Studies Relating to the Mode of Action of Methotrexate

III. Inhibition of Thymidylate Synthetase in Tissue Culture Cells and in Cell-Free Systems

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SUMMARY

The effect of methotrexate on thymidylate synthesis in mammalian cells *in vitro* has been studied by using the incorporation of deoxyuridine-6-^{3}H into DNA as an assay for thymidylate synthesis. The results are consistent with the interpretation that inside the living cell methotrexate can inhibit thymidylate synthetase directly. In studies on the effects of this drug on thymidylate synthetase activity in cell-free preparations, using both mammalian and bacterial enzyme sources, inhibition of the synthetase was observed. The inhibition yielded Lineweaver-Burk plots that were uncompetitive with respect to deoxyuridylic acid and competitive with respect to tetrahydrofolate. Values of 1.4 ($\pm$0.1) $\times$ 10^{-5} M and 4.3 ($\pm$1.2) $\times$ 10^{-6} M were measured for $K_{i,\text{intercept}}$ and $K_{i,\text{slope}}$, respectively. The possible significance of these findings is discussed.

INTRODUCTION

The results in the accompanying report (1) suggest that methotrexate or some metabolic product of it is capable of directly inhibiting either thymidylate synthetase or the folate coenzyme interconversion enzymes in mammalian cells. Because this conclusion contradicts currently accepted models for the action of MTX[2] (2), and because the inhibition of thymidylate synthetase may be the basis for its chemotherapeutic action (3), we have studied its effects on partially purified thymidylate synthetase and on the incorporation of tritium-labeled deoxyuridine by way of thymidylic acid into DNA (4). Both the cellular incorporation studies and the studies using partially purified enzymes extracted from both mammalian and bacterial cells indicated that thymidylate synthetase is directly inhibited by MTX.

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[2] The abbreviation used is: MTX, methotrexate.

MATERIALS AND METHODS

Radioactive labeling experiments. Labeling experiments were carried out on cultures of L-cells, hamster embryo cells, and mouse embryo cells.

Deoxyuridine-6-^{3}H (3.2 Ci/mmole) and deoxyuridine-5-^{3}H (17.0 Ci/mmole) were purchased from Schwarz BioResearch, Inc., Orangeburg, New York. Both ^{3}H-thymidine (17.1 Ci/mmole) and ^{3}H-uridine (2.18 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, England. Methotrexate and N^5-formyltetrahydrofolate were purchased from Lederle Products Department, Cyanamid of Canada, Ltd., Montreal.

Prior to labeling, L-cells were grown in 200-ml suspension cultures in growth medium CMRL 1066 (5), from which the nucleosides and cofactors had been omitted

but to which had been added 10% (v/v) exhaustively dialyzed fetal calf serum and 10^{-5} g of deoxyadenosine per milliliter. Under these conditions all of the MTX-induced inhibitory effects on cell proliferation are due to inhibition of thymidylate synthesis (1). Aliquots, usually 5 ml, of exponentially growing cells at a concentration of approximately 1.6 x 10^5 cells/ml were put into 10-ml plastic tubes (Falcon Plastics, Los Angeles) and treated with MTX and/or N^5-formyltetrahydrofolate. In some experiments the cells were loaded with N^5-formyltetrahydrofolate by incubation for 15 min before the addition of MTX. The MTX and N^5-formyltetrahydrofolate were added in 0.10-ml volumes of phosphate-buffered 0.9% NaCl solution (6). ^{3}H-Labeled deoxyuridine was added to a final activity of 1.2 μCi/ml, and the cultures were incubated for specified periods at 37°. The incorporation was terminated by adding 5 ml of ice-cold phosphate-buffered 0.9% NaCl containing 10^{-4} g of thymidine (General Biochemicals) per milliliter and 10^{-5} g of fluorodeoxyuridine (Hoffmann-La Roche) per milliliter.

In the experiments with ^{3}H-labeled uridine and thymidine, the amounts of label added were 1.0 μCi/ml for the former and 0.5 μCi/ml for the latter. Incorporation was terminated by adding 5 ml of ice-cold phosphate-buffered NaCl containing 10^{-4} g of uridine or 10^{-4} g of thymidine per milliliter, respectively.

Hamster embryo and mouse embryo cells were grown in monolayer cultures in 2-oz Brockway bottles in CMRL 1066 supplemented with 10% fetal calf serum. Cells were used between the second and eighth subculturings following the establishment of the cultures from minced embryos. Prior to labeling, the medium from each culture was removed and the cells were washed once with 5 ml of CMRL 1066 lacking coenzymes and nucleosides but supplemented with 10^{-5} g of deoxyadenosine per milliliter. Then 2 ml of this same medium with 10% dialyzed fetal calf serum were added to the cultures along with N^5-formyltetrahydrofolate, if required. After 15 min at 37°, MTX was added as needed. Each drug was added in 10 μl of phosphate-buffered 0.9% NaCl solution. The use of deoxyadenosine as a source of purines assured that any effect of MTX would be due only to thymidylate depletion. Labeled deoxyuridine was added to a final concentration of 2.0 μCi/ml, and the cultures were incubated at 37° for 60 min. Incorporation was terminated by pouring off the medium and chilling the monolayer in an ice bath.

Acid-precipitable radioactivity was determined by a Millipore technique. For L-cells, the labeled cultures were first sedimented by centrifugation and the radioactive supernatant fraction was poured off. The pellet was washed once by resuspension in 5 ml of ice-cold phosphate-buffered NaCl, followed by centrifugation, after which the pellet was resuspended in 5 ml of the ice-cold buffer; 5 ml of 20% (w/v) ice-cold trichloracetic acid were added, and the entire culture was filtered through a Millipore filter (47-mm diameter; 0.45-μ pore). Each filter was washed with 30 ml of ice-cold 5% trichloracetic acid, placed in a counting vial (Wheaton Glass Company, Millville, New Jersey), and dried. Scintillation fluid (24 g of 2,5-diphenyloxazole and 0.2 g of bis[2-(4-methyl-5-phenyloxazolyl)]benzene per 4 liters of toluene) was added, and the samples were counted in a Nuclear-Chicago liquid scintillation counter.

In the case of hamster and mouse embryo cultures, the labeled monolayers were removed from the glass surface by trypsinizing for 20 min at 4°, using 5 ml of trypsin solution [1% (w/v) trypsin (Difco Laboratories) in phosphate-buffered NaCl] per bottle. The dispersed cells were sedimented by centrifugation in a clinical centrifuge, the supernatant fluid was discarded, and the pellet was resuspended in 5 ml of ice-cold phosphate-buffered NaCl to which were added 5 ml of a 20% solution of trichloracetic acid. The entire culture was then washed on Millipore filters and counted in the same manner as described above.

