

Threshold Methotrexate Concentration for In Vivo Inhibition of DNA Synthesis in Normal and Tumorous Target Tissues

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ABSTRACT The suppression of DNA synthesis in host and tumor tissues by methotrexate has been monitored in mice by determining the in vivo incorporation of tritium-labeled deoxyuridine ($[^3\text{H}]\text{UdR}$) into DNA. The duration of inhibition of $[^3\text{H}]\text{UdR}$ incorporation in normal tissues was related to the dose of methotrexate and was a direct function of plasma drug concentration. $[^3\text{H}]\text{UdR}$ incorporation recovered to 50% of pretreatment levels in bone marrow when plasma methotrexate concentration was 10^{-8} M or less, irrespective of the dose administered, while 50% recovery of DNA synthesis in intestinal epithelium was not observed until plasma methotrexate levels were 5×10^{-9} M or less. Ascitic L1210 leukemia cells did not fully return to pretreatment levels of $[^3\text{H}]\text{UdR}$ incorporation at any time, although a partial recovery of incorporation was noted at methotrexate ascitic fluid concentrations of approximately 10^{-8} M.

Methotrexate did not suppress the incorporation of tritium-labeled thymidine ($[^3\text{H}]\text{TdR}$) into bone marrow and duodenal mucosa, confirming the specificity of its action in inhibiting thymidylate synthesis in host tissues. In the ascites tumor a gradual decline in $[^3\text{H}]\text{TdR}$ incorporation was seen after methotrexate, indicating that the tumor tissue depression of $[^3\text{H}]\text{UdR}$ incorporation is not solely due to inhibition of thymidylate synthesis.

These studies indicate that host tissues are inhibited by extremely low concentrations of methotrexate, and indicate the importance of the slow final phase ($t_3 = 12$ h) of drug elimination from plasma in producing a prolonged exposure of sensitive host tissues to inhibitory drug concentrations.

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INTRODUCTION

Many aspects of the pharmacology of antineoplastic agents have been studied in depth, including mechanisms of action, metabolism, transport, and pharmacokinetics. However, little is known at present about the relationship between plasma concentrations of these agents and their pharmacologic effect on normal and neoplastic tissue.

The antineoplastic agents in current clinical use are primarily inhibitors of various steps in DNA synthesis and exert their main effects on rapidly dividing cells such as bone marrow, intestinal epithelium, and neoplastic tissue. Methods for the quantitation of drug effect on target tissues have recently been developed, based on the mechanisms of action of agents such as methotrexate (1) and cytosine arabinoside (2). Hryniuk and Bertino (1) showed that the folate antagonist methotrexate, which blocks the conversion of deoxyuridylate to thymidylate (3), specifically suppressed the incorporation of tritium-labeled deoxyuridine ($[^3\text{H}]\text{UdR}$)¹ into DNA in sensitive leukemic cells in vitro. Margolis, Philips, and Sternberg (4) employed sequential determinations of $[^3\text{H}]\text{UdR}$ incorporation to monitor the inhibitory effect of methotrexate on DNA synthesis in mouse intestinal epithelium in vivo and thus were able to demonstrate the sequence of biochemical and histologic changes induced by methotrexate toxicity in this target tissue. In this study, a close relationship was found between the duration of inhibition of DNA synthesis in mouse intestine and the presence of free intracellular methotrexate, but the final phase of methotrexate disappearance from plasma was not studied, and the relationship of inhibition of DNA

¹ Abbreviations used in this paper: $[^3\text{H}]\text{TdR}$, tritium-labeled thymidine; $[^3\text{H}]\text{UdR}$, tritium-labeled deoxyuridine.

synthesis to plasma methotrexate remained to be established. In the following studies, we have utilized serial observations of [^3H]UdR incorporation into the DNA of bone marrow, intestinal epithelium, and tumorous ascites to define the relationship between plasma methotrexate concentrations and inhibition of DNA synthesis *in vivo*.

