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# Rate-limiting Steps in the Interactions of Fluoropyrimidines and Methotrexate*

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**Abstract**—*Rate-limiting steps are defined between methotrexate (MTX) and 5-fluorouracil (FU) or 5-fluorodeoxyuridine (FUdR) and [$^{14}$C]-formate incorporation into RNA, DNA and protein as a function of the basal rate of dTMP synthesis. When Ehrlich cells are incubated with* 0.1 μM *FUdR,* 1 μM *FU and* 50 μM *MTX for* 1–35 min, [$^{3}$H]*-deoxyuridine (UdR) incorporation into DNA is maximally inhibited within* 1, 10 *and* 15 min *respectively. The delay in suppression of* [$^{3}$H]*-UdR incorporation into MTX-exposed cells compared to cells exposed to FU or FUdR is related to the slow transport of MTX and the increasing free intracellular MTX levels. Influx of MTX is* 4 *and* 10 *times slower than FU and FUdR respectively. At* 2.5, 5, 10 *and* 15 min *the free intracellular MTX levels* (nmol/g *dry wt) are* 5.8, 7.4, 8.7 *and* 8.8 *respectively. Free intracellular FdUMP is identified* 1 min *after exposure of cells to FU and FUdR. Antagonism to MTX-suppression of* [$^{14}$C]*-formate incorporation into RNA, DNA and protein occurs when cells are simultaneously exposed to MTX and FU or FUdR. However,* [$^{14}$C]*-formate incorporation into RNA, DNA and protein is maximally inhibited when Ehrlich tumor cells are incubated with 50* μM *MTX for* 10 min *and then exposed to* 1 μM *FU for* 1 min *(a time in which free intracellular MTX is maximal and* [$^{3}$H]*-UdR incorporation is maximally suppressed). Hence the sequence and time of administration of FU or FUdR and MTX inhibition of formate incorporation into RNA, DNA and protein is related to the rate of (a) FU, FUdR and MTX transport, (b) FU and FUdR metabolism to FdUMP and (c) generation of maximal free intracellular MTX.*

## INTRODUCTION

THE INTERACTION of fluoropyrimidines and methotrexate (MTX) has been studied extensively [1–10]. Previous studies from this laboratory [4, 6, 7] illustrated the importance of the basal rate of thymidylate (dTMP) synthesis as a critical determinant of MTX and fluoropyrimidine cytotoxicity. Our studies also clarified the role of fluoropyrimidine-induced antagonism to free intracellular MTX and tightly bound MTX on tetrahydrofolate (THF)-dependent nucleic acid and protein synthesis [6]. These studies indicated that the basal rate of dTMP synthesis can significantly modify the inhibitory action of MTX on nucleic acid and protein synthesis by affecting the rate at which folate coenzymes are utilized for these processes. The basal rate of dTMP synthesis can affect the inhibitory action of MTX on nucleic acid and protein synthesis by controlling the availability of reduced folates for purine and amino acid synthesis. When cells are pretreated with fluoropyrimidines to reduce the basal rate of dTMP synthesis, the portion of 5, 10-methylene tetrahydrofolate (5, 10-$CH_2$-THF) that is not part of the fluorodeoxyuridylate (FdUMP)–5-10-$CH_2$-THF–thymidylate synethetase (TS) ternary complex [11] will be available in the THF pool for purine and amino acid synthesis [12]. By preventing the oxidation of the 5, 10-$CH_2$-THF, fluoropyrimidines can antagonize the effects of MTX on RNA, DNA and protein synthesis. This mechanism has been proposed as evidence against the use of administering first the fluoropyrimidine 5-fluorouracil (FU) and then MTX in the treatment of malignancy.

Tattersall *et al.* [1] observed that, when cells are exposed simultaneously to FU and MTX, FU reversed the toxicity of MTX. The reversal of MTX toxicity by FU has been attributed to a

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decrease in the depletion of deoxypurines induced by MTX. In rodents, synergism between MTX and FU does not occur when these agents are given simultaneously or when FU administration precedes MTX [2].

