mol) for 2 h, harvested, washed free of exogenous radioactivity with five washes of 2 ml PBS, and extracted with 0.2 N PCA. An aliquot of PCA-soluble fraction was assayed for radioactivity in Aquasol. The remainder of this fraction was freed of perchlorate with KOH. The supernatant fraction was concentrated under vacuum, applied to cellulose thin-layer chromatography plates, and developed in butanol:ammonium hydroxide:water (86:4:10). Separated compounds detected by ultraviolet light, were cut out, eluted with water, and assayed for radioactivity.

RESULTS

Standard experimental protocol in control lymphocytes. The standard deoxyuridine suppression test assays the percent effect of exogenous deoxyuridine on the incorporation of [^{3}H]thymidine into DNA. When this experiment was repeated in cells that we term "control cells" (i.e., PHA-stimulated lymphocytes harvested from 72-h cultures), the original observation of Killmann (12) was readily confirmed (Table I). Preincubation of cells with 1 mM deoxyuridine reduced (or supressed) the incorporation of [^{3}H]thymidine into DNA to 10% of the control value.

Systematic variations of the standard protocol. To explore the mechanism of the suppressive effect of deoxyuridine on [^{3}H]thymidine incorporation, we modi-

[2] W. S. Beck, K. Soren, and T-T. Pelliniemi. Manuscript in preparation.

TABLE I

Relative Effects of Exogenous Deoxyribonucleosides on Incorporation of Various Labeled Precursors into DNA in Control PHA-Stimulated Lymphocytes

Exogenous unlabeled deoxyribonucleoside*	Labeled DNA precursor				
	[^{3}H]dThd	[^{3}H]dUrd	[^{3}H]dAdo	[^{3}H]dCyd	[^{3}H]dGuo
	% of control				
None	100	100	100	100	100
dUrd	10.0±5.0 (18)	—	55.0±16.5 (6)	98.6±14.2 (6)	70.8±16.8 (6)
dCyd	38.9±5.2 (4)	38.7±11.1 (2)			
dAdo	26.6±9.8 (5)	16.8±1.9 (2)			
dGuo	145±38 (7)	144±57 (3)			
dThd	—	0.32±0.14 (3)			

* Concentration, 1 mM, except dGuo, which was 0.1 mM. Unlabeled deoxyribonucleosides were added at 70 h, labeled precursors at 71 h, and cells were harvested at 72 h. Number of experiments is given in parenthesis. Figures are mean±SD when more than two experiments were performed; mean and range when two experiments were performed.

fied the Killmann protocol systematically. First, we tested the effects on [^{3}H]thymidine incorporation of exogenous deoxyribonucleosides other than deoxyuridine (Table I). It was found that [^{3}H]thymidine incorporation was decreased to 26.6% of control levels by exogenous deoxyadenosine and to 38.9% by exogenous deoxycytidine. Exogenous deoxyguanosine increased [^{3}H]thymidine incorporation to 145% of control levels, no doubt reflecting a decrease in endogenous thymidylate synthesis.

Different labeled DNA precursors were then substituted for [^{3}H]thymidine. In an incubation protocol inverse to that of the standard Killmann experiment (Table I), exogenous 1 mM thymidine decreased [^{3}H]deoxyuridine to 0.32% of control levels.[3] Exogenous deoxyuridine decreased the incorporation of [^{3}H]deoxyadenosine, and [^{3}H]deoxyguanosine to 55.0%, and 70.8% of control levels, respectively without much effect on [^{3}H]deoxycytidine incorporation. These results indicate that (*a*) exogenous deoxynucleosides other than deoxyuridine also suppress [^{3}H]thymidine incorporation, and (*b*) deoxyuridine suppresses incorporation of labeled precursors other than [^{3}H]thymidine.

Effect of exogenous deoxyuridine on intracellular dTMP and dTTP pools. According to the conventional interpretation of the standard Killmann experiment, unlabeled dTMP arising by *de novo* synthesis from dUMP formed from exogenous deoxyuridine dilutes the radioactivity of the labeled dTMP formed from [^{3}H]thymidine. If this were the only explanation for deoxyuridine suppression (which decreases [^{3}H]thymidine to 10% of the control level), the size of the dTMP pool should increase 10-fold in the presence of deoxyuridine.

To test this inference we performed direct assays of the dTMP and dTTP pools in control cells incubated in the presence and absence of deoxyuridine. As shown in Table II, dTTP levels increased with rising concentrations of exogenous deoxyuridine. The standard deoxyuridine concentration (1 mM) increased dTMP 3.2-fold and dTTP 2.2-fold.