Some of the studies with L-cells required separate determinations of the radioac-

tivity in DNA and RNA. For this purpose a modified Schmidt-Thannhauser procedure (7) was employed. Labeled L-cells (10-ml aliquots) were spun down and resuspended in 10 ml of ice-cold phosphate-buffered NaCl. After a second centrifugation, the washed pellet was resuspended in 5 ml of ice-cold 10% trichloracetic acid and placed in an ice bath for 30 min. The cell suspension was then sedimented and washed twice more in ice-cold phosphate-buffered NaCl by resuspension and centrifugation. This was followed by a wash with 5 ml of ice-cold absolute ethanol containing 10% (w/v) potassium acetate, after which the pellet was resuspended in 1 ml of 0.3 N KOH for 1 hr at 37° to hydrolyze the RNA. The samples were then cooled and the DNA was precipitated by the addition of 5 ml of 10% ice-cold trichloracetic acid. The precipitate was collected on Millipore filters, washed with 5% trichloracetic acid, dried, and counted as described above.

Chromatography of MTX. MTX was purified on a DEAE-cellulose column using a slight modification of the chromatographic technique described by Oliverio (8). Fifty milligrams of the commercial MTX preparation dissolved in 5 ml of 0.01 M phosphate buffer, pH 7.7, were adsorbed on a column (1 × 30 cm) which had been equilibrated with this same buffer. Elution was carried out at room temperature with a linear gradient (0.01–0.30 M) of phosphate buffer, pH 7.7. Five peaks were eluted, and these were designated A through E (as shown in Fig. 3).

Absorption spectra of the various peaks were measured in 0.1 N NaOH and in 0.1 N HCl in a Cary model 14 recording spectrophotometer. These were compared with the published spectra for MTX (9).

Peak D, containing 85% of the total absorbance at 254 mμ, was identified as MTX on the basis of its absorption spectrum and by comparison of its migration rate on ascending paper chromatography, in two different solvent systems, with an independently synthesized sample of ^{3}H-labeled MTX (Radiochemical Centre, Amersham, England). Solvent I consisted of a 1% (w/v) aqueous solution of K_2HPO_4; solvent II consisted of a 70:10:20 mixture of 2-propanol, NH_3, and H_2O. Chromatograms were developed at room temperature for approximately 2 hr with solvent I and for about 6 hr with solvent II. The R_F values for the radioactive MTX and for the material from peak D were both 0.6 in solvent I and less than 0.1 in solvent II. The two spots were coincident in both solvent systems.

Assay for enzyme activity in cell-free extracts. Thymidylate synthetase activity was measured by the spectrophotometric assay of Wahba and Friedkin (10) as modified by Reyes and Heidelberger (11). Reactions were carried out in quartz cuvettes of approximately 1.5-ml capacity, directly in the sample holder of a Gilford multiple sample absorbance recorder in a 37° room. Four reactions were run simultaneously. Three of the cuvettes contained complete reaction mixtures, while the fourth, lacking dUMP, served as a control to establish the background rate of change of absorbance at 340 mμ. Initial slopes of the plots of absorbance vs. time were used to calculate reaction velocities. The absorbance change was a linear function of time for at least 10 min in most cases.

Each reaction mixture of 1.0 ml contained the following reagents: 100 μmoles of phosphate buffer (pH 6.7), 50 μmoles of 2-mercaptoethanol, 3.3 μmoles of formaldehyde, 16 μmoles of sodium bicarbonate, variable amounts of tetrahydrofolate cofactor [N^5,N^{10}-methylene-*dl* (L)-tetrahydrofolate], and varying amounts of dUMP, MTX, and enzyme. Reaction mixtures were prepared, with only the dUMP omitted, and were allowed to equilibrate at 37° for 5–10 min, by which time all four cuvettes showed identical rates of change of absorbance at 340 mμ. The reaction was then initiated by the addition of dUMP in 10 μl of distilled water. An equivalent volume of distilled water was added to the control cuvette.

Enzyme preparation. Extracts were prepared from Ehrlich ascites carcinoma cells and from *Escherichia coli.* To extract the enzyme from ascites cells, the ascitic fluid

from Swiss mice, which had received 1 ml of ascitic fluid intraperitoneally 7 days previously, was drained and pooled. The ascitic cell suspension was centrifuged at 4° for 10 min at 500 × *g*. The supernatant fluid was discarded, and the pellet was washed repeatedly with ice-cold phosphate-buffered NaCl. After washing, the packed cells were resuspended in 3 volumes of ice-cold phosphate buffer, 0.05 M, pH 6.7, and were disrupted at 20 kc/sec with a Branson model S125 sonicator. Cellular debris was removed by centrifuging the disrupted cell suspension at 4° for 10 min at 10,000 × *g*. The supernatant was then spun for 1 hr at 105,000 × *g* in a Beckman model L-2 ultracentrifuge. The resulting supernatant was freed of nucleic acids by precipitation with protamine sulfate (Sigma Chemical Company), which was added dropwise as a 10 mg/ml aqueous solution, and centrifugation. Usually two precipitations were sufficient. For fractionation of the extract, ammonium sulfate was added to the desired degree of saturation (12), and the precipitate was collected by centrifugation at 10,000 × *g* for 10 min. The precipitate was resuspended in 0.05 M phosphate buffer, pH 6.7, and dialyzed overnight against 100 volumes of the same buffer. All the enzyme activity was found in the 30–50% saturated ammonium sulfate fraction. When unfractionated 105,000 × *g* supernatant was used as the enzyme source, it was dialyzed before use in the manner described above.

Bacterial enzyme was extracted from *E. coli* cells (General Biochemicals, Chagrin Falls, Ohio). About 30 g of frozen cells and 60 g of alumina were worked into a paste with a mortar and pestle by grinding vigorously for 10 min. Alumina and cellular debris were removed by centrifugation for 10 min at 10,000 × *g*. The extract was spun for 1 hr at 105,000 × *g*; the supernatant was freed of nucleic acids and dialyzed as described above, and used as the source of enzyme.

Protein concentration was determined quantitatively by means of the Folin-Ciocalteu reagent (13), using bovine serum albumin as a standard.