Recently, Cadman *et al.* [5, 9] have shown a biochemical basis for synergism between MTX and FU when MTX precedes FU administration. These authors established a correlation between the sequence of administration of MTX and FU, FU incorporation into RNA and synergism in terms of tumor cell kill. When MTX administration precedes FU, MTX blocks purine biosynthesis and phosphoribosylpyrophosphate (PRPP) not utilized there (for purine biosynthesis) becomes available for the conversion of FU to FUMP, with subsequent incorporation into RNA. In addition, pretreatment of L1210 cells with 5-fluorodeoxyuridine (FUdR) prevented the effects of MTX on PRPP pools, i.e. the PRPP pools did not increase. Similar results also occurred when L1210 cells were simultaneously exposed to FUdR and MTX [9].

Antagonistic interactions, upon the simultaneous administration of FU or FUdR and MTX, or when FU or FUdR administration precedes MTX, are based upon their relative rates of transport and the basal rate of dTMP synthesis [13]. Hence the sequence of administration of MTX and the fluoropyrimidines, FU and FUdR, and the biochemical perturbations caused by the combination of these agents should depend, at least in part, upon the rate of their transport, metabolism and interactions with dihydrofolate reductase (DHFR) and thymidylate synthetase. This report defines the relationship between the rate at which MTX, FU and FUdR inhibited dTMP synthesis to (a) MTX, FU and FUdR transport; (b) the generation of free intracellular MTX; and (c) the formation of FdUMP. Hence rate-limiting steps are defined between MTX and fluoropyrimidine interactions with their target sites and THF-dependent RNA, DNA and protein synthesis to determine the proper sequence of administration for biochemical synergism to occur from these agents.

## MATERIALS AND METHODS

*Chemicals*

[$^{14}$C]-Formate, sodium salt (4–53 mCi/mmol) and [$^{3}$H]-UdR (24.5 mCi/mmol) were obtained from New England Nuclear Corporation (Boston, MA, U.S.A.). [$^{3}$H]-FU (1.4 Ci/mmol) and [$^{3}$H]-FUdR (2.3 Ci/mmol) were supplied by Amersham (Arlington Heights, IL, U.S.A.). [$^{3}$H]-FdUMP was obtained from Moravek Biochemicals (Brea, CA, U.S.A.). [$^{3}$H]-MTX (20 Ci/mmol) or non-labeled MTX, obtained from Amersham and Lederle Laboratories (Pearl River, NY, U.S.A.) respectively, were purified by fractionation on DEAE-cellulose ion exchange column as previously described [14]. Non-labeled FU and FUdR were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A.

*Cells, media and incubation techniques*

Ehrlich ascites tumor cells were obtained from CF-1 mice 6–12 days after i.p. injection of 0.2 ml undiluted ascitic fluid and prepared for experimentation as previously described [4, 14]. Cells were suspended in modified Eagle's medium [15] free of folates, serum or methionine, with the following electrolyte composition: 135 mM NaCl, 4.4 mM KCl, 16 mM $NaHCO_3$, 1.1 mM $Na_2HPO_4$, 1.9 mM $CaCl_2$ and 1.0 mM $MgCl_2$. The cell suspension was stirred continuously with a motor-driven Teflon paddle as reported previously [14]. A pH of 7.2–7.4 was maintained during incubation by passing warmed and humidified 95% $O_2$–5% $CO_2$ over the cell suspension at 37°C.

Detailed information on the uptake of radiolabeled MTX, FU and FUdR were determined according to methodology described in previous reports [14, 16, 17]. Specific procedures employed are indicated briefly in the legends to the figures.

[$^{14}$C]-Formate incorporation into RNA, DNA and protein and [$^{3}$H]-UdR incorporation into DNA was determined by measuring $^{14}$C or $^{3}$H in a dilute acid extract of a perchlorate precipitate as reported previously [18]. Data are expressed as means ± S.E.