To verify this small increase in the dTMP pool relative to the anticipated 10-fold increase we analyzed the distribution of radioactivity arising from [^{3}H]thymidine in the three major thymidine phosphate esters (Table III). Application of these percentages to direct assay data on the dTTP pool showed that the 2.2-fold increase in the dTTP pool caused by exogenous deoxyuridine was associated with a 2.7-fold increase in the dTMP pool, in general agreement with direct dTMP assays. Added confirmation came from [^{3}H]thymidine uptake studies based on assays of radioactivity in the

TABLE II

Effects of Exogenous Deoxyuridine on dTMP and dTTP Pools in Control PHA-Stimulated Lymphocytes

Experiment	Addition*	dTMP		dTTP	
	mM dUrd	*pmol/10^6 cells*	*% of control*	*pmol/10^6 cells*	*% of control*
1	None (control)			19.0	100
	0.01			21.2	112
	0.1			27.4	144
	1.0			41.8	220
	10.0			63.6	335
2	None (control)	1.3	100	20.7	100
	1.0	4.3	320	44.6	215

* dUrd was added 2 h before harvesting the cells.

[3] Except where the use of [5-^{3}H]deoxyuridine is specified, all references to [^{3}H]deoxyuridine denote [6-^{3}H]deoxyuridine.

TABLE III
Distribution of Radioactivity from [³H]Thymidine among Thymidine Phosphate Derivatives

Addition	dTMP	dTDP	dTTP
	% of total radioactivity		
None	22.4±7.0	27.1±4.6	50.5±6.3
dUrd	23.9±8.3	32.2±6.1	43.9±3.4

Effect of exogenous deoxyuridine on the relative levels of dTMP, dTDP, and dTTP was estimated by measuring the distribution of radioactivity arising from [³H]thymidine in the three thymidine derivatives. After exposure of appropriate cultures to 1 mM deoxyuridine for 1 h, [³H]thymidine (10 μCi/ml, 20 Ci/mmol) was added and the incubation continued for another hour, after which the cells were extracted with 60% methanol. Thymidine phosphates were separated by anion exchange chromatography on poly(ethyleneimine)-cellulose thin-layer plates in 1 M LiCl. Spots detected by ultraviolet light were cut out and assayed for radioactivity in Aquasol. Values are mean±SD of six independent determination.

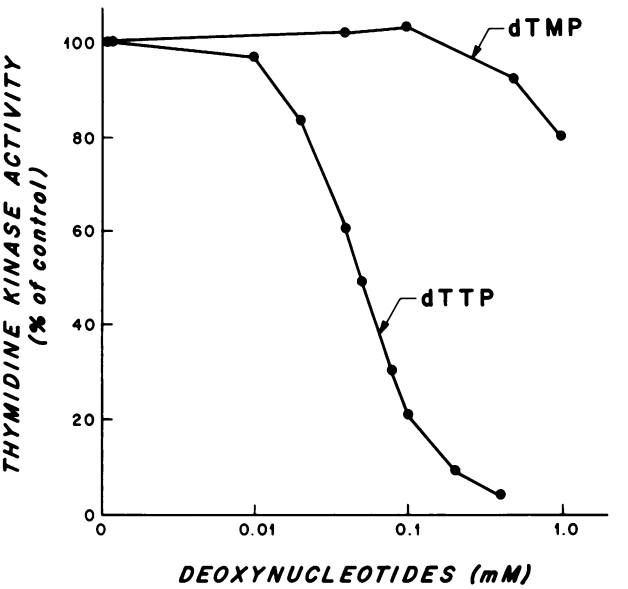

FIGURE 1 Inhibition of thymidine kinase extracted from normal PHA-stimulated human lymphocytes (72 h in culture) by dTTP and dTMP added to standard assay mixture.

PCA-soluble fraction. In cells incubated with 1 mM deoxyuridine PCA-soluble radioactivity deriving from [³H]thymidine was 19.4±3.1% (mean±SD) of the level in controls incubated without deoxyuridine. DNA radioactivity was 10.1±3.7% of corresponding controls. The ratio between PCA-soluble and DNA radioactivity was 2.1±0.6. This measure of diluted specific radioactivity further indicates the relatively moderate effect of exogenous deoxyuridine on the dTMP pool.