RESULTS

Studies on Intracellular Inhibition of Thymidylate Synthesis by MTX

Characteristics of radioactive deoxyuridine incorporation into acid-precipitable material. To evaluate the possibility of utilizing the incorporation of labeled deoxyuridine into acid-precipitable material as a measure of thymidylate synthesis in cells growing in cell culture, a series of control experiments were performed. These were designed to establish that labeled deoxyuridine is incorporated into DNA only, specifically via the thymidylate synthesis pathway.

Figure 1 shows the kinetics of deoxyuridine-6-^{3}H incorporation into acid-precipitable material in exponentially growing L-cells, measured using the technique described in MATERIALS AND METHODS. The incorporation proceeded linearly for at least 45 min, following a lag of approximately 2 min. In this experiment the presence

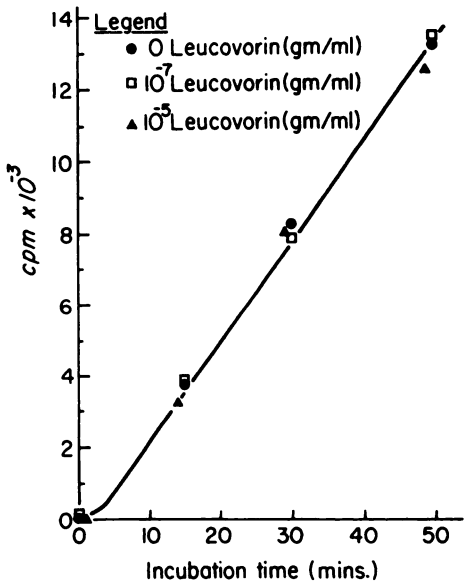

FIG. 1. *Kinetics of incorporation of radioactivity from deoxyuridine-6-^{3}H into acid-precipitable material in L-cells*

Total counts incorporated were determined as described in MATERIALS AND METHODS, and are plotted against time of incubation at 37°. Leucovorin is N^5-formyltetrahydrofolate.

TABLE 1
Incorporation of labeled deoxyuridine into DNA and RNA

Isotope	Total activity	Activity after alkaline hydrolysis	Background (no incubation)[a]
	cpm	cpm	cpm
Thymidine-^{3}H	23,505	18,100	198
	21,906	17,843	187
Uridine-^{3}H	4,309	165	147
	4,604	158	153
Deoxyuridine-6-^{3}H	8,340	6,549	173
	7,987	6,214	184

[a] Counter background = 39 cpm.

of 10^{-5} or 10^{-7} g/ml of N^5-formyltetrahydrofolate did not affect the incorporation to any significant extent, although in some experiments the presence of N^5-formyltetrahydrofolate at 10^{-7} g/ml slightly increased the rate of incorporation.

To show that the observed counts were in DNA and not in RNA, an incorporation experiment was performed in which four cultures were labeled with deoxyuridine-6-^{3}H, four were labeled with deoxythymidine-^{3}H, and four were labeled with uridine-^{3}H. The cultures were labeled for 30 min, as described in MATERIALS AND METHODS. Two of the four cultures exposed to each isotope were processed to determine total radioactivity incorporated into acid-precipitable material, while the remaining six cultures were subjected to the modified Schmidt-Thannhauser procedure to yield alkali-resistant radioactivity in DNA. The results are given in Table 1. The "total activity" column indicates that good incorporation of all three isotopes was achieved. After alkaline hydrolysis, the counts in the uridine-^{3}H-labeled cultures were similar to background, indicating that the RNA was completely hydrolyzed by the treatment. The counts in the cultures labeled with thymidine-^{3}H and those labeled with deoxyuridine-6-^{3}H were lowered an equivalent fraction by the alkaline hydrolysis. Since thymidine-^{3}H is a specific label for DNA (14–16) while, under the conditions of incorporation, uridine-^{3}H is specific for RNA (17), it can be concluded on the basis of the counts resistant to alkaline hydrolysis that deoxyuridine-6-^{3}H is incorporated specifically into DNA.

To establish that the incorporation of deoxyuridine-6-^{3}H into DNA is a valid measure of thymidylate synthesis, it was essential to rule out the possibility that this precursor is incorporated via some alternative pathway, such as the addition of an amino group to the 4-position to form deoxycytidine-6-^{3}H. Three separate lines of evidence indicate deoxyuridine-6-^{3}H is incorporated specifically via the thymidylate synthesis pathway. The first is the effect of 5-fluoro-2′-deoxyuridine on the labeling. This substance is known to

TABLE 2
Incorporation of labeled deoxyuridine into acid-precipitable material in the presence of 5-fluoro-2′-deoxyuridine and thymidine, and effect of using deoxyuridine-5-^{3}H as label

Label	Addition	Acid-precipitable radioactivity after 60-min incubation	Background (no incubation)
		cpm	*cpm*
Deoxyuridine-6-^{3}H	None (control)	5426	198
		5293	174
Deoxyuridine-6-^{3}H	Fluorodeoxyuridine (10^{-6} g/ml)	180	151
		179	172
Deoxyuridine-6-^{3}H	Thymidine (10^{-5} g/ml)	204	186
		217	164
Deoxyuridine-5-^{3}H	None	255	299
		282	290

result in inhibition of thymidylate synthetase (11, 18); hence, if deoxyuridine-6-^{3}H were incorporated into DNA via the thymidylate synthesis pathway, fluorodeoxyuridine should prevent the labeling. The second line of evidence is provided by the effect of excess thymidine on the incorporation. Excess thymidine present in the medium should dilute the labeled dTMP synthesized via thymidylate synthetase from dUMP-6-^{3}H. Since, in the synthesis of dTMP, the methylation of dUMP takes place at the 5-position, the ^{3}H from the 5-position should be lost and no label from deoxyuridine-5-^{3}H should be incorporated into DNA if the incorporation occurs via this pathway. Table 2 shows the results of incorporation experiments under these conditions. Whereas in the control culture deoxyuridine-6-^{3}H was incorporated readily, this incorporation was completely prevented by the presence of fluorodeoxyuridine or thymidine in the culture medium, and radioactivity from deoxyuridine-5-^{3}H was not incorporated. These results, along with those in Table 1, indicate that deoxyuridine-6-^{3}H is incorporated specifically into DNA, and specifically via the thymidylate synthesis pathway. Hence, the incorporation of counts from deoxyuridine-6-^{3}H into acid-precipitable material may be used as a valid assay of thymidylate synthetase activity in tissue cultures. In all experiments to be reported, controls were carried out with excess thymidine and fluorodeoxyuridine in the culture medium.

