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The Mechanism of Action of Methotrexate

I. Interaction with a Low-Affinity Intracellular Site Required for Maximum Inhibition of Deoxyribonucleic Acid Synthesis in L-Cell Mouse Fibroblasts

I. DAVID GOLDMAN[1]

Departments of Medicine and Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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SUMMARY

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L-cell mouse fibroblasts were loaded with methotrexate (MTX) *in vitro* to a level which exceeded the capacity of high-affinity intracellular binding sites, following which MTX in excess of the tightly bound fraction was eliminated from the intracellular and extracellular compartments. Although binding of MTX was irreversible over the short interval of these experiments and the medium was free of folates and serum, cells continued to incorporate deoxyuridine into DNA at a rate which was depressed by only 27 %. Upon further exposure of cells to MTX there was increased inhibition of deoxyuridine incorporation into DNA, which was a hyperbolic function of the extracellular and intracellular MTX concentrations, with 50 % inhibition at 0.2 μM and 0.2–0.4 μM, respectively. The net cellular uptake of radioactivity after exposure of cells to deoxyuridine was characterized by an initial rapid uptake of label, following which the net cellular uptake slowed to approximately the rate of incorporation of label into cellular constituents which do not penetrate the cell membrane. The net cellular uptake of label over 5 min was not decreased by a reduction of temperature from 37° to 23.5°. A 5-min exposure of cells to MTX at 37° markedly inhibited net cellular uptake of radioactivity, but this process was unaffected by MTX at 23.5° (when influx of MTX was markedly reduced) unless the cells had first been loaded with MTX at 37°. MTX inhibited net cellular uptake of radioactivity under conditions in which incorporation of deoxyuridine into DNA was already negligible, and inhibited incorporation of label into the trichloracetic acid supernatant fraction. MTX was not metabolized by L-cells. MTX did not accelerate the initial rate of efflux of a rapid-exit component of radioactivity from cells loaded with N^5-methyl[^{14}C]tetrahydrofolate, but quickly displaced (in less than 17 min) a small fraction of a slow-exit component. However, even after exposure of cells to 12 μM MTX (sufficient for complete suppression of deoxyuridine incorporation into DNA) for 30 min (an interval which should be sufficient to eliminate displaceable endogenous tetrahydrofolates for this MTX level), and under conditions in which the medium should have been cleared of

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displaced folates, exchangeable intracellular MTX in the range of 0.2–0.4 μM still produced marked inhibition of deoxyuridine incorporation into DNA in comparison to cells in which all exchangeable intracellular MTX was eliminated. These studies suggest that in addition to tight binding to dihydrofolate reductase, MTX inhibits a lower-affinity receptor site necessary for the maintenance of deoxyuridine metabolism.

INTRODUCTION

Previous studies from this laboratory characterized aspects of the mechanism by which the folate compounds and their synthetic analogue methotrexate traverse mammalian cell membranes (1–6). The energetics of the uptake process were described (2, 5), and manifestations of heteroexchange diffusion between MTX[2] and the folates were evaluated and quantitated (1, 3–5). In this report similar techniques have been utilized to assess the relationship between the inhibition of DNA synthesis by MTX, as determined from the effect of this agent on the incorporation of deoxyuridine into DNA, and the physical state of MTX within the cell. This experimental approach quantitates the interaction between MTX and its intracellular target site(s) within the milieu of the intact cell, which is neither spatially nor biochemically perturbed, and provides data which complement studies that define the interaction between the drug and isolated target enzymes in cell-free systems. Since an analysis of the effect of MTX on DNA synthesis involves interactions between this agent and the naturally occurring folates, both within the cell, and heteroexchange phenomena at the level of their common membrane carrier, experimental designs were developed to discriminate between these factors.

MTX inhibition of DNA synthesis has been related to its tight binding to dihydrofolate reductase, blocking the regeneration of tetrahydrofolate from dihydrofolate produced in the synthesis of deoxythymidylate from deoxyuridylate (7–9). According to this scheme, (a) intracellular MTX beyond that necessary for saturation of high-affinity dihydrofolate reductase sites should not contribute directly to the inhibition of DNA synthesis, although it may be a factor in the duration of inhibition by inactivating new enzyme as it is synthesized, and (b) since influx of MTX across the cell membrane is rate-limiting to subsequent binding within the cell (1, 10), and cytotoxicity requires only saturation of these sites, influx is considered a major determinant of the efficacy of this agent (7). However, resistance to MTX in some cells in which MTX influx is decreased (11) cannot be accounted for by the alteration in influx per se; rather, the crucial change may be a depression in the intracellular electrochemical potential for MTX, a parameter controlled by the membrane transport system, which may change in parallel with changes in influx (12). Studies have suggested that free intracellular MTX may be required for maximum inhibition of DNA synthesis (13–15), and the data reported in this and the accompanying paper (16) indicate that maximum suppression of DNA synthesis by MTX, at least over the short intervals of these experiments, requires the continuous exposure of cells to extracellular MTX in excess of 6 μM, a phenomenon which is related to the accumulation of intracellular MTX in excess of that required for complete association with high-affinity intracellular binding sites (to be referred to as "exchangeable" MTX).[3] This is of particular interest, since metabolic poisons increase this component of intracellular MTX, presumably as a result of inhibition of energy-dependent processes which limit the accumulation of exchangeable intracellular MTX (2, 17). Vincristine

[2] The abbreviation used is: MTX, methotrexate, 4-amino-N^{10}-methylpteroylglutamic acid.

[3] "Exchangeable" MTX will refer to that component of intracellular MTX in excess of the tightly bound fraction. This includes MTX which is osmotically active and MTX which is loosely bound within the cell. "Free" MTX will refer to that component which is osmotically active within the intracellular water and contributes to the intracellular electrochemical potential.

sulfate appears to act as a metabolic poison (18) to increase MTX uptake into tumor cells (18, 19) and increase MTX cytotoxicity (19). In the accompanying paper (16) it is suggested that the basis of the augmented cytotoxicity of MTX by vincristine may be related to the increased suppression of DNA synthesis which accompanies the vincristine-induced augmentation of MTX accumulation within the cell. A brief report on these studies has been published (20).

MATERIALS AND METHODS

Cells, media, and incubation techniques. L-cell mouse fibroblasts were grown in suspension culture as previously described (18). In preparation for experiments, cells in log phase growth were separated from their culture medium by centrifugation, washed twice in 0° buffer (composed of the electrolytes from the following medium), then resuspended in Eagle's medium (21) without serum, modified to contain 10 mM glucose, no folic acid or phenol red, and the following salts: NaCl, 117 mM; KCl, 5.2 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 13 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 2.1 mM. The cell suspensions were incubated in specially designed flasks which permit warmed, humidified 95% O_2–5% CO_2 to pass over the surface of the suspension. Cells were continuously dispersed by gentle mixing with a Teflon paddle. The pH of the suspension was maintained at 7.0–7.4 over a 1.5-hr incubation, and the temperature was 37° unless otherwise indicated. Cytocrits were less than 4%. Cell counts were in the range of 5–15 × 10^6 cells/ml.

Experimental techniques and analytical procedures. For measurement of radioactivity associated with intact cells, cell suspensions were exposed to labeled MTX, N^5-methyltetrahydrofolate, or deoxyuridine. At specified intervals, portions of the cell suspensions were injected into 10 volumes of 0° buffered 0.85% NaCl, pH 7.4 (to be referred to as NaCl solution). The cells were separated by centrifugation at 2000 × *g* for 30–60 sec, then washed twice with 5 ml of the 0° NaCl solution. The cell pellets were aspirated into the tip of a Pasteur pipette, extruded onto polyethylene tares, and dried overnight at 70°. The dried cells were peeled off the polyethylene tares and weighed directly on a Beckman LM800 automatic microbalance, then transferred to 20-ml liquid scintillation vials tilted in a specially designed rack so that the subsequent addition of 0.1 ml of 1 N KOH would completely encompass the cell pellet. After a 1-hr digestion at 70°, the vials were cooled, 10 ml of a methanol-toluene fluor solution (1) were added, and radioactivity was determined on a liquid scintillation spectrometer.