Effect of dTMP and dTTP on thymidine kinase activity. To evaluate the possibility that an increase in endogenous pools of dTMP or dTTP may influence [³H]thymidine incorporation by affecting the level of thymidine kinase activity, the experiments summarized in Fig. 1 were performed. It is seen that thymidine kinase was strikingly inhibited by dTTP. When dTTP and substrate thymidine were present in equal concentrations (50 μM), thymidine kinase was inhibited by 40%. Substrate concentrations of dTMP were not inhibitory although there was a slight decrease in thymidine kinase activity at higher dTMP concentrations. In concentrations of 1 mM dTDP and dUMP had no effect on thymidine kinase activity. We conclude that exogenous deoxyuridine decreases [³H]thymidine incorporation into DNA by diluting the specific activity of [³H]dTMP derived from [³H]thymidine and by promoting the inhibition of thymidine kinase activity by dTTP, thereby decreasing entry of isotope into the dTMP pool.

Effect of exogenous deoxyribonucleosides on precursor incorporation in cells in which de novo thymidylate synthesis has been blocked by FdUrd or MTX. As noted above, the suppressive effect of deoxyuridine on [³H]thymidine incorporation in control cells in the standard Killmann experiment has been considered to reflect in large part the extent to which unlabeled dUMP (arising from exogenous deoxyuridine) is converted to unlabeled dTMP. To test this concept we performed the standard deoxyuridine suppression test and variations thereof in cells in which *de novo* dTMP synthesis had been blocked by exposure to FdUrd or MTX. We term these as "drug-treated cells."

It was first necessary in these experiments to demonstrate that these metabolic antagonists did in fact inhibit *de novo* dTMP synthesis. Evidence on that question is shown in Table IV. Cells exposed to 100 μM FdUrd or MTX for 24 or 4 h before harvesting were almost completely incapable of incorporating [³H]deoxyuridine into DNA. It is of interest that FdUrd and MTX enhanced [³H]thymidine incorporation under all of the incubation conditions tested.

The standard Killmann experiment was then performed with cells blocked in *de novo* dTMP synthesis. As shown in Fig. 2, when 100 μM FdUrd or MTX was added to PHA-stimulated lymphocyte cultures 4 h before harvesting the blocking effect of exogenous deoxyuridine on [³H]thymidine incorporation into DNA was smaller than in control cells (i.e., not exposed to deoxyuridine). Nonetheless, exogenous deoxyuridine still decreased [³H]thymidine incorporation significantly. The degree of inhibition produced by deoxyuridine depends on the concentration and exposure time of FdUrd and MTX (Fig. 3). Irrespective of these small variations, it appears that a suppressive effect of deoxyuridine on [³H]thymidine incorporation still

TABLE IV

Effects of FdUrd and MTX on Incorporation of [³H]Deoxyuridine and [³H]Thymidine into DNA

Drug added	Labeled DNA precursor	
	[³H]dUrd	[³H]dThd
μM	% of control	
None (control)	100	100
FdUrd		
100, 24 h	0.13±0.05 (3)	130±46 (4)
0.1, 24 h	31.4±20.6 (3)	335±61 (5)
100, 4 h	0.24±0.22 (4)	201±77 (6)
0.1, 4 h	11.1±3.6 (7)	237±63 (4)
MTX		
100, 24 h	0.18±0.07 (3)	200±60 (3)
0.1, 24 h	2.5±0.4 (2)	204±13 (2)
100, 4 h	0.35±0.16 (3)	202±66 (4)
0.1, 4 h	61.2±13.0 (4)	137±23 (2)

Number of experiments is given in parentheses. Figures are mean±SD when more than two experiments were performed; mean and range when two experiments were performed.

takes place despite the nearly complete inhibition of *de novo* thymidylate synthesis. We again conclude that the level of *de novo* thymidylate synthesis is not the only biochemical parameter affecting results in the deoxyuridine suppression test.