Effect of MTX on thymidylate synthesis in L-cell cultures. In this section data will be presented which suggest that MTX, or some metabolic product of MTX formed inside the living cell, probably inhibits thymidylate synthetase directly. Aliquots (5 ml; 8×10^5 cells) of exponentially growing L-cells in medium supplemented with 10^{-5} g of deoxyadenosine per milliliter were treated with a series of concentrations of MTX and N^5-formyltetrahydrofolate [as a source of folate coenzymes; see (19)]. Deoxyuridine-6-^{3}H incorporation into acid-precipitable counts during a 45-min incubation at 37° was measured, and Fig. 2 shows the results, as counts per minute

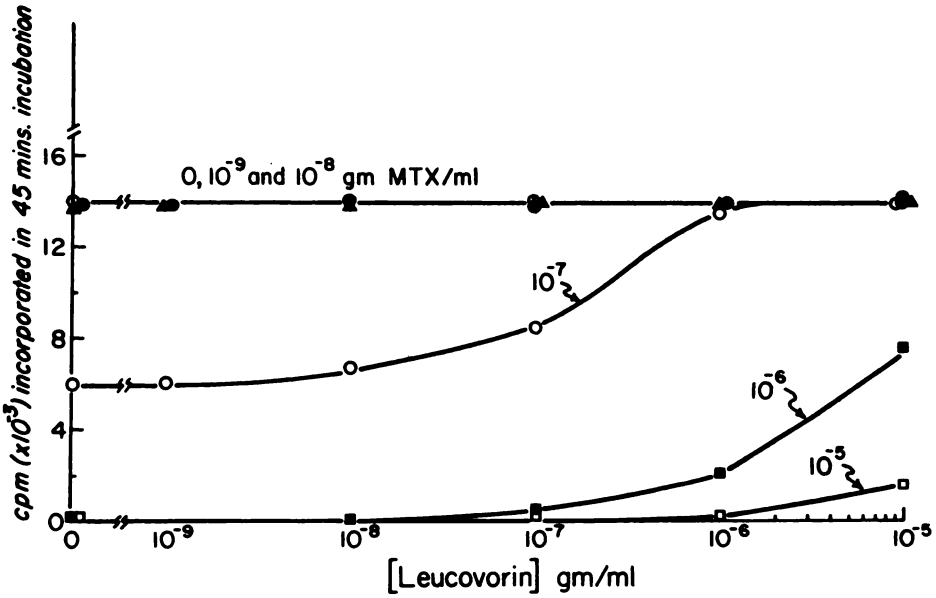

FIG. 2. *Incorporation of deoxyuridine-6-^{3}H into acid-precipitable material in L-cells as a function of MTX and N^5-formyltetrahydrofolate (leucovorin) concentration*

For each concentration of MTX, the total counts incorporated during a 45-min incubation are plotted as a function of N^5-formyltetrahydrofolate concentration.

incorporated per culture, as a function of N^5-formyltetrahydrofolate concentration for various concentrations of MTX. These data show that MTX inhibits the incorporation of deoxyuridine-6-^{3}H. In the absence of N^5-formyltetrahydrofolate, 10^{-7} g of MTX per milliliter reduces the incorporation to about 50% of that seen in the control culture, whereas MTX at 10^{-6} and 10^{-5} g/ml completely inhibits the incorporation. The addition of N^5-formyltetrahydrofolate to the cultures reverses the inhibition, but the concentration required increases with greater concentrations of MTX. This competitive reversal by N^5-formyltetrahydrofolate of the inhibitory action of MTX, together with the fact that even 10^{-6} g of N^5-formyltetrahydrofolate per milliliter (which should make the incorporation completely independent of the reductase) prevents incorporation in the presence of 10^{-5} g of MTX per milliliter, makes it highly unlikely that the observed inhibition arises from action of MTX on the reductase. When the cells were loaded with N^5-formyltetrahydrofolate by incubation for 15 min in the presence of this drug prior to the addition of MTX, the results were unchanged. This indicates that the observed competition does not arise at the level of drug transport. These results are consistent with the view expressed previously (1) that MTX acts at some site along the thymidylate synthesis pathway which is distal to folate and dihydrofolate reductase, probably at the level of thymidylate synthetase.

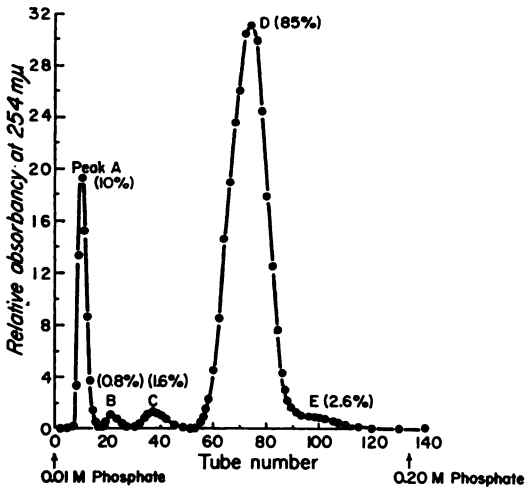

FIG. 3. *Chromatography on DEAE-cellulose of the commercial preparation of MTX used throughout these studies*

The relative absorbance of the fractions at 254 mμ is plotted against fraction number.

To rule out the possibility that the results attributed to MTX might have been caused by contaminants in the drug preparation, an inhibition experiment was carried out utilizing purified MTX.

On chromatography, as described in MATERIALS AND METHODS, the commercial preparation of MTX yielded five separate peaks, as indicated in Fig. 3. The material in the main peak, D, identified as MTX as described in MATERIALS AND METHODS, was tested for its ability to inhibit incorporation of radioactive deoxyuridine into acid-precipitable material in L-cells. The four minor peaks were also tested, at concentrations equivalent to those at which each would be present in 10^{-6} g of commercial MTX per milliliter. Table 3 shows that only peak D was effective in inhibiting the incorporation, which makes it unlikely that the results obtained in the viability and growth inhibition studies (1) can be attributed to the presence of various impurities in the commercial preparations of MTX used.

In the remaining studies, the normal commercial preparations of MTX were used without purification.