*Chromotographic techniques*

After incubation with [$^{3}$H]-FU or [$^{3}$H]-FUdR, cells were washed twice with 0.85% NaCl–dipyridamole at 0°C and resuspended in 0°C sodium phosphate (20 mM), pH 7.2. The cell suspension was sonicated four times for 15 sec on a Heat Systems ultrasonic oscillator and cell debris was removed by centrifugation (4°C, 1000 *g* for 5 min). Cell cytosol was then heated at 65°C for 15 min to dissociate the [$^{3}$H]-FdUMP–5-10-$CH_2$THF–thymidylate synthetase ternary complex [19]. The sample was lypholized to dryness, redissolved in $H_2O$ and filtered. [$^{3}$H]-FdUMP analysis was performed by high performance liquid chromatography (Altex model 110 A pump microprocessor-controlled system) using a 5 μm Sperisorb RP-18 column (Brownlee Labs, Santa Clara, CA, U.S.A.). Absorbance was recorded at 254 nm. Elution was carried out with 5 mM tetrabutyl ammonium hydrogen sulfate:3 mM potassium phosphate, pH 7, at a flow rate of 1 ml/min. Fractions were collected and analyzed for radioactivity by liquid scintillation counting.

Cellular [$^3$H]-FdUMP was identified by comparison to the retention time of standard [$^3$H]-FdUMP (32 min).

## RESULTS

The rate at which the fluoropyrimidines, FU and FUdR, inhibit dTMP synthesis can affect the level of reduced folates and determine whether the fluoropyrimidine–MTX combination is antagonistic or synergistic. If fluoropyrimidines inhibit dTMP synthesis faster or at the same rate as MTX, the fluoropyrimidines should decrease the consumption of 5, 10-methylene THF and antagonize MTX effects on nucleic acids and protein when these agents are given simultaneously.

Figure 1 illustrates an experiment which determined the rate of maximal inhibition of [$^3$H]-UdR incorporation into DNA by FU, FUdR and MTX. Ehrlich ascites tumor cells were incubated with 1 μM FU, 0.1 μM FUdR or 50 μM MTX for 50 sec to 35 min, following which cell fractions were resuspended into a medium containing [$^3$H]-UdR in the absence of FU and FUdR or presence of MTX. [$^3$H]-UdR incorporation into DNA is maximally inhibited by FUdR within 1 min, whereas maximal inhibition of [$^3$H]-UdR incorporation into DNA by FU and MTX does not occur until 10 min. The increasing suppression of UdR incorporation into the MTX-exposed cells is related to the increasing exchangeable intracellular level of MTX which accumulates into cells over this interval (Fig. 2). Inhibition of [$^3$H]-UdR incorporation into DNA by FUdR is attributed to FdUMP. FdUMP, which inhibits deoxyuridylate (dUMP) conversion to dTMP [20] and, hence, DNA synthesis, has been shown by Bowen *et al.* [17] to be present in Ehrlich ascites tumor cells 15 sec after exposure to FUdR. An analysis of intracellular [$^3$H]-FdUMP after incubation with [$^3$H]-FU is shown in Fig. 3.

Figure 2 is a representative experiment which illustrates (a) the uptake of [$^3$H]-MTX and subsequent retention of a fraction of labeled drug during efflux in MTX-free medium and (b) the time required for maximal accumulation of free intracellular MTX (inset). In the experiment

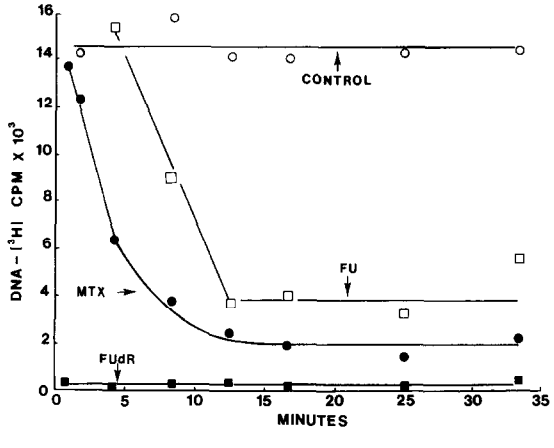


*Fig. 1. Time course of inhibition of* [$^3$H]-*UdR incorporation into DNA by FU, FUdR and MTX. Cells were exposed either to* 1 μM *FU* (□), 0.1 μM *FUdR* (■) *or* 50 μM *MTX* (●), *or in the absence of these agents* (○), *following which samples from each of the four suspensions were isolated by centrifugation and the supernatant fluid was thoroughly aspirated. After washing the cell fractions twice, the tubes were gassed with* 95% $O_2$–5% $CO_2$ *and capped. For an analysis of incorporation of* [$^3$H]-*UdR into DNA, the tubes were warmed in a* 37° C *bath, following which the cell pellets were dispersed in a medium containing* 0.5 μM [$^3$H]-*UdR only* (○□■) *or* [$^3$H]-*UdR and* 50 μM *MTX* (●).