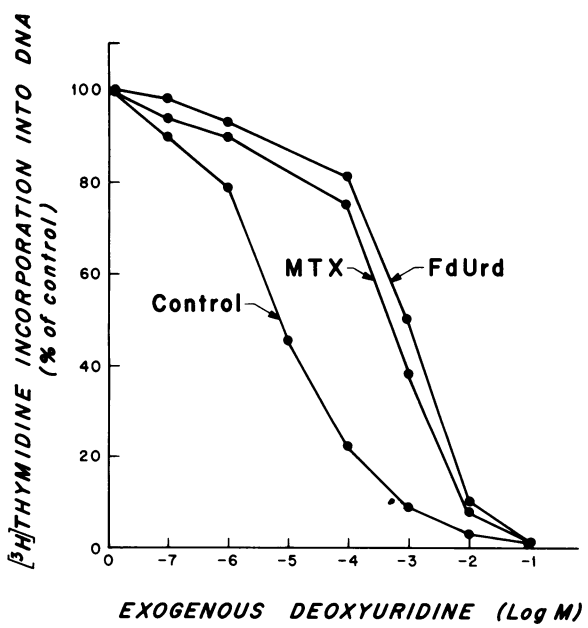

FIGURE 2 The effect of various exogenous dUrd concentrations on [³H]thymidine incorporation into DNA in normal and drug-treated (100 μM, for 4 h before harvesting) PHA-stimulated human lymphocytes.

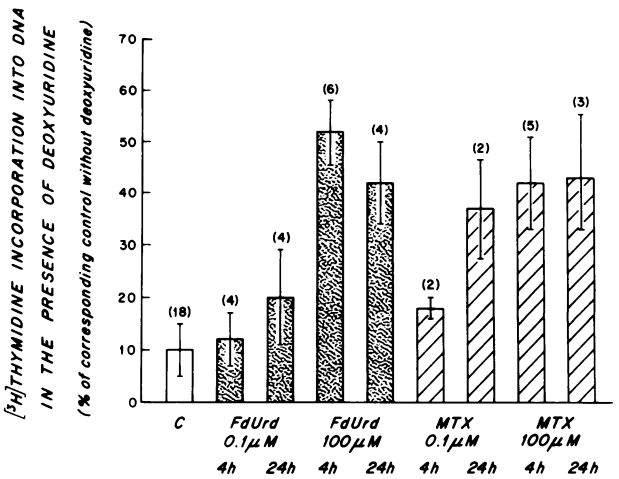

FIGURE 3 The effect of exogenous deoxyuridine (1 mM) on [³H]thymidine incorporation into DNA in normal (C) and drug-treated, PHA-stimulated human lymphocytes. Numbers of observations are given in parentheses. Values are mean±SD when more than two experiments were performed; mean and range when two experiments were performed.

Evidence that other exogenous deoxyribonucleosides suppress DNA synthesis in drug-treated cells. Data on the effect of DNA synthesis of exogenous deoxyribonucleosides other than deoxyuridine are shown in Table V. It is seen that exogenous deoxyadenosine suppressed the incorporation of both [³H]thymidine and [³H]deoxyuridine to about 17–27% of the control level, whether or not *de novo* thymidylate synthesis had been blocked by drugs. We assume this reflects the inhibitory effect on ribonucleotide reductase of an increasing intracellular concentration of dATP (35). Exogenous deoxycytidine was more inhibitory to [³H]-thymidine and [³H]deoxyuridine incorporation in control cells than in drug-treated cells. Presumably, the actions of deoxycytidine mimic those of deoxyuridine, of which it is a precursor (36). Exogenous deoxyguanosine stimulated [³H]thymidine and [³H]deoxyuridine incorporation in control cells and inhibited it slightly in drug-treated cells under most of the conditions studied.

These results show that the incorporation of labeled precursors into DNA is influenced by a number of factors that are unrelated to the level of *de novo* thymidylate synthesis.

Studies of the mechanism of deoxyuridine suppression of [³H]thymidine incorporation in cells blocked in de novo thymidylate synthesis. In seeking to account for the observation that exogenous deoxyuridine suppresses [³H]thymidine incorporation even when dTMP synthesis is blocked we considered two hypotheses: (*a*) by raising the intracellular concentration of dUMP exogenous deoxyuridine may stimulate *de novo* thymidylate synthesis, even though thymidylate syn-

TABLE V

Relative Effects of Various Exogenous Deoxyribonucleosides on DNA Synthesis

Added drug	Labeled precursor	Exogenous unlabeled deoxyribonucleoside*				
		None	dAdo	dCyd	dGuo	dThd
μM						
None (control)	[^{3}H]dThd	100	26.6	38.9	145	
FdUrd						
100, 24 h	[^{3}H]dThd	100	22.0	63.8	89.3	
0.1, 24 h	[^{3}H]dThd	100	29.7	93.3	69.0	
100, 4 h	[^{3}H]dThd	100	22.7	78.7	124.5	
0.1, 4 h	[^{3}H]dThd	100	17.2	65.1	77.5	
MTX						
100, 4 h	[^{3}H]dThd	100	25.2	41.9	90.6	
0.1, 4 h	[^{3}H]dThd	100	16.5	59.9	84.7	
None (control)	[^{3}H]dUrd	100	16.8	38.7	144	0.32
FdUrd						
100, 4 h	[^{3}H]dUrd	100	26.6	72.6	50.0	2.0