Comparison of the Results of Growth Inhibition and Deoxyuridine-6-^{3}H Incorporation

Inhibition studies on thymidylate synthetase activity. Two separate assays for thymidylate synthesis in the presence of various concentrations of MTX were used: the growth inhibition assay of cells cultured in medium supplemented with 10^{-5} g of deoxyadenosine per milliliter but lacking thymidine [see (1)], and the inhibition of incorporation of deoxyuridine-6-^{3}H into acid-precipitable material in cells in culture as described in this paper. Since both assays supposedly yield results pertaining to a

TABLE 3
Inhibition of deoxyuridine-6-^{3}H incorporation into acid-precipitable material in L-cell tissue cultures by the different components present in the commercial preparation of MTX used throughout these studies

Peak	Absorbance at 254 mμ	Concentration tested[a]	Activity incorporated in 30 min
	% total	M	*cpm*
A	10.0	2.3×10^{-7}	5426
			5050
B	0.8	1.9×10^{-8}	5041
			5293
C	1.6	3.8×10^{-8}	5336
			5026
D	85.0	2.0×10^{-6}[b]	252
			263
E	2.6	6.1×10^{-8}	5129
			4749
Control culture			5562
			4942
Zero time incubation control			198
			174

[a] The concentrations of the material in peaks A, B, C, and E were calculated on the assumption that their extinction coefficients at 254 mμ were the same as that of MTX. This is probably not a valid assumption, but their concentrations relative to the MTX peak will still be correct because these concentrations were adjusted to give the same relative absorbance at 254 mμ as that found for the peaks obtained from the column.

[b] Equivalent to approximately 10^{-6} g of MTX per milliliter.

single site of action of MTX (i.e., thymidylate synthetase), it was necessary to show that the results of the two assays led to compatible conclusions. Figure 4 shows thymidylate synthesis activity measured both ways, as a function of MTX concentration. Data from Fig. 2, obtained using the assay of deoxyuridine-6-^{3}H incorporation into acid-precipitable material in the absence of N^5-formyltetrahydrofolate, are plotted along with the growth inhibition data from Fig. 6 of the preceding paper (1). The two curves have been normalized so that the two plateau levels, indicating uninhibited thymidylate synthesis, correspond. From these curves it can be seen that thymidylate synthesis in the growth assay is much more sensitive to inhibition by MTX than in the radioactivity incorporation assay. Since these two assays are interpreted as measures of MTX inhibition of thymidylate synthetase, the discrepancy must be explained.

The major difference in the two techniques was the duration of time over which the assay was carried out. In the growth assay, the MTX was added to the cell cultures and any inhibitory effects were seen after 48 hr of growth. In the radioactivity incorporation assay, the MTX and deoxyuridine-6-^{3}H were added to the cell cultures and inhibition was measured during a 45-min incubation. Hence, experiments were carried out to measure thymidylate synthesis, as assayed by the deoxyuridine-6-^{3}H technique, as a function of time after the addition to exponentially growing spinner cultures of L-cells in growth medium supplemented with 10^{-5} g of deoxyadenosine per milliliter at zero time. At various times a 30-min pulse-label of deoxyuridine-6-^{3}H was given. It can be seen (Fig. 5) that after the addition of MTX to cell cultures, thymidylate synthesis was inhibited at a rate dependent on MTX concentration. At 10^{-6} g of MTX per milliliter, inhibition was complete essentially immediately upon addition of the drug, while at 10^{-8} g/ml, thymidylate synthesis was essentially unaffected 45 min after the drug was added but was essentially completely inhibited within 8 hr after addition of the drug. This explains the difference between the results obtained with the two different assay techniques used to measure thymidylate synthetase activity as a function of MTX concentration.

The possibility that the above differences may be attributable to varying degrees of cell killing is ruled out by the fact that over the 8-hr period involved essentially no cell killing occurred at any of the MTX concentrations used (3).

Studies on MTX inhibition of thymidylate synthesis in cultures of cells from embryonic mice and hamsters. Because L-strain mouse cells have been in cell culture for an extended period of time, they may

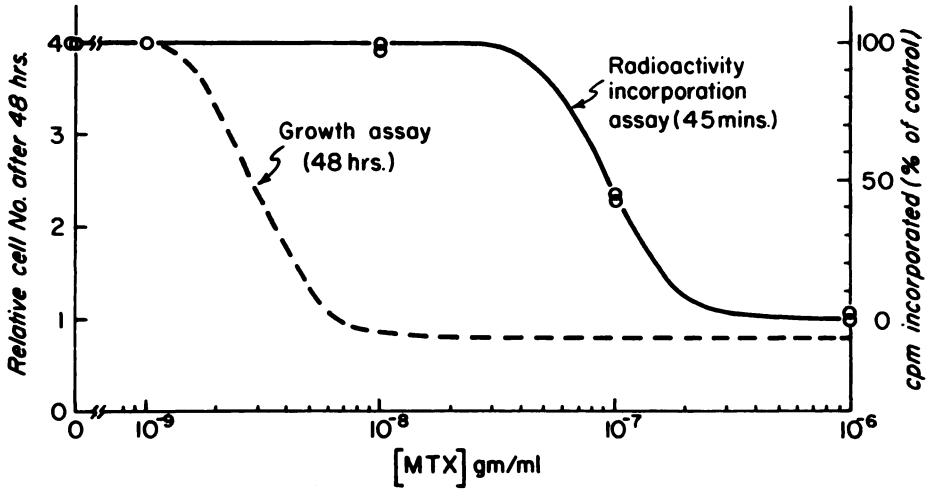

FIG. 4. *Comparison of sensitivity of thymidylate synthesis to inhibition by MTX as determined by two independent assay techniques*

For experimental details, see the text.

not be completely representative of mammalian cells in their response to MTX. Hence a series of experiments were performed, using cultures of cells from hamster and mouse embryos, in which the inhibition by MTX of thymidylate synthesis, as measured by uptake of deoxyuridine-6-^{3}H, was studied in both the presence and absence of 10^{-7} g of N^5-formyltetrahydrofolate per milliliter, a concentration sufficient to permit normal growth of L-cells in the presence of 10^{-8} g of MTX per milliliter. Since MTX at 10^{-8} g/ml completely inhibits L-cell growth in the absence of N^5-formyltetrahydrofolate, 10^{-7} g of N^5-formyltetrahydrofolate per milliliter must be a concentration sufficiently great to provide the cultures with all their folate coenzyme requirements for normal growth. With this level of N^5-formyltetrahydrofolate present in the culture medium, if MTX acted only on folate and dihydrofolate reductase, incorporation of label in the presence of MTX should be unaffected because MTX, if it does not act directly on thymidylate synthetase, cannot interfere with the *substrate* function of tetrahydrofolic acid in the synthesis of dTMP. By addition of N^5-formyltetrahydrofolate to the culture medium, the incorporation of deoxyuridine-6-^{3}H into DNA via thymidylate synthetase is made essentially independent of folate and dihydrofolate reductase. Hence, the occurrence of inhibition under these conditions would strongly suggest that MTX directly inhibits thymidylate synthetase.