The incorporation of deoxyuridine into DNA was determined by measurement of radioactivity in the 0° trichloracetic acid precipitate after exposure of cells to labeled deoxyuridine. Cells were first washed twice with 0° NaCl solution. The cell pellet was then dispersed in 0.5 ml of this solution, to which 0.5 ml of 10% trichloracetic acid at 0° was added. After 5 min at 0°, the acid precipitate was separated by centrifugation, washed twice with 5 ml of 5% trichloracetic acid at 0°, then transferred to polyethylene tares and processed as described above except for an additional incubation of the KOH digest with 0.5 ml of "NCS" solubilizer (Amersham/Searle) at 65° for 30 min prior to the addition of the scintillation fluor. Final counting efficiencies established with [^{14}C]- and [^{3}H]toluene internal standards were in the range of 75% and 25%, respectively.

Intracellular water content was determined from the difference between the wet and dry weight of a cell pellet less the [^{14}C]-inulin space as reported previously (1, 6).

The experimental techniques employed in the measurement of uptake and efflux kinetics have been described (1, 2, 4); details of experimental design are outlined under RESULTS. Because the extracellular compartment is very large in comparison to the intracellular space, changes in the level of extracellular substrate during uptake into cells were negligible. Likewise, because of the low cytocrit and frequent replacement of the medium, accumulation of appreciable levels of substrate in the extracellular compartment during unidirectional efflux or related studies was excluded.

Data are expressed as nanomoles of radioactive label (or nonmetabolized substrate,

when that was established) per gram of cells, dry weight, or per gram of trichloracetic acid precipitate, dry weight, or as micromoles per liter of intracellular water. Statistical analysis of differences between experimental conditions employed a standard paired *t*-test. Numbers of experiments performed refer to experiments from different days. In each experiment at least five replicate determinations of each condition were made, and the average was employed for statistical analysis. When measurements involved slope analysis, each slope was determined from a least-squares evaluation of a minimum of six points.

Chemicals. [3′5′-^{3}H]MTX and N^{5}-methyl-[^{14}C]tetrahydrofolate were obtained from Amersham/Searle. Labeled or unlabeled MTX was purified by DEAE-cellulose ion-exchange column chromatography (1). The purity of N^{5}-methyl[^{14}C]tetrahydrofolate was verified by a modification of the method of Noronha and Silverman (22) as previously described (4). N^{5}-Formyltetrahydrofolate and unlabeled MTX were obtained from Lederle Laboratories. Both N^{5}-methyl- and N^{5}-formyltetrahydrofolate are racemic mixtures, and indicated concentrations refer only to the "active" L isomer. [2-^{3}H]Deoxyuridine, [6-^{14}C]deoxyuridine, and [^{14}C]inulin were obtained from New England Nuclear Corporation.

Recovery of intracellular radioactivity. Cells were incubated with [^{3}H]MTX for 50 min, then washed twice with 0° NaCl solution and suspended in 0.1 N NH_4HCO_3. The cells were disrupted by sonic oscillation, then centrifugated at 9000 × *g* for 20 min at 4°, following which the supernatant fluid was fractionated on a DEAE-cellulose column (1). By this technique more than 98% of the intracellular label in three experiments was recovered in the MTX-containing fractions.

To determine the composition of radioactivity in the acid precipitate after exposure to deoxyuridine, cells were incubated with 0.1 μM [2-^{3}H]deoxyuridine for 1 hr, following which the cell fraction was washed twice with 0° NaCl solution and then exposed to 0.2 N $HClO_4$ or 5% trichloracetic acid, each at 0°, as described above. In three studies, 90–98% of the radioactivity associated with the washed trichloracetic acid precipitate or a washed 0.2 N $HClO_4$ precipitate was extracted by a 30-min incubation with 0.4 N $HClO_4$ at 70°. Hence, in agreement with other investigators (14), the major portion of radioactivity associated with the acid precipitate of L-cells is incorporated into DNA, and this technique appears to be a reliable indicator of the rate of utilization of deoxyuridine by the metabolic pathways leading to the synthesis of DNA.

RESULTS

Kinetics of dissociation of MTX from L-cells. Figure 1 illustrates an experiment in which L-cells were loaded with MTX to a level which exceeded the capacity of the high-affinity intracellular binding sites, following which the cell fraction was separated and resuspended in MTX-free medium, and the time course of the unidirectional efflux of MTX was monitored. As observed for several other cell systems (1, 6, 16), two intracellular MTX components can be distinguished by this technique. One component rapidly leaves the cell with a half-time in the range of 2 min (the "exchangeable component"); the other intracellular MTX component appears to be tightly bound within the cell. To evaluate the reversibility of MTX binding to high-affinity intracellular sites over short intervals under the condi-

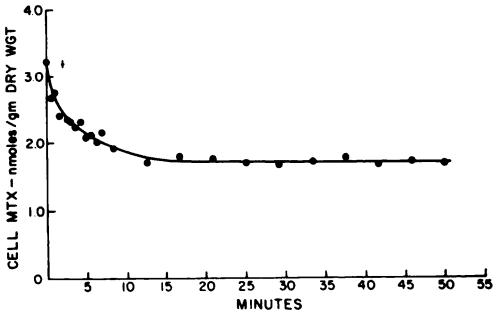

FIG. 1. *Time course of unidirectional efflux of MTX*

Cells were incubated with 6 μM MTX for 5 min at 37°. The cell fraction was separated by centrifugation, washed in 0° buffer, then resuspended in a large volume of 37° MTX-free medium, and the fall in the intracellular MTX level was monitored. The arrow indicates the half-time for efflux of the exchangeable intracellular component.

tions of these experiments, the rate of change in the intracellular MTX level was monitored for 25–55 min following resuspension of cells in MTX-free medium. In eight such experiments there was no significant loss of intracellular MTX ($p > 0.5$), and over an interval of at least 30 min following resuspension in MTX-free medium association of MTX with high-affinity intracellular binding sites was more than 99% complete.

MTX inhibition of deoxyuridine metabolism; role of exchangeable MTX. In the experiment of Fig. 2, cells were incubated with 6 μM MTX for 5 min as described in Fig. 1, then divided into two portions. The cell fractions were separated and resuspended in media containing deoxyuridine in the presence or absence of 6 μM MTX. Even though association of MTX with high-affinity intracellular binding sites is more than 99% complete under both conditions, within 5 min following resuspension a difference in the rate of deoxyuridine incorporation into DNA

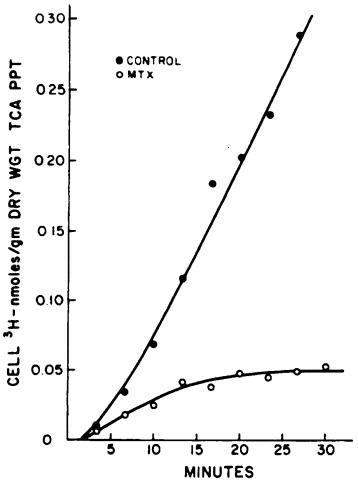

FIG. 2. *Deoxyuridine incorporation into DNA under conditions in which exchangeable intracellular methotrexate is changing*

Cells were incubated with 6 μM MTX at 37° for 5 min, then divided into two portions. The cell fractions were separated by centrifugation, washed with 0° buffer, and resuspended in medium containing 0.05 μM deoxyuridine in the presence or absence of 6 μM MTX. The time course of the incorporation of label into the trichloracetic acid (TCA) precipitate was monitored (representative of five experiments).

can be observed, with a marked depression in the cells resuspended into the MTX-containing medium. There is a similar difference between the rates of deoxyuridine incorporation into the trichloracetic acid supernatant fraction. The increasing rate of deoxyuridine incorporation over the initial 10 min in cells resuspended in MTX-free medium is related to the falling level of exchangeable intracellular MTX (Fig. 1) and the rising level of intracellular deoxyuridine and its metabolites over this interval (see next section). The increasing suppression of deoxyuridine incorporation into DNA in the cells resuspended in MTX-containing medium is related to the continued cellular uptake of MTX and the increasing exchangeable intracellular level.