* Concentration 1 mM except dGuo, which was 0.1 mM. Experimental procedures are specified in Table I and in Methods.

thetase has been substantially inhibited by FdUrd or MTX, and (*b*) thymidine kinase, the first enzyme on the salvage pathway that converts [^{3}H]thymidine to [^{3}H]dTMP, may be inhibited not only by dTTP but also by deoxyuridine. Experiments were performed to test these postulations.

Several types of evidence indicated that exogenous deoxyuridine increases *de novo* thymidylate synthesis in the presence of FdUrd or MTX. In one experiment the effect of deoxyuridine on thymidylate synthesis was tested in the presence and absence of FdUrd or MTX by assaying its effects on the incorporation of [^{3}H]deoxyadenosine, [^{3}H]deoxycytidine, and [^{3}H]deoxyguanosine, the assumption being that any enhancement of their incorporation in drug-treated cells would reflect a deoxyuridine-induced increase in the availability of dTTP. The results in Table VI show that in drug-treated cells in which incorporation of these labeled deoxyribonucleosides was decreased, exogenous deoxyuridine regularly stimulates their incorporation into DNA relative to incorporation rates in the absence of deoxyuridine. These results are in contrast with the suppressive effect of deoxyuridine on the incorporation of [^{3}H]deoxyadenosine and [^{3}H]deoxyguanosine in control cells (i.e., cells that were not treated with FdUrd or MTX). Deoxyuridine had little effect on [^{3}H]-deoxycytidine incorporation in control cells but increased it moderately in drug-treated cells. These results suggest that one effect of deoxyuridine in drug-treated cells is to enhance *de novo* dTMP synthesis despite the actions of thymidylate synthetase inhibitors.

The conclusion that a high concentration of exogenous deoxyuridine can substantially overcome the block caused by FdUrd or MTX received further support from studies of the effect of increasing concentrations of exogenous deoxyuridine on the rate of [^{3}H]-deoxyuridine incorporation into DNA (Fig. 4). Because of the low isotope incorporation rates with high drug concentrations this effect could be studied only at low drug concentrations. It is seen that with rising concentrations of exogenous deoxyuridine incorporation rates of [^{3}H]deoxyuridine in both control and FdUrd-treated lymphocytes increased. Similar results were obtained with MTX-treated cells (0.1 μM). The relative increase was far greater in drug-treated cells, almost reaching the control level at 100 μM deoxyuridine in FdUrd-treated cells (and at 1 μM deoxyuridine in MTX-treated cells.)

Direct evidence of the ability of exogenous deoxyuridine in adequate concentrations to overcome the blocking effect of FdUrd or MTX on *de novo* thymidylate synthesis was found in studies on the dTTP pools under similar conditions. It is seen in Table VII that FdUrd and MTX both decreased the dTTP pool substantially, the extent of the decrease ranging from 97.7% (with FdUrd, 100 μM, 4 h) to 75.0% (FdUrd, 0.1 μM, 24 h) and from 93.8% (MTX, 0.1 μM, 24 h) to 44.3% (MTX, 0.1 μM, 4 h). However, addition of 1 mM deoxyuridine in every case substantially increased the dTTP pool, effects ranging from five- to sevenfold increases in the most inhibited cells to full or nearly full restoration to normal of the dTTP pool in less severely inhibited cells. This increase in the dTTP pool decreases [^{3}H]thymidine incorporation by inhibition of thymidine kinase with resulting decrease in specific activity of the radiolabel, whereas its stimulatory effect on DNA synthesis tends to increase the deoxyuridine suppression test value.