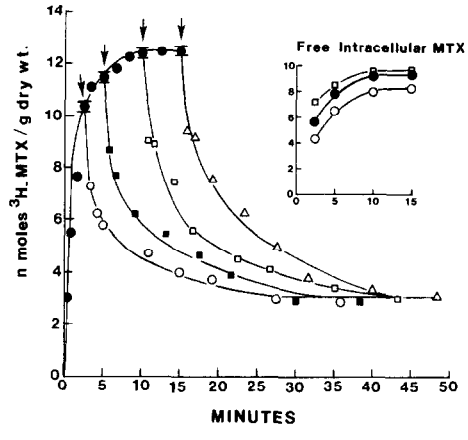


*Fig. 2. Time required for maximal accumulation of free intracellular MTX. Cells were incubated with* 50 μM *MTX for* 15 min *at* 37°C. *At the arrows* (2.5, 5, 10 *and* 15 min) *portions of the cell suspension were separated by centrifugation, washed twice with* 0°C *buffer, resuspended into large volumes of* 37°C *MTX-free medium and the fall in the intracellular MTX level monitored. The intracellular level (at the arrows) prior to resuspension is the mean ± S.E. of three determinations. The difference between the total intracellular MTX and the non-exchangeable component represents free intracellular MTX (inset). The different symbols in the inset represent experiments performed on separate days.*

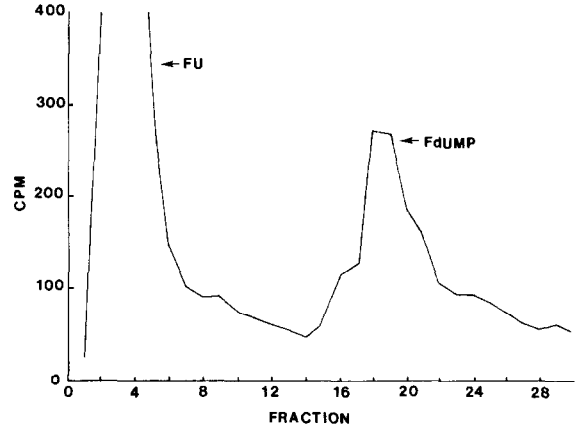


*Fig. 3. High-performance liquid chromatographic analysis of intracellular FdUMP after exposure of cells to FU for* 1 min.

shown, cells were exposed to 50 μM MTX for 1 min to 15 min. At 2.5, 5, 10 and 15 min (at the arrows) cells were resuspended into a large extracellular volume of MTX-free medium and MTX was allowed to efflux for 30 min until a stable plateau of intracellular [$^3$H]-MTX was attained (identified as MTX bound to DHFR over this interval [21]). Intracellular MTX levels declined to the same plateau content of 3.09 nmol/g dry wt from cells incubated with this drug for 2.5–15 min. The difference between the total intracellular MTX and the stable plateau or non-exchangeable fraction represents free intracellular MTX. At 2.5, 5, 10 and 15 min the free intracellular MTX levels are 5.8, 7.4, 8.7 and 8.8 nmol/g dry wt respectively. The inset represents three different experiments which illustrate the time required for cells to accumulate maximal free intracellular levels of MTX. Maximal free levels of intracellular MTX appear within 15 min after exposure to 50 μM MTX, which is similar to the time required for maximal inhibition of [$^3$H]-UdR incorporation into DNA.