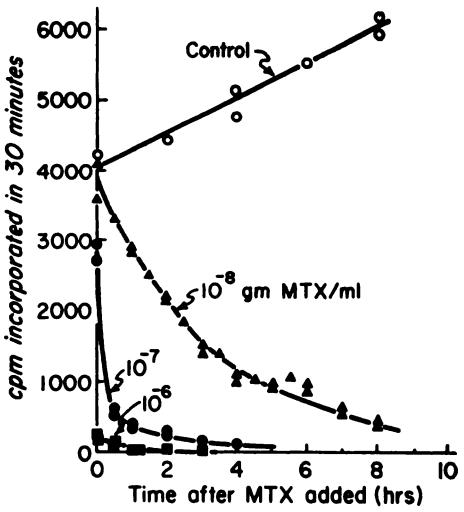

FIG. 5. *Kinetics of inhibition of thymidylate synthesis by various concentrations of MTX, as measured by the radioactivity incorporation assay*

For experimental details, see the text.

Table 4 shows the results of two experiments on hamster embryo cultures and one experiment on mouse embryo cultures. The

TABLE 4

Inhibition by MTX of deoxyuridine-6-^{3}H incorporation into acid-precipitable material in hamster and mouse embryo cell cultures

The data indicate the counts per minute incorporated per culture during a 60-min incubation.

Culture	MTX (10^{-6} g/ml)					
	No MTX	No N^5-formyltetrahydrofolate	N^5-Formyltetrahydrofolate (10^{-7} g/ml)	Fluorodeoxyuridine (10^{-6} g/ml)	Thymidine (10^{-5} g/ml)	Zero time control
	cpm	*cpm*		*cpm*	*cpm*	*cpm*
Hamster embryo 1	5750	243	375	213	313	165
	6040	276	402	194	282	155
Hamster embryo 2	2490	205	340	190	227	139
	2280	231	292	179	213	157
Mouse embryo	563	80	72	74	91	100
	551	92	111	87	80	87

mouse embryo cultures used in this experiment were in late log phase and were nearly confluent, so that the control cultures incorporated fewer counts than did the hamster embryo cultures, which were in the early log phase of growth. The second column indicates deoxyuridine-6-^{3}H incorporation by the control cultures, to which no MTX was added. An MTX concentration of 10^{-6} g/ml inhibited incorporation essentially completely, in both the presence and absence of N^5-formyltetrahydrofolate (10^{-7} g/ml), as shown in the next two columns. The remaining columns indicate that incorporation of label was inhibited by 10^{-6} g of fluorodeoxyuridine per milliliter and by the presence of 10^{-5} g of thymidine per milliliter in the culture medium. These data suggest that MTX directly inhibits thymidylate synthetase in the various cultures and that the observed inhibition is not unique to L-cells.

Studies on Inhibition of Thymidylate Synthetase by MTX in Cell-Free Systems

General properties of the reaction catalyzed by the enzyme extracted from Ehrlich ascites carcinoma cells. Up to this point the inhibition studies had all been carried out on intact cells *in vitro*. However, it seemed desirable to observe the effects of MTX on thymidylate synthetase in cell-free systems, and for this purpose enzyme was extracted from Ehrlich ascites cells as described in MATERIALS AND METHODS.

TABLE 5

Requirements for thymidylate synthesis

The complete reaction mixture contained 200 mμmoles of dUMP, 400 mμmoles of tetrahydrofolate cofactor, enzyme extract (5.0 mg of protein), and other reagents as specified in MATERIALS AND METHODS

System	dTMP synthesized	Relative activity
	mμmoles/ 5 mg/5 min	%
Complete	6.3	100
−Tetrahydrofolate cofactor	0	0
−dUMP[a]	0	0
Complete + $MgCl_2$ (1 μmole)	6.1	97
Complete, using boiled enzyme extract	0	0
Complete, using 50% less enzyme	3.3	53
Complete + fluorodeoxyuridine (200 mμmoles)	5.9	94

[a] The rate of change of absorbance at 340 mμ after the addition of 10 μl of distilled water (no dUMP) was usually slightly less than, and never greater than, the rate of change prior to its addition.

Table 5 lists the reaction velocities measured in experiments designed to test the requirements of the reaction using the

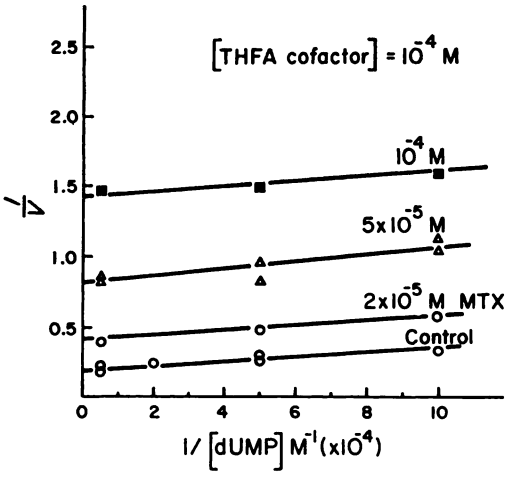

FIG. 6. *Inhibition of thymidylate synthetase by MTX in a cell-free system*

Each reaction mixture contained 100 mμmoles of tetrahydrofolate (THFA) cofactor, 5 mg of protein, varying amounts of dUMP and MTX, and other reagents as specified in MATERIALS AND METHODS. From the data a $K_{i,\text{intercept}}$ value of 1.4 (±0.1) × 10^{-5} M was calculated for MTX. A value of 1.1 (±0.2) × 10^{-5} M was calculated for the apparent K_m for dUMP.