TABLE VI

Evidence that Exogenous Deoxyuridine Enhances Incorporation into DNA of [^{3}H]Deoxyadenosine [^{3}H]Deoxycytidine, and [^{3}H]Deoxyguanosine in Cells in which De Novo Thymidylate Synthesis is Substantially Blocked

Drug added	Nucleosides in incubation					
	[^{3}H]dAdo (control A)	[^{3}H]dAdo + dUrd*	[^{3}H]dCyd (control A)	[^{3}H]dCyd + dUrd*	[^{3}H]dGuo (control A)	[^{3}H]dGuo + dUrd*
None						
Relative dUrd effect	100 →	55.0±16.5	100 →	98.6±14.2	100 →	70.8±16.8
FdUrd, 100 μM, 24 h						
Percentage of control A	3.9	11.0	79.5	97.0	9.9	29.3
Relative dUrd effect	100 →	283	100 →	122	100 →	296
FdUrd, 100 μM, 4 h						
Percentage of control A	9.1	16.7	16.0	17.3	11.6	25.3
Relative dUrd effect	100 →	184	100 →	108	100 →	218
FdUrd, 0.1 μM, 4 h						
Percentage of control A	22.5	59.9	13.6	84.3	30.2	91.8
Relative dUrd effect	100 →	266	100 →	620	100 →	304
MTX, 100 μM, 24 h						
Percentage of control A	21.2	95.2	38.6	112	39.1	61.4
Relative dUrd effect	100 →	449	100 →	291	100 →	157
MTX, 0.1 μM, 24 h						
Percentage of control A	51.2	60.4	59.4	127	28.2	51.0
Relative dUrd effect	100 →	118	100 →	213	100 →	181
MTX, 100 μM, 4 h						
Percentage of control A	18.8	60.5	7.5	20.7	13.8	27.7
Relative dUrd effect	100 →	322	100 →	276	100 →	201

* 1 mM.

Experimental procedures are specified in Table I and in Methods.

To test the second hypothesis outlined above, which seeks to explain deoxyuridine suppression of [^{3}H]thymidine incorporation in cells inhibited in *de novo* thymidylate synthesis, we studied thymidine kinase activity in control and drug-treated cells with and without exogenous deoxyuridine. Thymidine kinase activity increased 100-fold in the course of a 72-h culture of PHA-stimulated lymphocytes. Activity increased further when such cells were treated with FdUrd or MTX for 24 h (Table VIII). However, short-term exposures to these drugs did not increase thymidine kinase activity. The studies shown in Table IX indicate that thymidine kinase from control cells was inhibited by deoxyuridine. Inhibition was competitive, the enzyme having a greater affinity for thymidine than for deoxyuridine. Other deoxyribonucleosides, uridine and uracil had negligible effects. Enzyme extracted from drug-treated cells was similarly inhibited by deoxyuridine. After 2 h exposure to 1 mM deoxyuridine the intracellular deoxyuridine content varied from 140 to 240 pmol/10^6 cells, which is 50–100 times higher than the estimated intracellular thymidine pool (37). This further suggests that intracellular deoxyuridine inhibits thymidine kinase in cells exposed to a high concentration of exogenous deoxyuridine.

DISCUSSION

We have shown that exposure of normal PHA-stimulated lymphocytes to exogenous deoxyuridine (1 mM) expands the dTMP and dTTP pools only two- to three-fold (Tables II, III, and VII). (These data contrast with results obtained under different conditions [38] showing no consistent rise in the dTTP pool after exposure of cells to deoxyuridine). We conclude that an increase in the thymidylate pools is indeed one of the biochemical events leading to suppression of [^{3}H]thymidine incorporation by exogenous deoxyuridine. Although the DNA polymerase used in the assay of dTTP does not discriminate between dTTP and dUTP (39), normal cells contain a specific dUTPase that effectively hydrolyses dUTP (40) and we doubt that dUTP is present. We also found in cells exposed to [5-^{3}H]deoxyuridine that no radioactivity appears in DNA from control and drug-treated cells (41), even when cells had been incubated with 20 mM uracil to inhibit uracil-DNA glycosylase (42, 43). This is further evidence against the accumulation of dUTP.

The fact that deoxyuridine still inhibited [^{3}H]thymidine incorporation when thymidylate synthetase was inhibited with FdUrd of MTX (Fig. 2) implied