METHODS

Methotrexate concentrations. Methotrexate concentrations in plasma and ascites were determined by the dihydrofolate reductase inhibition assay as described by Bertino and Fischer (5). Dihydrofolate reductase purified by affinity chromatography (6) from L1210 leukemia cells and of specific activity 200 U/mg protein was used in the assay. The lower limit of sensitivity of this assay is 2×10^{-10} M methotrexate in the assay cuvette. This sensitivity allows determination of plasma methotrexate levels as low as 1×10^{-9} M.

[^3H]UdR incorporation studies. [^3H]UdR (1.9 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. 100 μCi was injected i.p. into male BDF₁ mice weighing 18–23 g, and at specified time intervals, animals were killed by cervical dislocation. A 5-cm segment of duodenum was obtained and slit longitudinally; the epithelium was stripped from the underlying muscularis by firm scraping with the edge of a glass microscopic slide and dispersed in iced phosphate-buffered saline. Bone marrow cells were removed from the tibias by inserting a hypodermic needle into one end of the medullary cavity and expelling the marrow content with a jet of iced buffered saline. In tumor-bearing mice, ascitic L1210 leukemia cells were obtained by lavage of the peritoneal cavity with iced buffered saline. DNA was extracted from each tissue specimen by the method of Schneider (7). A portion of the final supernate was dissolved in Aquasol (New England Nuclear) for measurement of ^3H radioactivity in the DNA, and a second portion was used for determination of the concentration of DNA by Burton's method (8). Results were expressed as counts per minute ^3H per microgram DNA.

Preliminary studies of [^3H]UdR incorporation into DNA in both normal and tumor-bearing animals indicated that deoxynucleoside incorporation in both bone marrow and duodenal mucosa increased linearly for 20 min after isotope injection before approaching a maximum value at 1 h, and declining slowly thereafter (Fig. 1). In the ascitic tumor, [^3H]UdR incorporation into DNA occurred more rapidly, reaching a maximum value within 10 min and persisting at that level for 90 min. Because of the multiple tissues to be sampled from each animal, an interval of 1 h after [^3H]UdR injection was chosen for sacrifice of the animals, this length of time allowing the maximum level of incorporation achieved in all three tissues.

Correlation of [^3H]UdR incorporation and plasma methotrexate concentration. At time 0, male BDF₁ mice were given 5, 50, or 350 mg/kg of methotrexate by i.p. injection in a volume of 0.01–0.015 ml/g of body weight. In experiments on tumor-bearing mice, $1\text{--}2 \times 10^6$ L1210 leukemia cells were injected i.p. on day 0, and methotrexate was given on day 6 at 9:00 a.m.

At selected intervals after methotrexate administration, duplicate groups of three mice were each given 100 μCi [^3H]UdR i.p., and 1 h later, the animals were sacrificed

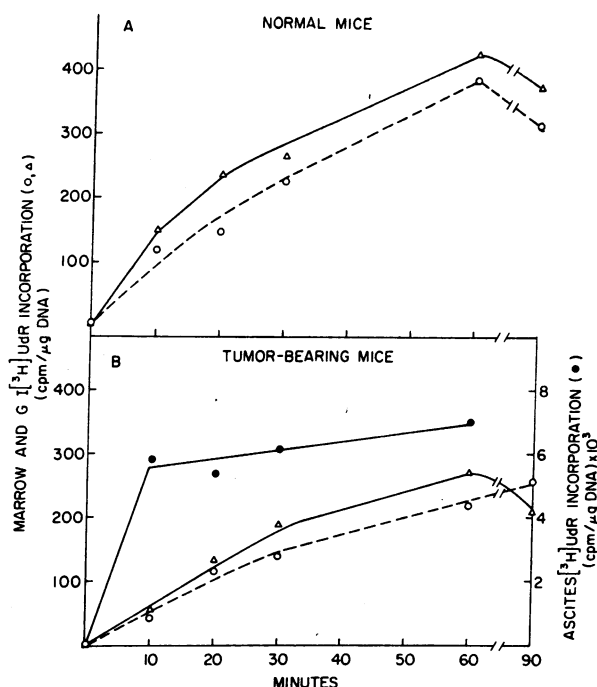


FIGURE 1 [^3H]UdR incorporation into DNA of bone marrow (O--O), duodenal epithelium (Δ--Δ), and ascitic L1210 cells (●--●) as a function of time after i.p. injection of 100 μCi of the deoxynucleoside.