The experiment of Fig. 3 compares the incorporation of deoxyuridine into the trichloracetic acid precipitate of cells not exposed to MTX (line *A*), cells in which the high-affinity intracellular binding sites were saturated with MTX but negligible exchangeable MTX was present (line *B*), and cells in which high-affinity binding sites were saturated with MTX but, in addition, increasing levels of exchangeable MTX were present within the intracellular and extracellular compartments (lines *C–E*). Deoxyuridine was added to the cells 30 min after resuspension in the test media to allow sufficient time for exchangeable intracellular MTX to exit from cells resuspended in MTX-free medium (*B*) and to ensure that the intracellular MTX level had achieved a steady state in those cells resuspended in MTX-containing medium (*C–E*). It is clear that deoxyuridine metabolism continues when high-affinity binding sites are associated with MTX but exchangeable intracellular MTX is negligible (line *B*). In five such experiments the mean depression of deoxyuridine metabolism under these conditions was 27.4 ± 10.4% (the high standard error reflects the variability from day to day). This suggests that high-affinity binding sites may not be of major importance to the incorporation of deoxyuridine into DNA over this interval of exposure to MTX. When MTX was added to the extracellular compartment there was a progressive reduc-

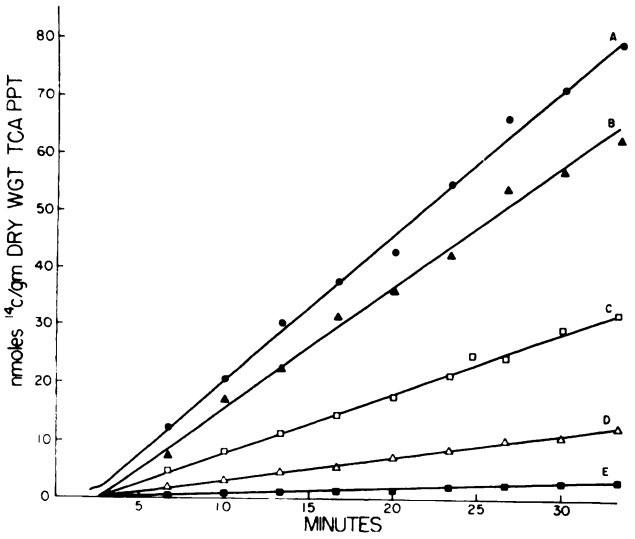

FIG. 3. *Effect of methotrexate on deoxyuridine incorporation into DNA under steady-state conditions for exchangeable intracellular methotrexate*

A cell suspension was divided into two portions. One was exposed to medium containing 6 μM MTX for 5 min, and the other, a control, to MTX-free medium. The cell fractions were separated by centrifugation, washed with 0° buffer, and resuspended in fresh medium. The control cells (*A*) were again suspended in MTX-free medium, while the other cells were suspended in media in the absence of MTX (*B*) or in the presence of 0.12 μM (*C*), 0.6 μM (*D*), or 6.0 μM MTX (*E*). After 15 min the cells were separated, washed, and resuspended in fresh medium of the same composition. After an additional 15 min of incubation, when steady-state conditions for intracellular MTX had been achieved, deoxyuridine was added, and the rate of incorporation of label into the trichloracetic acid precipitate was monitored. The final deoxyuridine level was 1.3 μM. The results were similar over a deoxyuridine concentration range of at least 0.05–1.3 μM.

tion in the rate of deoxyuridine metabolism as the extracellular MTX level was raised from 0.12 to 6.0 μM. However, at each level of extracellular MTX, the rate of deoxyuridine incorporation into DNA was perfectly constant with time. The results of Fig. 3 were unaltered by 0.35 mM cycloheximide, a level sufficient to abolish protein synthesis. In view of the brief duration of these experiments, the constancy of deoxyuridine incorporation into DNA when high-affinity binding sites are saturated with MTX (Fig. 3, line *B*), and the failure of cycloheximide to alter the results, the possibility that new synthesis of dihydrofolate reductase contributes to the sustained metabolism of deoxyuridine in these experiments can be excluded.

Figure 4 is derived from a composite of four experiments similar to Fig. 3 and illustrates the percentage inhibition of deoxyuridine incorporation into DNA by exchangeable MTX (inhibition in excess of that observed when high-affinity binding sites are saturated but no exchangeable MTX is present) as a function of the extracellular MTX level. The dashed line indicates that 50% inhibition of this component of deoxyuridine metabolism occurs at an extracellular MTX level of 0.2 μM. The relationship between exchangeable intracellular MTX and inhibition of deoxyuridine metabolism is more complex. Previous studies have shown that exchangeable intracellular MTX is a hyperbolic function of extracellular MTX in L1210 leukemia cells (1). The data in Table 1 indicate that the ratio of the exchangeable intracellular to extracellular MTX level at the steady state in L-cells also falls as extracellular MTX is increased. The kinetics of this relationship was not quantitated precisely, so that the kinetics of inhibition of deoxyuridine metabolism as related to exchangeable intracellular MTX

is not defined; however, this relationship approximates a hyperbolic form similar to that of Fig. 4, in which the intracellular level of exchangeable MTX which results in 50% inhibition of deoxyuridine metabolism is in the range of 0.2–0.4 μM.

Nature of association of labeled deoxyuridine with L-cells and effect of temperature on MTX inhibition of net cellular uptake of label. The data of Fig. 2 and 3 suggest that maximum suppression of deoxyuridine metabolism by MTX requires exchangeable intracellular MTX. In this and the following section alternative possibilities are evaluated. Studies were undertaken to determine whether inhibition of deoxyuridine metabolism by exchangeable MTX may be due to inhibition of deoxyuridine transport into the cell by extracellular MTX, limiting the availability of deoxyuridine for subsequent intracellular metabolism, rather than to an interaction between exchangeable intracellular MTX and an intracellular target site. These studies were also necessary to understand better the nature of the association of deoxyuridine with L-cells and the significance of alterations in that process. Figure 5 illustrates the time course of the net cellular uptake of label on exposure of cells to [^{14}C]- or [^{3}H]deoxyuridine. At the arrows, portions of the cells were separated and resuspended in deoxyuridine-free medium to determine the exit characteristics of the label (this is representative of four such experiments). Intracellular radioactivity that can leave the cell appears within the intracellular compartment early in the uptake process. However, the major fraction of

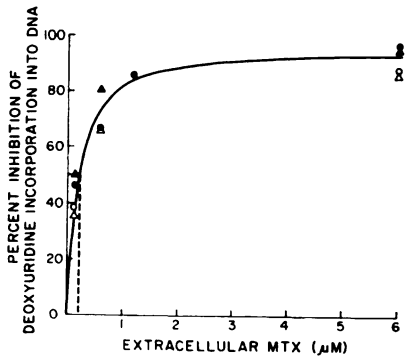

FIG. 4. *Percentage inhibition of deoxyuridine incorporation into DNA in the presence of exchangeable intracellular MTX, as a function of extracellular MTX level*

This was determined from the percentage depression of deoxyuridine incorporation into DNA in cells containing exchangeable MTX (lines *C–E*, Fig. 3) as compared to cells in which high-affinity binding sites were saturated with MTX but exchangeable intracellular MTX was not present (line *B*, Fig. 3). The different symbols represent four experiments performed on different days.