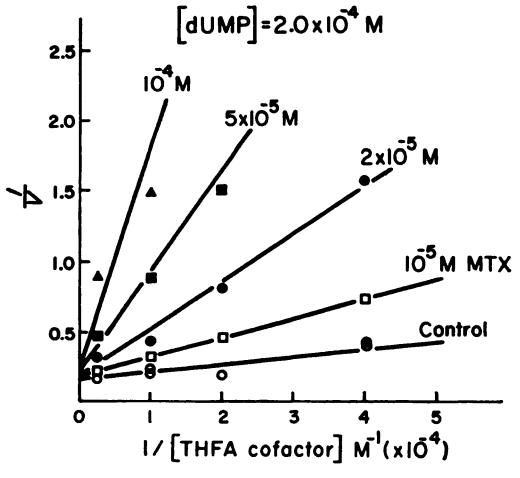

FIG. 7. *Inhibition of thymidylate synthetase by MTX in a cell-free system*

Each reaction mixture of 1.0 ml contained 200 mμmoles of dUMP, varying amounts of tetrahydrofolate (THFA) cofactor, and other components as described for Fig. 6. From the data a $K_{i,\text{slope}}$ value of 4.3 (±1.2) × 10^{-6} M was calculated. A value of 1.5 (±0.7) × 10^{-5} M was calculated for the K_m for N^5,N^{10}-methylene-*l*(L)-tetrahydrofolate.

30–50% saturated ammonium sulfate precipitate as the source of enzyme, and shows that the reaction was absolutely dependent on the presence of tetrahydrofolate cofactor, dUMP, and enzyme. The reaction rate was proportional to enzyme concentration. Magnesium ions did not increase the reaction rate, in agreement with the finding of others (11). Fluorodeoxyuridine did not inhibit the reaction, probably because of a lack of phosphorylating enzyme for its conversion to fluorodeoxyuridylate.

Inhibition by MTX of thymidylate synthetase from Ehrlich ascites carcinoma cells. To determine whether MTX exerted any inhibitory effect on thymidylate synthetase in a cell-free system, a series of experiments was carried out in which the reaction velocity was measured as a function of concentration of dUMP, tetrahydrofolate cofactor, and MTX, using the 30–50% saturated ammonium sulfate precipitate fraction. In Fig. 6 a Lineweaver-Burk plot (20) is shown, with the reciprocal of the measured velocity plotted against the reciprocal of dUMP concentrations. The uninhibited reactions yield a curve which intersects the negative abscissa to yield a value of 1.1 (±0.2) × 10^{-5} M for the apparent K_m for dUMP. This is in good agreement with the value of 1.4 (±0.2) × 10^{-5} M reported by Reyes and Heidelberger (11). The upper curves in Fig. 6 are for reactions carried out in the presence of the indicated concentrations of MTX. It is clear that MTX inhibits the reaction. The mechanism of inhibition appears to be uncompetitive (21) with respect to dUMP. The data were fitted with curves corresponding to the equation which defines reaction velocity of an uncompetitively inhibited reaction,

$$\frac{1}{v} = \frac{1}{V_{\max}}\left(1 + \frac{I}{K_{i,\text{intercept}}}\right) + \frac{K_m}{V_{\max}} \cdot \frac{1}{S}$$

where v = observed velocity
$V_{\max}$ = maximum velocity at infinite substrate concentration
S = substrate concentration
I = inhibitor concentration
$K_{i,\text{intercept}}$ = inhibition constant
K_m = Michaelis constant for the substrate

From the data a value of 1.4 ($\pm$0.1) $\times$ 10^{-5} M for $K_{i,\ \text{intercept}}$ was obtained.

Similar plots were obtained for a series of tetrahydrofolate cofactor concentrations. Data obtained with a dUMP concentration of 200 μM are shown in Fig. 7, where the reciprocal of the measured velocity is plotted against the reciprocal of tetrahydrofolate cofactor concentration. The curve for the uninhibited reactions intersects the negative abscissa to yield a value of 1.5 ($\pm$0.7) $\times$ 10^{-5} M for the apparent K_m for N^5,N^{10}-methylene-*l*(L)-tetrahydrofolate. In making this calculation it was assumed that the concentration of N^5,N^{10}-methylene-*l*(L)-tetrahydrofolate is one-half the concentration of N^5,N^{10}-methylene-*dl*(L)-tetrahydrofolate. The above value for K_m agrees well with the 1.4 ($\pm$0.2) $\times$ 10^{-5} M reported by Reyes and Heidelberger. The upper curves in Fig. 7 are for reactions carried out in the presence of the indicated concentrations of MTX. From the data it is evident that MTX inhibits the reaction, in a manner which appears to be competitive (21) with respect to tetrahydrofolate cofactor. Fitting the data with curves corresponding to the equation which defines reaction velocity of a competitively inhibited reaction (21),

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{I}{K_{i,slope}}\right)\cdot\frac{1}{S}$$

where v = observed velocity
V_{max} = maximum velocity at infinite substrate concentration
K_m = Michaelis constant for the substrate concentration
$K_{i,slope}$ = inhibition constant
I = inhibitor concentration

yields a value of 4.3 ($\pm$1.3) $\times$ 10^{-6} M for $K_{i,\ slope}$. Methotrexate-induced inhibition of thymidylate synthetase via a mechanism competitive with respect to tetrahydrofolate cofactor is precisely the result predicted by the studies of growth inhibition and reversal (1) and inhibition of the incorporation of radioactivity presented above. It is also what would be expected if MTX were capable of competing with the folate coenzymes as well as with folic acid.

It is well known (10, 22, 23) that reduced derivatives of the 4-aminofolic acid antagonists, tetrahydroaminopterin and tetrahydromethotrexate, are potent inhibitors of thymidylate synthetase. This suggests that the results observed here could have been due to the presence of tetrahydromethotrexate as an impurity in the MTX. Even after storage for up to 7 days, as an aqueous solution freely exposed to air at temperatures fluctuating between 4° and 37°, however, the inhibitory power of MTX was undiminished from its value immediately upon preparation from the dry powder. Since tetrahydromethotrexate, like tetrahydrofolate, is known to be unstable (22), this treatment should certainly have altered very significantly the potency of the inhibition if the inhibition were due to such an impurity.

The possibility exists that the observed inhibition could have been due to contamination of the cofactor with folic acid and dihydrofolic acid, and the presence of reductase in the enzyme preparation. Two arguments make this possibility highly improbable. (a) The absorption spectrum of the cofactor solution had a single maximum at 294 mμ, with no evidence of a shoulder in the region of the dihydrofolate maximum at approximately 282 mμ. The spectrum agreed perfectly with that reported by Wahba and Friedkin (10) for methylenetetrahydrofolate. (b) For any folate cofactor concentration the concentration of MTX could be adjusted to result in essentially 100% inhibition of the reaction. Since from (a) we know that the folate cofactor solution had, at worst, only very small amounts of folate and dihydrofolate contaminants, the only way in which complete inhibition of the reaction could have occurred would be interference by MTX with the utilization of the methylenetetrahydrofolate present.