by cervical dislocation. Plasma for methotrexate determination was obtained by cardiac puncture. Bone marrow, duodenal mucosa, and ascitic cells from the three animals in each group were pooled for determination of [^3H]UdR incorporation into DNA. The duplicate values for [^3H]UdR incorporation for each time point, representing determinations performed on tissues from the two groups of animals, were in close agreement: the standard deviation was 10.1%, and duplicates were averaged to obtain the experimental value.

Studies of the incorporation of tritium-labeled thymidine ([^3H]TdR) into DNA after methotrexate administration were performed in a manner identical to those with [^3H]UdR.

Ascitic fluid cell counts. The number of ascitic cells remaining at various time intervals after 5, 50, or 350 mg/kg of methotrexate was determined by sacrificing groups of 10 mice at each time point. The abdominal cavity of each animal was exposed, free ascitic fluid aspirated, and the cavity rinsed repeatedly with iced buffered saline until the washing solution was clear. The fluid from each group of 10 animals was pooled, and cells were counted in a Coulter Model F (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). The cell counts were expressed as the average number of cells per animal.

autoradiographic studies. 50 μCi of [^3H]UdR were injected i.p. into groups of three mice bearing L1210 ascites tumors; 1 h later the mice were killed by cervical dislocation, and the ascites fluid was aspirated and pooled. Tumor cells were fixed in a methanol:glacial acetic acid solution (4:1), which was then spread on gelatinized slides, allowed to dry, and covered with AR-10 stripping

film (Kodak Limited, London, England). The slides were stored in light-tight plastic boxes at 4°C for 14–21 days and developed with Kodak 19D developer. After fixation and drying, the slides were stained with Giemsa stain for 27 min. The labeling index was determined by counting 1,000 ascites tumor cells and given by the ratio of labeled to total cells (9).

RESULTS

Initial studies were directed at determining the effects of methotrexate on [³H]UdR incorporation into DNA of bone marrow, duodenal mucosa, and ascitic tumor cells. As shown in Fig. 2, 5 mg/kg of methotrexate markedly inhibited [³H]UdR incorporation for 6 h in all three tissues, bone marrow recovered fully by 12 h, and duodenal mucosa recovered to only 50% of pretreatment level by this time. Ascitic tumor [³H]UdR incorporation also showed evidence of recovery between 6 and 12 h, although never fully returning to pretreatment levels.

A 10-fold higher dose of methotrexate, 50 mg/kg (Fig. 3), inhibited DNA synthesis for a longer period