TABLE 1

Relationship between exchangeable intracellular MTX and extracellular concentration under steady-state conditions

Values are the means ± standard errors of three experiments performed on 3 different days. In each experiment the steady-state intracellular MTX level, the tightly bound fraction, and the cell volume are the averages of five replicate measurements obtained 40–75 min after exposure to MTX.

$[MTX]_e$	Cellular MTX		$[MTX]_i$[b]	$[MTX]_i/[MTX]_e$[c]
	Total	Exchangeable[a]		
μM	*nmoles/g, dry wt*		μM	
0.12	2.41 ± 0.28	0.88 ± 0.18	0.26 ± 0.05	2.01
1.2	5.18 ± 1.29	3.77 ± 0.76	1.12 ± 0.22	0.93
6.0	10.60 ± 1.65	8.86 ± 1.5	2.62 ± 0.47	0.43

[a] Determined from the difference between total cell MTX and the tightly bound fraction as quantitated from unidirectional efflux measurements as in Fig. 1.

[b] Determined by dividing exchangeable cell MTX by the ratio (3.38) of the intracellular water (microliters) to dry weight (milligrams) of a cell pellet.

[c] The distribution ratio for MTX, or ratio of concentration of exchangeable intracellular MTX, $[MTX]_i$, to extracellular MTX, $[MTX]_e$.

intracellular radioactivity cannot leave the cell, and increases rapidly with time. From the dotted line which passes through points *A*, *B*, and *C* (these points indicate the levels of this component of intracellular radioactivity at the time the cell fractions were isolated from the parent suspension), it may be seen that the rate of increase of this intracellular component approximates the rate of net cellular uptake of radioactivity. The time course of the net cellular uptake of label falls slowly over the interval of observation, and any line tangent to the net uptake curve intercepts the ordinate above the point of origin. Within 3 min after exposure of cells to deoxyuridine, the level of intracellular radioactivity that can leave the cell is more than half the extracellular deoxyuridine concentration. These data are consistent with a very rapid initial uptake (unidirectional influx) of label, which is free to leave the cell (presumably osmotically active deoxyuridine), with the rapid buildup of a backflux (unidirectional efflux) of this component of intracellular radioactivity which slows the resultant net uptake process. The subsequent net cellular uptake rate is comparable to the rate of incorporation of radioactivity into intracellular components which must represent at least in part, metabolites of deoxyuridine which do not penetrate the cell membrane. Hence, the data suggest that after a brief interval of exposure to deoxyuridine under these conditions, the intracellular metabolism of deoxyuridine represents to a major extent the net rate of uptake of radioactivity into the cell.

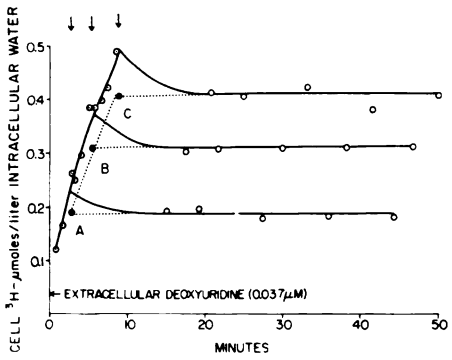

FIG. 5. *Net uptake and subsequent unidirectional efflux of radioactivity in cells exposed to 0.037 μM deoxyruidine at zero time*

At the arrows, portions of the cell suspension were separated by centrifugation, washed with 0° buffer, and resuspended in a large volume of deoxyuridine-free medium. Points *A*, *B*, and *C* represent the extrapolation of the level of intracellular label which does not penetrate the cell membrane, indicated by the horizontal dotted lines, to the time the cell fractions were isolated from the parent suspension. The diagonal dotted line is the slope through points *A*, *B*, and *C* and indicates the rate of incorporation of radioactivity into this intracellular component.

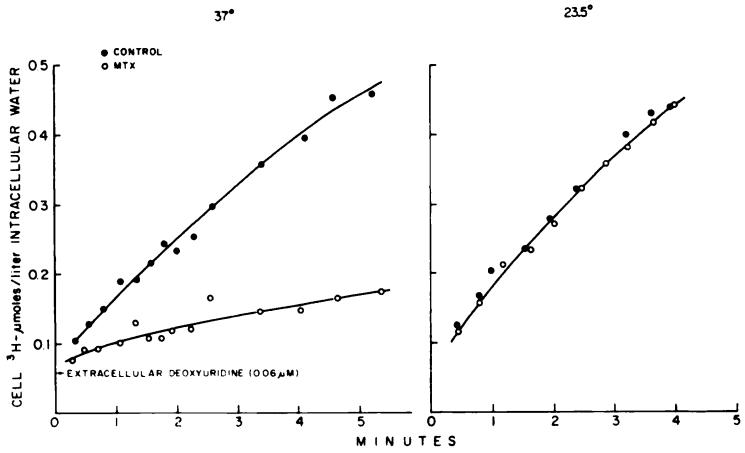

FIG. 6. *Time course of net uptake of radioactivity on exposure of cells to 0.06 μM deoxyuridine at 37° (left) or 23.5° (right) in the presence* (○) *or absence* (●) *of 6 μM MTX*

Cells were exposed to MTX 5 min prior to addition of deoxyuridine.

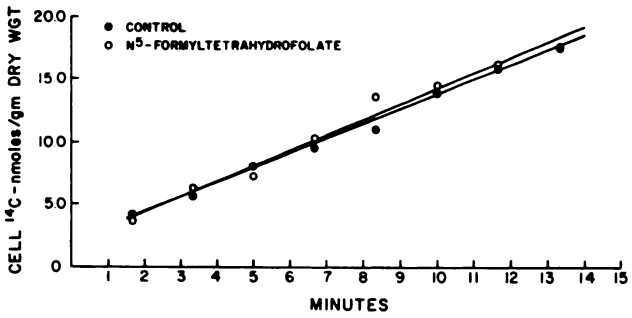

FIG. 7. *Time course of net cellular uptake of radioactivity on exposure of cells to 0.46 μM deoxyuridine in the presence or absence of 6 μM N^5-formyltetrahydrofolate*

N^5-Formyltetrahydrofolate was added 5 min prior to deoxyuridine. In five similar experiments 6 μM N^5-formyltetrahydrofolate did not alter the net uptake of 0.02 μM deoxyuridine (ratio of uptake slope of control to slope of N^5-formyltetrahydrofolate = 0.98 ± 0.10; $p > 0.5$).

When cells at 37° are exposed to MTX 5 min prior to addition of deoxyuridine, net uptake of radioactivity is inhibited. Although the net cellular uptake of radioactivity over 5 min is unchanged or slightly increased by a reduction in temperature to 23.5°, this process is unaffected by MTX (Fig. 6). The failure of MTX to inhibit net uptake of radioactivity at 23.5° suggests that MTX does not inhibit the membrane transport of deoxyuridine. The absence of an effect of MTX at the lower temperature is related to the high temperature coefficient for the MTX influx process ($Q_{23.5-37°} = 7.1$) and a rate of penetration of MTX into the cell which is so slow that saturation of the high-affinity binding sites with the accumulation of sufficient exchangeable intracellular MTX to inhibit deoxyuridine metabolism does not occur over the interval of observation. Over longer intervals of incubation, however, sufficient intracellular MTX accumulates to result in inhibition of net uptake of radioactivity. The lack of an effect of MTX on deoxyuridine transport is further supported by the observation that N^5-formyltetrahydrofolate, which has a high affinity for the same transport carrier as MTX (see next section) but is not an inhibitor of deoxyuridine metabolism, does not alter the net uptake of radioactivity at 37° (Fig. 7).