Inhibition by MTX of thymidylate synthetase from E. coli. To extend the validity of the results described above to at least one other system, some preliminary experiments were carried out on the inhibition by MTX of thymidylate synthetase from *E. coli*. A 105,000 $\times$ *g* supernatant, as described in MATERIALS AND METHODS, was used as the enzyme source. Table 6 shows the results

TABLE 6

Effect of MTX on thymidylate synthetase from E. coli

The complete reaction mixture contained 100 mμmoles of tetrahydrofolate cofactor, 200 mμmoles of dUMP, enzyme (5.0 mg of protein), and other reagents as specified in MATERIALS AND METHODS.

System	dTMP synthesized	Inhibition	Expected inhibition
	mμmoles/5 mg/5 min	%	%
Complete	4.0		
−Tetrahydrofolate cofactor	0		
−dUMP	0		
Complete, using boiled enzyme	0		
Complete, using 50% less enzyme	2.1		
Complete			
+MTX (5×10^{-5} M)	2.1	47	79
+MTX (10^{-4} M)	1.5	63	88

[a] Expected inhibition was calculated on the assumption that K_m and $K_{i,slope}$ for the bacterial enzyme are the same as those found for the enzyme extracted from Ehrlich ascites carcinoma cells, and that the inhibition is competitive with respect to tetrahydrofolate cofactor.

of experiments in which tests for inhibition of thymidylate synthetase were made in the presence of two concentrations of MTX (5×10^{-5} and 1×10^{-4} M) and fixed concentrations of tetrahydrofolate cofactor (100 μM) and dUMP (200 μM). The reaction was absolutely dependent on tetrahydrofolate cofactor, dUMP, and enzyme, and the velocity was proportional to enzyme concentration. Methotrexate inhibited the reaction, with 5×10^{-5} M producing 47% inhibition and 10^{-4} M producing 63% inhibition. In the "expected inhibition" column is shown the degree of inhibition which would have resulted, with the given concentrations of MTX and tetrahydrofolate cofactor, if the K_m and K_i values were the same as found for the Ehrlich ascites enzymes. It appears from these data that the bacterial enzyme may be more resistant to inhibition by MTX than is the mammalian enzyme. Assuming that the K_m for the tetrahydrofolate cofactor is unchanged (which may not be a valid assumption) and that the observed inhibition is competitive with respect to tetrahydrofolate cofactor, the observed degrees of inhibition would result from a $K_{i,\,slope}$ value of approximately 2.3×10^{-5} M. This value is more than 5-fold greater than that measured for the mammalian enzyme. These comparisons are complicated by the fact that in the two studies enzyme preparations of different degrees of purity were used. Further, detailed kinetic studies will be required before valid comparisons can be made between the enzymes from different sources. It is of interest that several authors (24, 25) have reported species differences in at least one other folate enzyme, folate and dihydrofolate reductase.

DISCUSSION

The experimental results presented here, together with those on the growth inhibition studies (1), constitute strong evidence that MTX directly inhibits thymidylate synthetase from both mammalian (mouse and hamster) and bacterial sources. This conclusion is in direct contradiction to the currently accepted view as expressed by several authors (2, 26), and the reason for this contradiction must be ascertained.

The most frequently quoted study with respect to the lack of action of MTX on thymidylate synthetase is that of Wahba and Friedkin (10). These authors, using a bacterial enzyme, tested the inhibitory effects of MTX, aminopterin, tetrahydro-MTX, and tetrahydroaminopterin on

thymidylate synthetase. Under their conditions each of the tetrahydro derivatives was a potent inhibitor of the enzyme, while aminopterin did not inhibit the reaction at all. Methotrexate, on the other hand, did inhibit the reaction slightly, but Wahba and Friedkin interpreted the results as indicating that significant inhibition did not occur. Using the values of K_m and $K_{i,\ slope}$ measured by us with the Ehrlich ascites enzyme system, it can be calculated that for the concentrations of tetrahydrofolate and MTX employed by Wahba and Friedkin there should have been approximately 70% inhibition of the enzyme. Only 20% inhibition was observed. This discrepancy may arise from the fact that Wahba and Friedkin in their inhibition studies did not compare initial velocities, but looked at the increase in absorbance at 340 mμ after 40 min of incubation, which might tend to obscure any differences in initial velocities. A second report by Friedkin *et al.* (27) suffers from the limitation that the concentration of MTX employed (0.8×10^{-6} M), with a bacterial enzyme, is considerably less than the $K_{i,\ slope}$ value of MTX for mammalian enzyme we have found.

Several reports indicate that aminopterin does not inhibit thymidylate synthetase in cell-free systems (10, 28–30). Kisliuk and Levine (23), on the other hand, have demonstrated inhibition of thymidylate synthetase by aminopterin in a cell-free system. The discrepancy between these findings and those of the other reports concerning aminopterin, referred to above, most probably is due to too low a ratio of aminopterin to tetrahydrofolate used in the latter studies, and to differences in experimental procedure employed.

Although our studies on thymidylate synthetase in a cell-free system show that MTX is capable of inhibiting this enzyme under the conditions employed, the extrapolation of this finding to the situation in the living cell is somewhat uncertain. Our studies in tissue cultures, using both the cell growth and the radioactivity assay for thymidylate synthesis, indicate that inhibition of dTMP synthesis occurs at MTX concentrations between 100- and 1000-fold less than those required to inhibit thymidylate synthetase in a cell-free system. This indicates that straightforward extrapolation of the enzyme results from the cell-free system to the enzyme situation *in vivo* is invalid. In addition, the concentrations of N^5-formyltetrahydrofolate required to maintain normal cell proliferation are also between 100- and 1000-fold less than the concentration of tetrahydrofolate cofactor employed in the cell-free system studies.

The chance that MTX and aminopterin differ quantitatively with respect to their ability to inhibit thymidylate synthetase offers interesting possibilities. If such a difference is real, the structural difference between MTX and aminopterin must be involved in effecting the inhibition and should be modified in an attempt to increase both the efficacy and specificity of the inhibition of thymidylate synthetase. In particular, one can speculate that the attachment of a suitable reactive group to the methyl group carbon of MTX, since it may well approximate the position of the 5,10-methylene group of the normal coenzyme relative to the enzyme surface, may result in an irreversible inhibitor of the type proposed and being developed by Baker (31, 32). The achievement of such increased efficacy and specificity should, on the basis of the viability studies presented separately (3), result in a superior agent for chemotherapy.

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