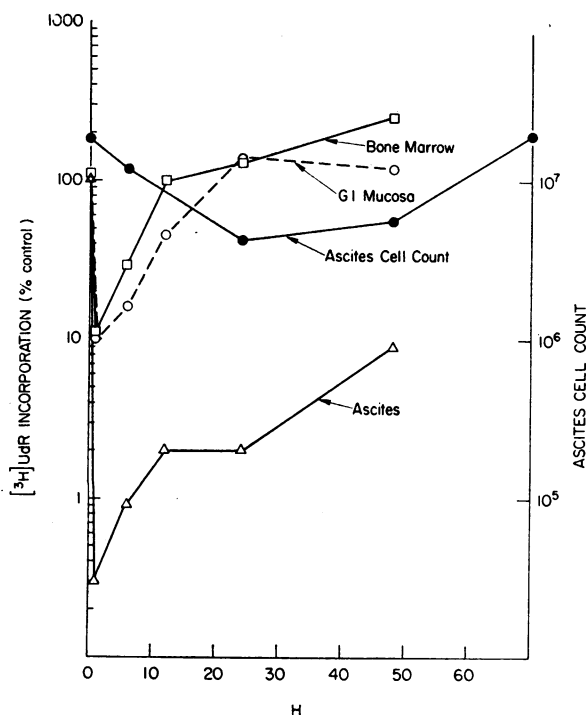


FIGURE 2 [³H]UdR incorporation into DNA of bone marrow, duodenal epithelium (GI mucosa), and ascitic L1210 cells at specific times after administration of methotrexate, 5 mg/kg, at time 0. Simultaneous determination of ascites cell count (●—●) is given on the right hand scale. Control incorporation levels 60 min after [³H]UdR injection were: bone marrow, 215 cpm/μg DNA; duodenal epithelium, 270 cpm/μg DNA; and ascites tumor, 5,500 cpm/μg DNA.

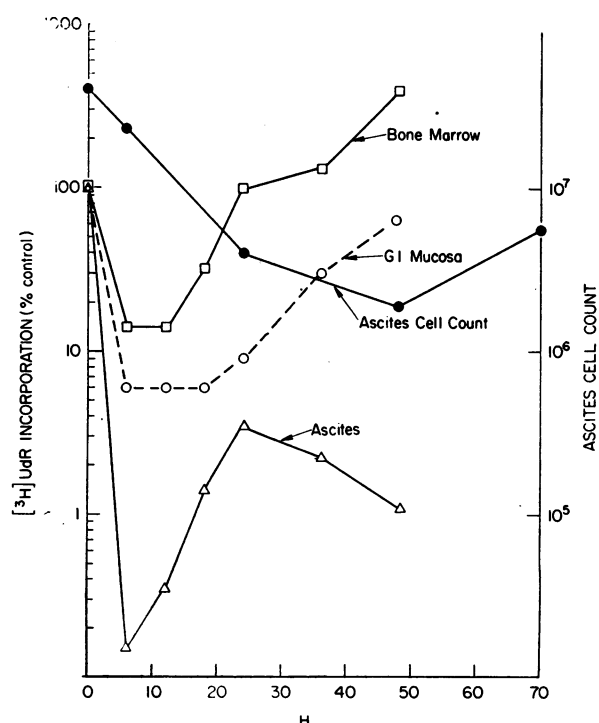


FIGURE 3 [³H]UdR incorporation into DNA and ascites cell count after administration of methotrexate, 50 mg/kg, at time 0.

in all three tissues. Bone marrow incorporation of [³H]UdR recovered fully by 24 h, but intestinal mucosa remained inhibited for 36 h. Tumor cell [³H]UdR incorporation appeared to recover partially after 18 h but again failed to reach pretreatment levels for the duration of the study.

After 350 mg/kg of methotrexate, an LD₅₀ dose (Fig. 4), inhibition of [³H]UdR incorporation into bone marrow DNA persisted for 36 h, while duodenal mucosa recovered only after 48 h. A partial recovery of ascitic tumor incorporation was seen at 36 h.

Identical studies were performed with [³H]TdR as the labeled deoxynucleoside to determine the specificity of the effect of methotrexate on thymidylate synthesis. All three doses of methotrexate (5, 50, and 350 mg/kg) produced an initial rise in [³H]TdR incorporation into bone marrow DNA, but the incorporation of [³H]TdR into duodenal mucosa remained unchanged (Table I). These findings contrast with the rapid fall in [³H]UdR incorporation seen in both tissues after methotrexate dosage, confirming the selective inhibitory effect of methotrexate on the conversion of dUMP to dTMP. However, in the ascites tumor, a gradual decline in [³H]TdR incorporation was seen after methotrexate dosage, reaching a nadir of 7% of the pretreatment level 72 h after 350 mg/kg. Lesser changes in tumor

cell [^3H]TdR incorporation were seen with smaller doses of methotrexate.