The critical role of exchangeable intracellular MTX is further suggested by the experiment of Fig. 8. Here cells were divided into three portions. Portions A and B were

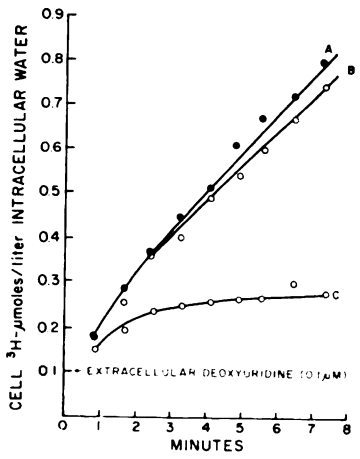

FIG. 8. *Role of exchangeable intracellular methotrexate in the inhibition of deoxyuridine incorporation into DNA*

Cells were divided into three portions, A, B, and C. Portions A and B were suspended in MTX-free medium, while C was exposed to medium containing 6 μM MTX. Following a 5-min incubation at 37°, the cell fractions were separated by centrifugation and washed with 0° buffer, and all portions were resuspended in fresh media at 25°. Portion A was continued in MTX-free medium, while B and C were suspended in media containing 6 μM MTX. At zero time the cells were exposed to 0.1 μM deoxyuridine, and the net cellular uptake of radioactivity was monitored.

suspended in MTX-free medium while C was exposed to 6 μM MTX. Following a 5-min incubation at 37°, during which high-affinity binding sites in portion C were saturated with MTX and exchangeable intracellular

MTX accumulated, all the cell fractions were separated and resuspended in fresh media at 25°. Portion A was continued in MTX-free medium, while B and C were exposed to 6 μM MTX, and the net uptake of radioactivity after addition of deoxyuridine was monitored over the next 7 min. Marked suppression of net uptake of radioactivity occurred only in those cell first incubated with MTX at 37°; i.e., those cells which would contain exchangeable MTX in the intracellular compartment. However, the possibility that this phenomenon is related to an increased loss of intracellular tetrahydrofolates during the 37° prior incubation with MTX rather than to the subsequent presence of exchangeable intracellular MTX during exposure of cells to deoxyuridine cannot be excluded, and is evaluated in the next section. The very prominent early fall in the rate of net cellular uptake of radioactivity in the presence of MTX (Figs. 6 and 8) is attributed to the rapid buildup and backflux of radioactivity, under conditions in which the metabolism of deoxyuridylate is reduced, and radioactivity which is free to leave the cell represents a larger than usual component of total cellular radioactivity over the interval of observation.

Although changes in the net uptake of radioactivity are negligible with a reduction of temperature to 23.5° (Fig. 6), incorporation of radioactivity into the trichloracetic acid precipitate is completely abolished at this temperature over an interval of exposure to deoxyuridine of at least 5 min. This has also been reported for other cell systems (23, 24). Indeed, over this interval incorporation of radioactivity into the acid precipitate is a small component of the total cellular radioactivity even at 37°, and accounts for only a small portion of the intracellular radioactivity which does not penetrate the cell membrane (an average of 16% in three experiments). Hence inhibition of net cellular uptake of radioactivity by MTX at 25° (Fig. 8) or over brief intervals of exposure to deoxyuridine at 37° (Fig. 6) must be due to a block in the metabolism of a labeled derivative of deoxyuridine prior to the incorporation of deoxythymidylate into DNA.

Heteroexchange and displacement phenomena. Studies were undertaken to exclude the possibility that MTX inhibition of deoxyuridine metabolism is related to the presence of extracellular MTX with MTX-folate heteroexchange phenomena at the level of their common membrane carrier and to explore the mechanism by which exchangeable intracellular MTX inhibits deoxyuridine metabolism.

MTX, N^5-formyltetrahydrofolate, and N^5-methyltetrahydrofolate share a common carrier transport system in several mammalian cells (1, 4, 6, 25). This was also observed for the L-cell. Extracellular N^5-formyltetrahydrofolate inhibits the unidirectional influx of MTX and results in a fall in the electrochemical potential for intracellular MTX when added to cells at the steady state with MTX (countertransport). Extracellular MTX or N^5-formyltetrahydrofolate inhibits the unidirectional influx of N^5-methyltetrahydrofolate. The following studies evaluate whether MTX depression of deoxyuridine metabolism is related to a transconcentration phenomenon, in which an acceleration of the carrier-mediated efflux of tetrahydrofolate cofactor stores from within the intracellular compartment is induced by the simultaneous entry of extracellular MTX into the cell via the same carrier. N^5-Methyl[^{14}C]tetrahydrofolate was employed as the tracer compound (see DISCUSSION). Cells were loaded in the presence of 1 μM N^5-methyltetrahydrofolate for 1 hr, following which the cell fraction was separated and resuspended in a large volume of N^5-methyltetrahydrofolate-free medium in the presence or absence of 1.2 μM MTX, a level at least 10 times that necessary to augment suppression of deoxyuridine metabolism (Fig. 3, line *C*). Exit of radioactivity is characterized by a rapid and a slow component (Fig. 9). In five such experiments, 1.2 μM MTX did not significantly alter the exit of either components ($p > 0.5$, $p > 0.1$, respectively). In five other experiments (Fig. 10) the 100-sec initial unidirectional efflux of radioactivity was not altered by 6 μM MTX ($p > 0.5$), a level of MTX which completely inhibits deoxyuridine incorporation into DNA (Fig. 4).

Figure 11 illustrates the effect of 6 μM

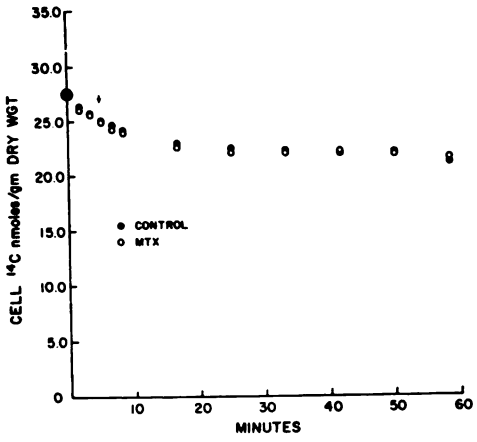

FIG. 9. *Effect of 1.2 μM methotrexate on the unidirectional efflux of label from cells loaded with N^5-methyl[^{14}C]tetrahydrofolate*

Cells were incubated with 1 μM N^5-methyl[^{14}C]-tetrahydrofolate at 37° for 1 hr. The cell fraction was then separated by centrifugation, washed twice with 0° buffer, and resuspended in a large volume of N^5-methyltetrahydrofolate-free medium at 37° in the presence (○) or absence (●) of 1.2 μM MTX, and the unidirectional efflux of radioactivity was monitored. The arrow indicates the half-time for efflux of the rapid-exit component. The zero-time point indicates the cellular radioactivity prior to resuspension.