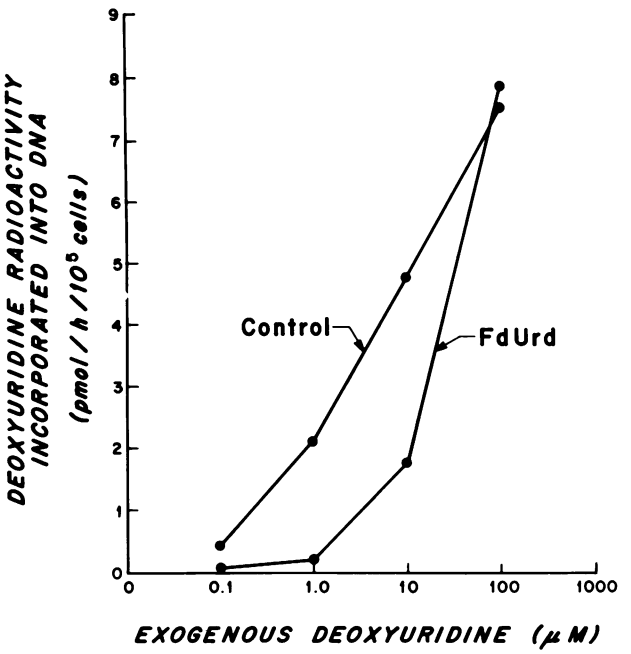

FIGURE 4 Effect of increasing amounts of exogenous deoxyuridine on the incorporation of [^{3}H]deoxyuridine into DNA in normal and FdUrd-treated human PHA-stimulated lymphocytes. Cells were exposed to FdUrd (0.1 μM) for 4 h and incubated in the presence of 0.1–100 μM deoxyuridine (0.5–50 μCi/ml culture) for 1 h. Calculation of the amount of radioactivity from [^{3}H]deoxyuridine incorporated into DNA is based on the specific activity of exogenous deoxyuridine. Results are means of three independent determinations.

TABLE VII

Evidence from Measurements of dTTP Pools that Exogenous Deoxyuridine Can in Part Overcome a Drug-induced Block in De Novo Thymidylate Synthesis

Drug added	dUrd added	Number of experiments	dTTP*
μM			*pmol/10⁶ cells*
None (controls)	0	9	17.6±3.5
	+	9	35.2±7.6
FdUrd			
100, 4 h	0	3	0.4±0.4 (0.1–0.9)
	+	3	3.1±2.0 (0.8–4.7)
0.1, 4 h	0	6	3.4±2.9 (0.7–8.0)
	+	6	23.9±14.3 (4.6–42.2)
0.1, 24 h	0	3	4.4±4.0 (0.3–8.3)
	+	3	20.0±12.3 (6.1–29.3)
MTX			
100, 4 h	0	4	2.1±2.1 (0.1–5.1)
	+	4	6.4±2.7 (2.7–9.1)
0.1, 4 h	0	3	9.8±3.7 (6.1–13.5)
	+	3	18.3±9.5 (7.4–25.1)
0.1, 24 h	0	3	1.1±1.9 (0–3.3)
	+	3	5.2±1.5 (3.6–6.5)

* Figures are mean±SD. The range is given in parenthesis. Concentration of dUrd added 2 h before harvesting was 1 mM.

that other mechanisms operate in the deoxyuridine suppression test and led to experiments confirming that deoxyuridine as well as dTTP can inhibit thymidine kinase (44, 45). On a molar basis dTTP is a more potent inhibitor than deoxyuridine, but the intracellular deoxyuridine level in cells exposed to 1 mM exogenous deoxyuridine is four to seven times higher than the dTTP level. Thus, exogenous deoxyuridine in high concentrations probably inhibits thymidine kinase at least as much as does the twofold increase in the dTTP pool and such inhibition must be part of the explanation for deoxyuridine suppression of [^{3}H]thymidine incorporation. Because thymidine kinase and thymidine transport into the cell are not linked (46), inhibition of thymidine transport by exogenous deoxyuridine is still another mechanism for decreasing the specific radioactivity of [^{3}H]dTMP.

Thymidine kinase activity increases in bone marrow cells of patients with folate or cobalamin deficiency and repletion of these cells with folic acid or cobalamin significantly decreases this activity (47–49). We infer that such a fall in kinase activity may significantly contribute to the increased deoxyuridine suppression of [^{3}H]thymidine incorporation seen in vitamin-treated megaloblastic cells in vivo (19).

We found, in addition, that high exogenous deoxyuridine concentrations can partially overcome the inhibition of thymidylate synthetase (Fig. 4). In those experiments the exposure of cells to FdUrd or MTX was brief enough to avoid the induction of thymidylate synthetase reported after long exposures to MTX (50–

TABLE VIII

Effects of FdUrd and MTX on Thymidine Kinase Activity

Additions	Number of experiments	Thymidine kinase activity*	
μM		*nmol/min/10⁷ cells*	*% of control*
None (control)	5	0.67±0.19	100
FdUrd			
0.1, 24 h	4	2.33±0.69	345±78
0.1, 4 h	5	0.77±0.26	115±21
100, 4 h	4	0.87±0.30	128±28
MTX			
0.1, 24 h	4	1.40±0.64	198±54
0.1, 4 h	5	0.63±0.18	95±18
100, 4 h	4	0.70±0.25	103±21

* Figures are mean±SD.