In order to relate inhibition of [^3H]UdR incorporation to methotrexate pharmacokinetics, plasma concentration curves for each of the doses of methotrexate used in this study were determined by the dihydrofolate reductase inhibition assay (Fig. 5). Rapid disappearance of methotrexate was seen during the first 6 h after drug administration; thereafter a slow final phase of disappearance, characterized by a plasma half-life of 12 h, was found in both normal and tumor-bearing animals. Inhibition of DNA synthesis, as indicated by [^3H]UdR incorporation into DNA, was virtually complete in host tissues when the concentration of methotrexate in plasma was greater than 10^{-8} M for all doses of methotrexate studied (Figs. 6a and b). [^3H]UdR incorporation in bone marrow recovered to 50% of pretreatment levels only when plasma antifolate concentration fell below 10^{-8} M, but 50% recovery occurred in duodenal epithelium at plasma methotrexate concentrations of 5×10^{-9} M or less. A comparison of recovery times of bone marrow, duodenal mucosa, and ascitic tumor cells is given in Table II. The delayed recovery of intestinal mucosa as opposed to bone marrow has been confirmed in a second strain of mice (the CDF₁ strain) and was significant at $P < 0.05$ by rank analysis (10).

In the L1210 ascitic tumor, the interpretation of recovery after methotrexate was hampered by the failure of [^3H]UdR incorporation to return to pretreatment levels. However, a definite rebound in [^3H]UdR incorporation was observed at specific times for each dose level of methotrexate and was accompanied by a slowing in the rate of decrease of the ascitic cell population, as seen in Figs. 2-4. An actual increase in tumor

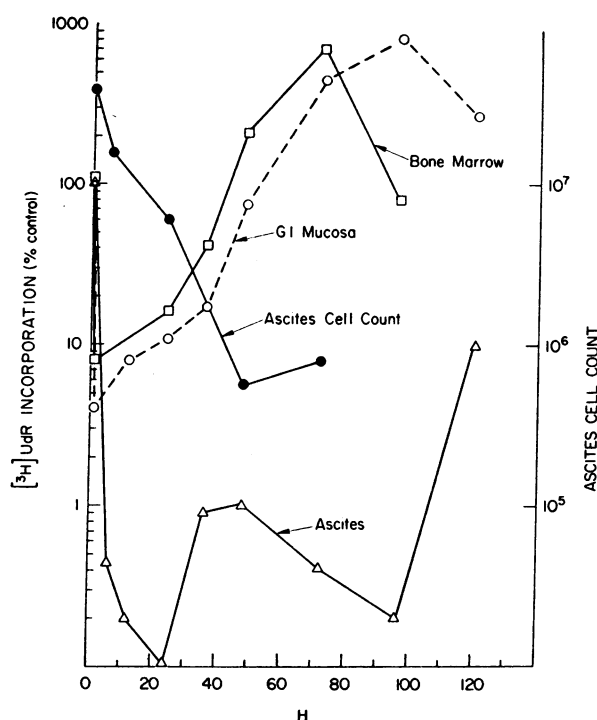


FIGURE 4 [^3H]UdR incorporation into DNA and ascitic cell count after administration of methotrexate, 350 mg/kg, at time 0.

cell count was observed 24-36 h after the initial upswing in [^3H]UdR incorporation. The partial recovery of tumor incorporation of deoxyuridine was seen at a methotrexate concentration of 10^{-8} M in the ascitic fluid for dosages of 5 and 50 mg/kg. At a dosage of 350 mg/kg, sufficient ascitic fluid could not be obtained at the time of partial recovery to determine the drug

TABLE I
[^3H]TdR Incorporation into DNA after Methotrexate

Dose	Tissue	Time						
		0	1	6	12	24	48	72
<i>cpm</i> $\times 10^3/\mu\text{g DNA}$								
5 mg/kg	Bone marrow	1.5	3.6	3.5	2.5*	—	—	
	GI mucosa	3.5	4.1	3.3	3.5*	—	—	
	Ascitic cells	140.0	110.0	46.0	68.0	—	—	
50 mg/kg	Bone marrow	1.5	3.5	3.5	—	4.4*	6.5	
	GI mucosa	3.5	5.5	3.4	—	2.1	7.4*	
	Ascitic cells	140.0	140.0	46.0	—	40.0	15.0	
350 mg/kg	Bone marrow	1.5	3.5	3.0	—	3.0	4.2*	6.2
	GI mucosa	3.5	4.3	3.0	—	2.5	2.6	8.5*
	Ascitic cells	140.0	132.0	38.0	—	50.0	12.0	10.5

* Recovery of [^3H]UdR incorporation 50% or greater at this time.