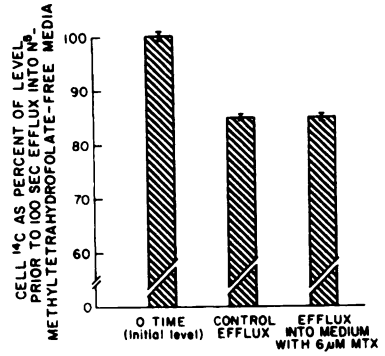

FIG. 10. *Evaluation of a possible MTX-tetrahydrofolate cofactor transconcentration phenomenon*

Cells were incubated with 2 μM N^5-methyl[^{14}C]-tetrahydrofolate for 1 hr, washed as described in Fig. 9, and divided into 15 equal portions in 15-ml conical centrifuge tubes. After centrifugation at 250 × *g* for 1 min, the supernatant fluid was aspirated completely, the tubes were gassed with 95% O_2–5% CO_2 and capped, and the tip of the tube containing the cell pellet was kept in an ice-water bath. The level of cell radioactivity prior to efflux was determined in five tubes. For measurement of the unidirectional efflux rates, the tube was removed from the ice bath and immersed in a 37° bath for 20 sec to warm the glass and pellet. Following this, 2 ml of medium at 37° (either control or containing 6 μM MTX) were rapidly injected, the cell pellet was thoroughly dispersed by three vigorous manual shakes and a Vortex mix, and the tube was mechanically agitated at high speed in an Eberbach shaker bath at 37° for 100 sec. The efflux reaction was stopped by injection of 10 ml of NaCl solution at 0°. Five replicate determinations were made with or without MTX. The bars indicate the means ± standard errors of the average values from five such experiments. The higher loading level of N^5-methyltetrahydrofolate was employed to increase the fraction of the total cellular radioactivity associated with the rapid-exit component to facilitate the recognition of small changes in this component (see DISCUSSION).

MTX on the slow-exit component of intracellular radioactivity. Following incubation of cells with N^5-methyltetrahydrofolate, the cell fraction was washed and incubated in fresh N^5-methyltetrahydrofolate-free medium for 17 min. The wash and 17-min incubation were repeated, following which the cells were washed, divided into two portions, and resuspended in media in the presence or absence of 6 μM MTX. MTX reduced the level of intracellular radioactivity by 1.134 ± 0.078 nmoles/g, dry weight ($p < 0.01$ in five experiments). The frequent washes, replacement of the medium, and long incubation prior to exposure to MTX should result in the loss of more than 99.9% of the rapid-exit component and eliminate the possibility that the loss of radioactivity was due to countertransport of free intracellular radioactivity which might remain in the cell in equilibrium with small amounts of residual extracellular N^5-methyltetrahydrofolate. The loss of cell radioactivity was not due to displacement from binding sites on the cell surface, since no loss of radioactivity resulted when cells were exposed to 6 μM MTX at 0° under the conditions of Fig. 11. In five such experiments at 0°, the difference between the cellular level of radioactivity with and without MTX was 0.069 ± 0.079 nmoles/g, dry weight, a value not significantly different from zero ($p > 0.2$) and only 6% of the amount lost at 37°. This loss of the slow component of intracellular radioactivity by

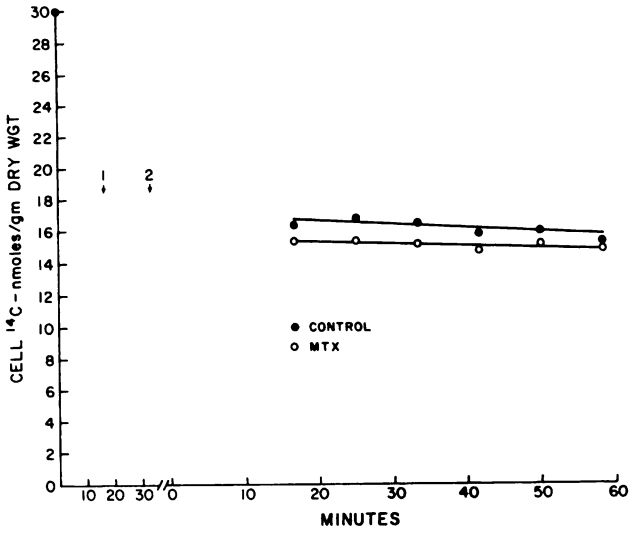

FIG. 11. *Displacement of 6 μM methotrexate of slowly exchanging label from cells loaded with N^5-methyl-[^{14}C]tetrahydrofolate*

Cells were incubated with 2 μM N^5-methyl[^{14}C]tetrahydrofolate for 1 hr then resuspended in fresh medium as described in Fig. 9. At arrow 1 the cells were separated, washed, and resuspended in fresh medium. At arrow 2 this was repeated, but now half the cells were resuspended in medium containing 6 μM MTX (○), while the other half remained in MTX-free medium (●). From 17 to 50 min later the level of cell radioactivity was measured.

MTX may have been due to displacement of labeled tetrahydrofolates from binding sites within the cell or to inhibition of the reutilization (and subsequent efflux) of a portion of the labile [^{14}C]methyl moiety when tetrahydrofolate synthesis was inhibited. In any event, the reduction of intracellular radioactivity by MTX was rapid and was essentially complete within 17 min after exposure to MTX, since the ratio of the slopes of the slow exit components, MTX/control, was 0.93 ± 0.28, a value not significantly different from 1($p > 0.5$ from five experiments such as Fig. 11).

The experiment of Fig. 12 is based upon the data of Figs. 4 and 11 and was designed to determine whether possible displacement of intracellular tetrahydrofolates from intracellular binding sites by exchangeable intracellular MTX could be an important factor in the inhibition of deoxyuridine metabolism by this agent. Cells were incubated with 12 μM MTX for 30 min to achieve total association of MTX with high-affinity binding sites and to generate an exchangeable intracellular level sufficient to produce maximum suppression of deoxyuridine metabolism (see Fig. 4). Displacement of intracellular tetrahydrofolates for this level of MTX should have been complete over this interval of incubation (Fig. 11; see DISCUSSION). The cells were then washed twice with 0° buffer to remove extracellular MTX and folates which had left the intracellular compartment. The cell fraction was divided into two portions and resuspended in fresh MTX-free medium (A) or medium containing 0.2 μM MTX (B). Incubation was continued for 20 min, during which exchangeable intracellular MTX left the A cells and the exchangeable intracellular MTX level fell to a steady state in the B cells. The cells were again washed twice with 0° buffer. Portion A cells were again suspended in fresh MTX-free medium. B was divided into two portions, one resuspended in MTX-free medium (B) and the other in medium with 0.2 μM MTX (C). After an additional 17 min of incubation the cells were washed and resuspended in fresh media of the same respective compositions, deoxyuridine was added, and metabolism was monitored over

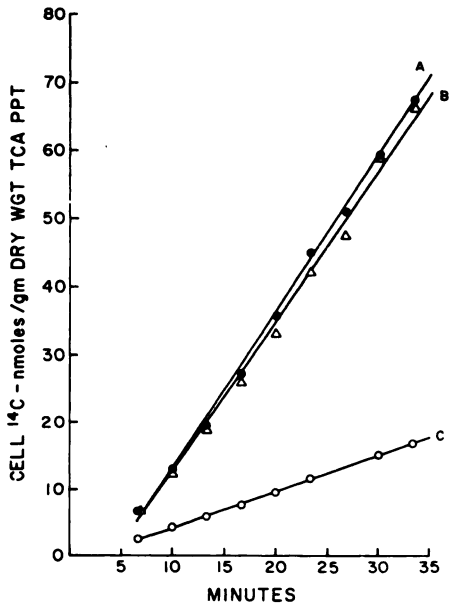

FIG. 12. *Evaluation of the role of displacement of intracellular tetrahydrofolates by methotrexate on methotrexate inhibition of DNA synthesis.*

Cells were incubated with 12 μM MTX for 30 min. The cells were washed twice with 0° buffer, divided into two portions, and resuspended in the presence (*B*) or absence (*A*) of 0.2 μM MTX. After a 20-min incubation the cells were washed again. Portion A was suspended in fresh MTX-free medium, while B was divided into two portions: B, which was now suspended in MTX-free medium, and C, suspended in fresh medium with 0.2 μM MTX. After 17 min the cells were washed again and resuspended in media of the same compositions; deoxyuridine (final level, 3 μM) was added, and the incorporation of radioactivity into the trichloracetic acid (TCA) precipitate was monitored.