TABLE IX
Effect of Nucleosides on Thymidine Kinase Activity

Addition*	Thymidine kinase activity
mM	*% of control*
None (control)	100
dUrd, 50	4.8
dUrd, 10	16.9
dUrd, 1.0	57.7
dUrd, 0.1	84.1
dUrd, 0.01	96.7
dUrd, 0.001	97.3
dAdo, 1.0	95.2
dCyd, 1.0	98.7
dGuo, 0.1	103.4
Urd, 10	88.6
Urd, 1.0	98.0
Urd, 0.1	90.9
Urd, 0.01	101.7
Uracil, 1.0	80.2

* Nucleosides were added to standard assay mixture in the beginning of 30-min incubation.

53). Present understandings of the mechanisms of action of FdUrd and MTX (54–57) suggest that the inhibitory effects of these drugs on thymidylate synthetase is not reversed by simple competition with the products of exogenous deoxyuridine. In our studies, FdUrd was added at least 2 h before deoxyuridine to allow covalent binding of FdUMP to thymidylate synthetase. These considerations make it likely that high concentrations of deoxyuridine can cause a strongly inhibited thymidylate synthetase to catalyze some conversion of dUMP to dTMP. The resulting increase in thymidylate pools (Table VI), albeit a small one, then increases DNA synthesis in cells previously starved for thymidylate (Table VI). Further studies are needed to show whether exogenous deoxyuridine similarly enhances DNA synthesis in folate and cobalamin-deficient cells.

The suppressive effect of exogenous deoxyuridine on [^{3}H]thymidine incorporation is not specific, inasmuch as [^{3}H]deoxyadenosine and [^{3}H]deoxyguanosine incorporation are also suppressed by deoxyuridine (Table I). Because exogenous deoxyuridine is not a general inhibitor of DNA synthesis, cell growth being unaffected by prolonged exposure to deoxyuridine (not shown in results), this effect of exogenous deoxyuridine might be mediated by an allosteric effect of dTTP on ribonucleotide reductase that promotes reduction of GDP and ADP (35, 58). The resulting increases in deoxyribonucleotide pools may dilute the radiolabel and thus decrease the incorporation of [^{3}H]deoxyadenosine and [^{3}H]deoxyguanosine without affecting [^{3}H]-deoxycytidine incorporation into DNA. In FdUrd and MTX treated cells exogenous deoxyuridine increases incorporation of [^{3}H]deoxyadenosine, [^{3}H]deoxyguanosine, and [^{3}H]deoxycytidine probably by increasing total DNA synthesis.

Use of the deoxyuridine suppression test has been recommended for two purposes: as a clinical diagnostic test for folate and cobalamin deficiency and as a tool for the investigation of *de novo* thymidylate synthesis (17, 22, 23, 59). Claims for the validity of the test have been based mainly on data showing the correction of abnormal deoxyuridine suppression test values by exogenous folates or cobalamins in vitro (13). Our results suggest that these vitamins (and other in vitro additions) correct only one of the metabolic mechanisms responsible for abnormal deoxyuridine suppression. The direct effects of deoxyuridine on thymidine kinase and presumably thymidine transport would remain unaffected. This might explain the frequent and unpredictable situation in which deoxyuridine suppression values are only partially corrected by in vitro supplements. If exogenous folates and cobalamin completely corrected deoxyuridine suppression in megaloblastic cells, these cells would undoubtedly be caused to show stronger suppression of [^{3}H]thymidine incorporation by deoxyuridine than normal cells, because their dUMP pool would be increased from both increased phosphorylation of exogenous deoxyuridine to dUMP and increased formation of dUMP from dCMP by dCMP deaminase, the activity of which is enhanced by lack of inhibition by dTTP (60). They also would contain increased amounts of thymidylate synthetase apoenzyme (61).

In sum, we consider the effects of exogenous deoxyuridine too complex and diverse to permit use of the suppression test as a reliable tool for investigating *de novo* thymidylate synthesis in all circumstances and we disagree with recent assertions that the deoxyuridine suppression test is the "final solution" to the problem of diagnosing cobalamin deficiency (62).

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