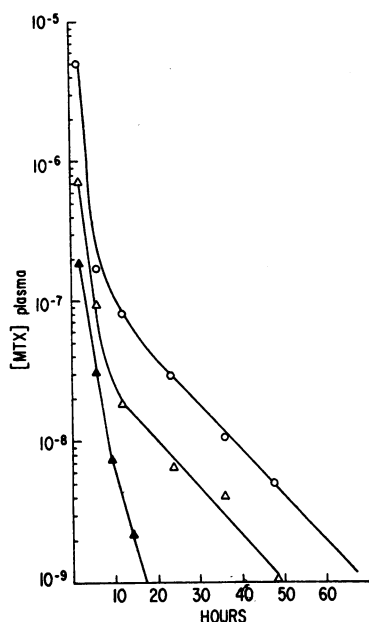


FIGURE 5 Plasma methotrexate concentration after i.p. injection of 5 (\blacktriangle — \blacktriangle), 50 (\triangle — \triangle), or 350 mg/kg (\circ — \circ) methotrexate (MTX) into BDF₁ male mice. Curves for normal and tumor-bearing mice were identical.

concentration, although plasma methotrexate, which was 50% of ascitic methotrexate for the lower doses, was 10^{-8} M at that time.

In order to elucidate the reason(s) for failure of [3 H]UdR to return to pretreatment levels after therapy, we studied the effect of methotrexate on the autoradiographic labeling index of ascites tumor cells exposed to a pulse dose of [3 H]UdR. Before therapy, 65.1% of L1210 leukemia cells were labeled by a single i.p. injection of [3 H]UdR, while at the time of partial recovery after methotrexate, the labeling indices were 24.1% (12 h after 5 mg/kg), 28% (24 h after 50 mg/kg), and 21.2% (120 h after 350 mg/kg). At these times, 78–89% of cells were viable as judged by trypan blue exclusion. Thus, only a fraction of the cells usually in DNA synthesis were actively synthesizing DNA during the recovery phase after methotrexate, which implies either an alteration in the cell cycle or a reduction in the growth fraction induced by chemotherapy.

DISCUSSION

The foregoing studies indicate that an important relationship exists between extracellular concentration of the antineoplastic agent methotrexate and its inhibitory effect on DNA synthesis in host tissues and ascites tumor. The duration of inhibition of [3 H]UdR incorporation in these tissues was directly related to dosage and in turn to the persistence of methotrexate in plasma and ascites during the final phase of drug elimination. This final phase of methotrexate disappearance, with a plasma half-time of 12 h, appears to contribute sig-