35 min. There was no significant difference ($p > 0.5$) in deoxyuridine incorporation into DNA in the B as compared to the A cells. There was, however, a marked depression in the rate of deoxyuridine metabolism in C as compared to B cells (71.68 ± 5.05% in five experiments; $p < 0.001$). This is related to the continued presence of exchangeable intracellular MTX in the C cells and the loss of exchangeable intracellular MTX from the B cells under conditions in which displacement of tetrahydrofolates should have been completed and the displaced tetrahydrofolates should have been eliminated prior to the division of cells into B and C media.

DISCUSSION

When cells are loaded with MTX to a level which exceeds the capacity of high-affinity intracellular binding sites, following which exchangeable intracellular and extracellular MTX is eliminated, there is only a small depression in the rate of incorporation of deoxyuridine into DNA. MTX binding to high-affinity sites is essentially irreversible over the interval of these studies, and saturation of these sites must be at least 99% complete. Folates or serum were not included in the medium, and the results precluded a role for new synthesis of dihydrofolate reductase (see RESULTS). Hence, sustained metabolism of deoxyuridine under these conditions is related to continued catalytic regeneration of tetrahydrofolates or the utilization of intracellular tetrahydrofolate cofactor stores. Complete suppression of deoxyuridine metabolism was achieved only after further addition of MTX to the medium and the accumulation of intracellular MTX in excess of the tightly bound component. The hyperbolic relationship of this further suppression of deoxyuridine metabolism by exchangeable intracellular or extracellular MTX with 50% inhibition at an intracellular MTX level of 0.2–0.4 μM indicates that this phenomenon is related to binding of MTX to a low-affinity intracellular site rather than to the further association of MTX with the less than 1% of the high-affinity sites which might remain free in the absence of exchangeable intracellular MTX. The possibility that a component of high-affinity dihydrofolate reductase is isolated in an intracellular compartment and is only slowly accessible to the loading levels of MTX is excluded. First, it is unlikely that after a 30-min incubation with 12 μM MTX the subsequent exposure of cells to 0.2 μM MTX would further penetrate a compartment to produce a large fall in the rate of deoxyuridine metabolism or that this effect would be rapidly reversible (Fig. 12). Second, if sustained exposure of cells to MTX is required for penetration of a cellular compartment, then, at a given extracellular

MTX level which produces a submaximal depression of deoxyuridine metabolism, the rate of incorporation of radioactivity into DNA should decrease as a function of time. However, under conditions in which cells are at a steady state with different levels of extracellular MTX, the rates of deoxyuridine metabolism are quite constant with time (Figs. 3 and 12).

The data suggest that free intracellular MTX interacts with a low-affinity target site to maximally inhibit deoxyuridine incorporation into DNA. However, to effect changes in the intracellular MTX level required comparable manipulations of the extracellular MTX level. In the accompanying paper (16) the crucial role of exchangeable intracellular MTX is supported by the observation that vincristine augments the net uptake of MTX into Ehrlich ascites tumor cells and enhances inhibition of deoxyuridine incorporation into DNA by MTX without changing either the unidirectional influx or the extracellular MTX level. The data for L-cells also appear to exclude several mechanisms by which extracellular MTX might inhibit deoxyuridine metabolism.

1. MTX does not alter the membrane transport of deoxyuridine. Inhibition by MTX of the net cellular uptake of radioactivity after exposure of cells to deoxyuridine is related to inhibition of incorporation of radioactivity into cellular constituents which do not penetrate the cell membrane.

2. Heteroexchange phenomena between extracellular MTX and tetrahydrofolates at their common membrane carrier could reduce the availability of tetrahydrofolate cofactors for thymidylate synthesis by several mechanisms: (a) inhibition of tetrahydrofolate cofactor influx into the cell, (b) depression in the net level of *free* intracellular tetrahydrofolates because of the initial asymmetrical distribution of MTX across the cell membrane—countertransport (1, 5, 26), or (c) acceleration of the carrier-mediated unidirectional efflux *rate* of free intracellular tetrahydrofolates as MTX enters the cell on the same carrier—transconcentration effect (4, 5, 25, 27). Mechanisms (a) and (b) are excluded because no exogenous folates were added to the medium and intracellular folates free to leave the cell were eliminated from the intracellular and extracellular compartments by the use of very large extracellular volumes in comparison to the intracellular space, frequent replacement of the medium, and long incubations prior to addition of deoxyuridine (Fig. 12). A transconcentration effect (c) is excluded by the demonstration that 6 μM MTX did not alter the initial unidirectional efflux of radioactivity from cells previously incubated with N^5-methyl-[^{14}C]tetrahydrofolate. Furthermore, a transconcentration effect would be of little importance, since the rate of efflux of the rapid exit component is so fast even in the absence of MTX ($t_{1/2} < 5$ min) that more than 99.9% of this fraction of intracellular tetrahydrofolates should have left the cells prior to the addition of deoxyuridine (see Fig. 12). Finally, a stimulatory effect by MTX on the unidirectional efflux rates of intracellular tetrahydrofolates either should be constant with time, since the extracellular MTX level remains constant, or should decrease as MTX enters the cell and competes with the tetrahydrofolates at the inner cell membrane for efflux on the membrane carrier. However, prior to the achievement of steady-state conditions for intracellular MTX, when the free intracellular MTX level is rising but the extracellular MTX level is constant, inhibition of deoxyuridine incorporation into DNA by MTX increases with time (Fig. 2). Trans-stimulation of the unidirectional efflux of radioactivity from L1210 leukemia cells loaded with N^5-formyltetrahydrofolate by higher levels (20 μM) of extracellular MTX has been reported (25). It is possible that conditions could be achieved to demonstrate this phenomenon in L-cells, but this would not be relevant to the conditions under which MTX inhibited deoxyuridine metabolism in these experiments.

Possible heteroexchange phenomena between MTX and the folates complicated interpretation of previous studies. The competitive nature of the protective effect of N^5-formyltetrahydrofolate on aminopterin toxicity to mice (28–30) or MTX toxicity

to L-cell mouse fibroblasts in cell culture (31) may be accounted for, at least in part, on the basis of competitive interactions at the cell membrane that limit, in a competitive way, the availability of tetrahydrofolate cofactors for thymidylate synthesis under conditions in which endogenous tetrahydrofolate synthesis is blocked. Likewise, the reduced toxicity of MTX to mice (32) and the reduced chemotherapeutic efficacy of MTX in L1210 leukemia-bearing mice when administered simultaneously with rather than prior to N^5-formyltetrahydrofolate (33) may be related in part to the competitive inhibition of MTX influx into the cell. The dose-dependent effects of MTX on mortality in mice (15) and on inhibition of deoxyuridine metabolism *in vivo* (13, 15) under conditions in which dihydrofolate reductase was presumed to be inactivated (see below) could be related to competitive inhibition by MTX of the influx of circulating tetrahydrofolates into and inhibition of their net accumulation within cells. The increased inhibition of deoxyuridine incorporation into DNA in mouse leukemia cells *in vitro* with increased extracellular levels of MTX under conditions in which the high-affinity binding sites should have been saturated with this agent could be related to inhibition of influx of tetrahydrofolate cofactors present in the 25% horse serum in the medium (13). Finally, the observation that MTX inhibited deoxyuridine incorporation into DNA in L-cells, even after the cells had been loaded with N^5-formyltetrahydrofolate for 15 min, could be accounted for by rapid countertransport of tetrahydrofolate cofactors from the intracellular compartment, with the subsequent marked inhibition of their influx into the cell (14).