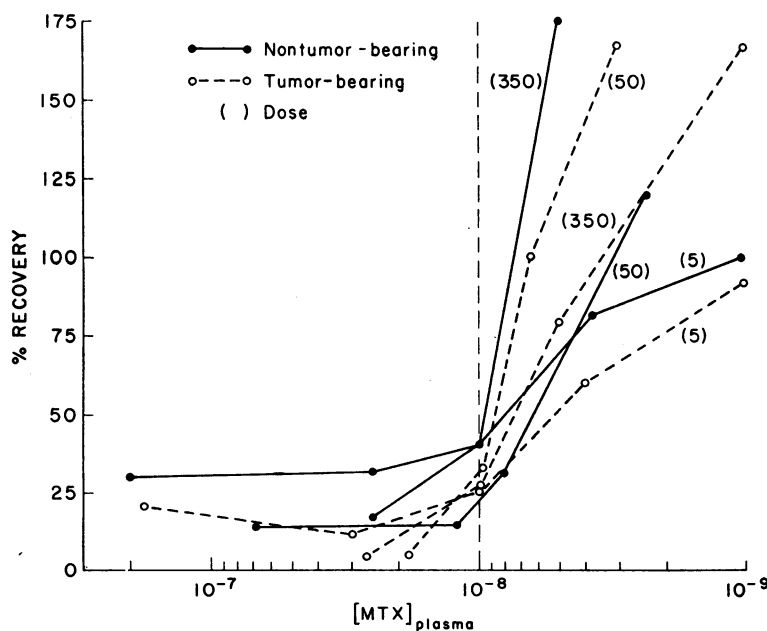


FIGURE 6a Inhibition of [3 H]UdR incorporation into DNA in bone marrow as a function of plasma methotrexate concentration. Inhibition is expressed as the percent of pretreatment incorporation. The dose which applies to the individual curves, expressed in mg/kg, is indicated in parentheses. The dashed line is drawn to separate inhibitory and noninhibitory plasma levels of methotrexate (MTX).

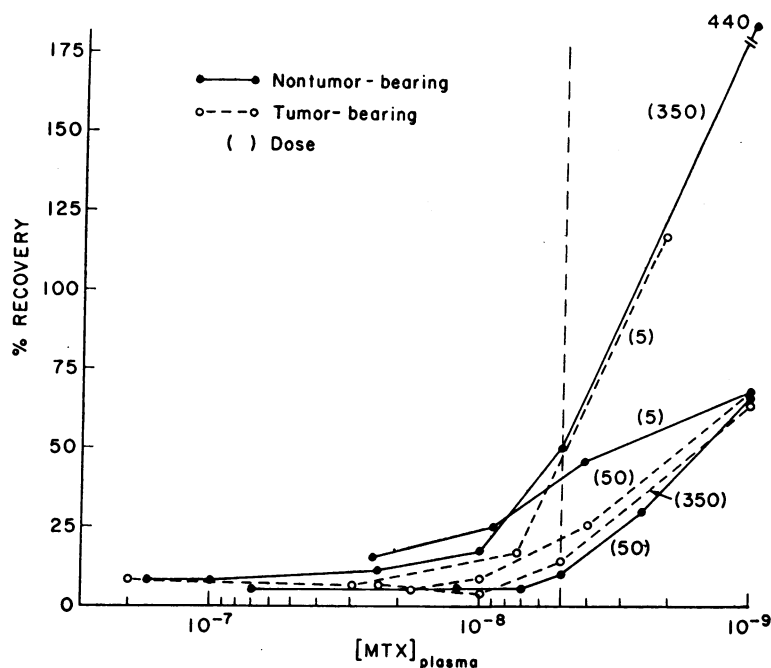


FIGURE 6b Inhibition of [^3H]UdR incorporation into DNA in intestinal epithelium as a function of plasma methotrexate concentration.

nificantly to the toxicity of large single doses or multiple dose regimens, both of which result in prolonged exposure of sensitive host tissues to low levels (10^{-8} to 10^{-7} M) of methotrexate. The present work demonstrates that, over a 70-fold range of methotrexate dosage, recovery of DNA synthesis in host tissues is found only when plasma methotrexate concentration is 10^{-8} M or less.

Other experimental evidence supports the concept that the persistence of low levels of methotrexate is responsible for host toxicity. Previous work from this laboratory has shown that the toxicity of otherwise lethal doses of methotrexate may be prevented by administration of a methotrexate-metabolizing enzyme, carboxypeptidase G₁, 24 h after the antifolate (11). In this instance, rescue from methotrexate toxicity was associated with eliminating low levels of residual methotrexate, less than 10^{-7} M, from extracellular fluid without affecting the established intracellular blockade of dihydrofolate reductase. Additional corroboration was furnished by Zaharko and Dedrick (12), who showed that repeated small doses of methotrexate, maintaining plasma methotrexate at 10^{-7} M for 30 h, were capable of producing lethal toxicity. Margolis, et al., (4) also found that a close relationship existed between the duration of inhibition of DNA synthesis in mouse intestine and the presence of free intracellular methotrexate after doses of 0.5, 5.0, and 50 mg/kg. However, plasma methotrex-

ate levels below 10^{-7} M (50 ng/ml) were not defined in their study, and the relationship of recovery of DNA synthesis to plasma methotrexate was not established.