The low-affinity intracellular target site with which MTX interacts to suppress deoxyuridine metabolism is uncertain, but the data are consistent with a block in the synthesis of deoxythymidylate from deoxyuridylate, since $N^{5,10}$-methylenetetrahydrofolate is required for this reaction and MTX inhibits deoxyuridine incorporation into the trichloracetic acid supernatant fraction and inhibits the net cellular uptake of radioactivity under conditions in which incorporation of deoxyuridine into the trichloracetic acid precipitate is negligible. There are several possible mechanisms by which exchangeable intracellular MTX may inhibit deoxyuridine metabolism.

1. It is possible that continued tetrahydrofolate synthesis under conditions in which high-affinity binding sites are saturated with MTX may be related to the presence of dihydrofolate reductase species which loosely bind MTX and are active in the absence of appreciable levels of free intracellular MTX. The constant rate of deoxyuridine incorporation into DNA with time under these conditions is more compatible with continued tetrahydrofolate synthesis than with the utilization and depletion of endogenous tetrahydrofolate cofactor stores. Multiple forms of dihydrofolate reductase have been isolated from bacteria (9, 34, 35) and mammalian cells (9, 36, 37). The affinity of dihydrofolate reductase for MTX (38–40) or irreversible inhibitors (41) is reduced in the absence of TPNH, but it is unclear whether low-affinity forms of this enzyme unassociated with TPNH are present within the intact mammalian cell. The measurement of tetrahydrofolate synthesis under these experimental conditions would clarify whether dihydrofolate reductase activity persists when high-affinity binding sites are saturated with MTX (42).

Following administration of MTX *in vivo*, deoxyuridine metabolism continues in gastrointestinal epithelial tissues (15, 43) and mouse leukemia cells (13), even under conditions in which dihydrofolate reductase activity (in cell homogenates) is negligible. This may be due to cellular tetrahydrofolate cofactors in the assay medium, or the conditions of the enzyme assay may not be comparable to the intact cell, i.e. dihydrofolate reductase activity *in vivo* remains. The observation that dihydrofolate reverses the lethality of MTX to mice *in vivo* suggested sustained dihydrofolate reductase activity (44); however, reduction of dihydrofolate may be related to isolation of unbound enzyme in cellular compartments or to tetrahydrofolate synthesis by intestinal flora. with subsequent absorption.

2. MTX may inhibit utilization of endogenous tetrahydrofolates by inhibiting their release from storage sites, or, as suggested in several reports, by inhibiting thymidylate synthetase. In crude enzyme preparations which contain both dihydrofolate reductase and thymidylate synthetase, aminopterin inhibits thymidylate synthesis only if tetrahydrofolate levels are low and a reductant nucleotide is present, suggesting that inhibition is related to depression of dihydrofolate reductase rather than thymidylate synthetase activity (45). Hence the basis of the inhibition of thymidylate synthesis in homogenates of CCRF-CEM cells is certain (46). MTX inhibition of deoxyuridylate conversion to deoxythymidylate was competitive with tetrahydrofolate in isolates of Ehrlich ascites tumor cells (K_i = 14 μM) and *Escherichia coli* (K_i = 23 μM), employing a dialyzed, 30–50% ammonium sulfate-precipitated fraction (14). Inhibition (20%) of thymidylate synthetase with 50 μM MTX was demonstrated in a further purified *E. coli* preparation, but 50 μM aminopterin had no effect (47). In another study 100 μM aminopterin resulted in 50% inhibition of *E. coli* thymidylate synthetase (48). If the low affinity of thymidylate synthetase for MTX in cell-free systems is comparable to the intact L-cell or the Ehrlich ascites tumor (16), then it is doubtful that this can account for the inhibition of deoxyuridine metabolism by the low levels of exchangeable intracellular MTX (0.2–0.4 μM) which resulted in 50% depression of deoxyuridine incorporation into DNA in these studies. It is possible, of course, that the affinity of this enzyme for MTX in the intact cell is greater.

3. MTX (6 μM) caused a loss of a slow-exit component of radioactivity from cells which had been loaded in the presence of N^5-methyl[^{14}C]tetrahydrofolate. This may represent displacement of tetrahydrofolate cofactors from binding sites, with their subsequent exit from the cell. However, it is unlikely that a displacement phenomenon could be an important factor in the inhibition of deoxyuridine metabolism by exchangeable intracellular MTX. At any level of MTX which produces submaximal inhibition of deoxyuridine metabolism the rate of incorporation of deoxyuridine into DNA is constant under steady-state conditions. Hence, if the basis of this inhibition is a displacement of intracellular tetrahydrofolate cofactors, this process must be completed rapidly at each MTX concentration, a conclusion which is compatible with the observation that displacement of intracellular radioactivity by MTX is complete within 17 min (Fig. 11). However, even after exposure of cells to 12 μM MTX (a level sufficient to inhibit deoxyuridine metabolism completely) for 30 min, which is long enough to complete displacement of endogenous tetrahydrofolates, under conditions in which the medium should have been cleared of displaced folates, an exchangeable intracellular MTX level in the range of 0.2 μM still produced marked inhibition of deoxyuridine incorporation into DNA (Fig. 12).

N^5-Methyl[^{14}C]tetrahydrofolate was employed as a "tracer" in these studies. It is the only commercially available tetrahydrofolate cofactor and is not an ideal tracer for efflux studies. It must be assumed that the rapid exit component represents efflux of unchanged N^5-methyltetrahydrofolate or related compounds. This is likely, since the major metabolites of N^5-formyl[^{14}C]tetrahydrofolate in L1210 cells over short incubations are other labeled tetrahydrofolates (25). Although $N^{5,10}$-methylenetetrahydrofolate is required for synthesis of thymidylate, it is a reasonable assumption from other studies (1, 4, 5, 25) that the tetrahydrofolate cofactors behave similarly in terms of their transport across the cell membrane. Finally, the [^{14}C]methyl moiety is labile and is probably incorporated into unidentified metabolites which penetrate the cell membrane slowly (5) and may represent the major portion of the intracellular radioactivity; their loss from the cell is related to the slow-exit component. Hence alterations in the rapid exit of radioactivity or a small portion of the slow-exit component require discrimination from the much larger total cell radioactivity.

These studies relate to the clinical use of MTX. The end point for therapy with MTX may not be the saturation of high-affinity binding sites with the generation of only a

small excess of free intracellular MTX to inactivate newly synthesized dihydrofolate reductase. Rather, the end point to achieve complete suppression of deoxyuridine incorporation into DNA may be the generation of much higher levels of intracellular MTX. The high levels of exchangeable intracellular MTX that would be maintained during prolonged infusions, as with the "rescue" protocols, may be an important factor in the efficacy of these procedures (49–53). Possible differences between free intracellular MTX levels in host and tumor tissues may be a determinant of the therapeutic index for this agent. These considerations may be relevant to the development of new folate analogues, in that the goal of structural design may be to enhance the electrochemical potential that an "antifol" achieves within the intracellular compartment or to enhance its interaction with target(s) in addition to high-affinity dihydrofolate reductase-binding sites. Finally, this and other studies continue to indicate that the mechanism by which MTX inhibits DNA synthesis is a complex process which is not yet clearly understood and warrants continued investigation. Likewise, the relationship between inhibition of DNA synthesis by exchangeable MTX and the cytotoxic effects of this agent, as well as the effects of exchangeable MTX on purine synthesis, require further clarification.

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