These findings are entirely in keeping with current knowledge of methotrexate's pharmacologic action as

TABLE II
Time Required for Recovery of [^3H]UdR Incorporation after Methotrexate

Dose MTX mg/kg	Time to 50% recovery of [^3H]UdR incorporation		
	Marrow	Intestinal epithelium	Ascites tumor*
Normal mice			
5	9-12	9-12	—
50	18-24	36-48	—
350	36-48	72	—
Tumor-bearing mice			
5	9-12	12	12
50	18-26	36-50	18
350	36	48-72	36-48

* Initial peak of recovery in ascites tumor did not reach 50% of control value. (See text and Figs. 2-4). Recovery here is defined as time to increase in DNA-labeling to greater than 100 cpm $^3\text{H}/\mu\text{g}$ DNA.

an inhibitor of DNA synthesis, with little effect on cells not in the S phase of the cell cycle (13). The duration of persistence of inhibitory levels of drug would determine the number of cells exposed as they enter S phase, and thus the number of cells killed.

Several factors may be responsible for reversal of methotrexate inhibition at 10^{-8} M. Although methotrexate acts as a titrating inhibitor of mammalian dihydrofolate reductase at pH 5.9, its binding at higher pH's is reversible, and at concentrations less than 10^{-8} M inhibition is likely to be only partial (14). Secondly, cellular uptake of methotrexate has been shown to take place by an active transport mechanism shared by certain other folates (15), including the predominant circulating compound, 5-methyl tetrahydrofolic acid, which in mice is present in concentrations of 10^{-7} M (16). Lesser concentration of methotrexate might thus be excluded from transport by the endogenous folates.

The delay in recovery of duodenal mucosa as compared to bone marrow observed in this study is consistent with previous work that showed that the lethality of high doses of the antifolate in mice is due to intestinal denudation rather than myelosuppression (17). This delay may indicate a greater sensitivity of intestinal mucosa or may result from the enterohepatic circulation of the drug, which produces a higher concentration of methotrexate in the lumen of small intestine as compared to plasma (18). Whether intraluminal methotrexate affects DNA synthesis in the intestinal crypts, where most reproductive activity takes place, is not known.

The effects of methotrexate on ascitic L1210 cells differed from that seen in host tissues. The duration of inhibition of [3 H]UdR incorporation in the L1210 cells was related to dose, but the initial recovery peak did not reach pretreatment levels after any of the doses examined. The gradual decline in [3 H]TdR incorporation in the ascitic tumor after methotrexate indicates that factors other than inhibition of thymidylate synthesis may be affecting [3 H]UdR incorporation in the tumor. Several of these factors may be (a) accumulation of intact but lethally injured, nondividing cells in the ascitic fluid, (b) prolongation of DNA synthesis by dihydrofolate reductase inhibition in injured but viable cells; (c) inhibition of purine biosynthesis by methotrexate (19), although the gradual nature of the fall in [3 H]TdR incorporation is against that possibility; or (d) selection of a residual tumor cell population with altered cell cycle characteristics, such as a reduced growth fraction, a more prolonged cell cycle, or a decreased rate of DNA synthesis. Available data does not allow a judgment as to the relative contribution of each of these factors, although the marked re-

duction in labeling index observed during the initial recovery period after methotrexate suggests that alterations in cell cycle characteristics are likely to be operative.

The foregoing study has yielded information about the sensitivity of mouse bone marrow and duodenal mucosa to methotrexate, and indicates the importance of the final slow phase of drug disappearance from plasma in producing host toxicity. It is likely that the sensitivity of various neoplasms to methotrexate will vary widely, as has been indicated by previous clinical (3, 20) and experimental observations (21, 22), and will have to be determined for each tumor individually. However, this type of information about plasma pharmacokinetics and host and tumor sensitivity, if obtained in individual patients, might prove of value in the design of improved drug administration schedules in clinical chemotherapy.

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