Figure 3 illustrates a chromatogram of intracellular $^3$H 1 min after exposure of cells to [$^3$H]-FU. At 1 min the free FdUMP levels represent ~1% of the total intracellular radiolabel. To determine total FdUMP levels in these cells, cell cytosol was subjected to heating at 65°C for 15 min prior to separation of the intracellular nucleotide pool. This procedure would release any FdUMP present as FdUMP-5-10-$CH_2$-THF-TS complex [19]. Following this treatment the cell cytosol was analyzed by HPLC and the fraction corresponding to authentic FdUMP was found to contain radioactivity.

Figure 4 is a representative experiment which compares the initial uptake rates of the fluoropyrimidines, FU and FUdR, and MTX

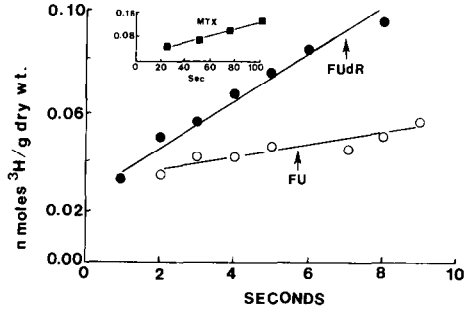


*Fig. 4. The rate of FU, FUdR and MTX uptake. Cell suspensions were added to capped tubes containing* [$^3$H]-*FU or* [$^3$H]-*FUdR, and* [$^3$H]-*MTX which had previously been gassed with* 95% $O_2$–5% $CO_2$. *The extracellular concentration of FU* (○), *FUdR* (●) *and MTX* (■; *inset*) *was* 0.1 μM. *The initial rates were obtained from the least-squares slope of* 4–7 *uptake points over an interval of* 10 sec *for FU and FUdR* [17, 23] *and* 100 sec *for MTX* [14, 16, 22].

(inset). Initial uptake rates of 0.1 μM FU, 0.1 μM FUdR and 0.1 μM MTX were measured over 10 and 100 sec respectively. Uptake over these intervals is a linear function of time and represents a unidirectional flux for these agents into the cell [14, 16, 17, 22, 23]. In 5 experiments the influx of MTX is 9.8 ± 1.9% and 3.8 ± 0.8% times slower than FUdR and FU respectively. The transport of FU, FUdR and MTX will determine, at least in part, the rates at which thymidylate synthetase and dihydrofolate reductase are inactivated.

Previous studies [6, 7] have demonstrated that pretreatment of Ehrlich cells with FUdR prevented the oxidation of 5, 10-$CH_2$THF and antagonized the inhibition of MTX on formate incorporation into RNA, DNA and protein. Similar effects were expected from the simultaneous administration of FU and MTX or FUdR and MTX since FU and FUdR transport and metabolism to FdUMP was faster than (a) MTX transport and (b) the generation of free intracellular MTX. Figure 5 illustrates the effects of simultaneous exposure of cells to FU and MTX on [$^{14}$C]-formate incorporation into RNA, DNA and protein respectively. A cell suspension of Ehrlich ascites tumor cells was divided into four portions, resuspended into an FU–MTX-free medium and a medium containing FU plus MTX, or MTX or FU only. Thirty minutes later, when MTX exceeded the capacity of the high affinity intracellular binding sites, [$^{14}$C]-formate incorporation was monitored. Antagonism to MTX-suppression of [$^{14}$C]-formate incorporation into RNA, DNA and protein occurred when cells were simultaneously exposed to FU and MTX (Fig. 5). In four separate experiments the rate of [$^{14}$C]-formate incorporation into RNA, DNA and protein, in the presence of MTX alone, was decreased by 39.42 ± 8.70%, 35.27 ± 10.47% and 39.47 ± 13.20% respectively, as compared to FU plus MTX. A similar effect was observed after the simultaneous exposure of cells to FUdR plus MTX (Fig. 6).

To further assess the effect of MTX and FU sequence of administration on formate incorporation into RNA, DNA and protein, Ehrlich tumor cells were first exposed to 50 μM MTX for 10 min and then to 1 μM FU for 1 min. These time factors were chosen since (a) intracellular FdUMP was identified within 1 min after FU and FUdR exposure and (b) approximately 10 min was required for the generation of maximal free intracellular MTX and maximal suppression of UdR incorporation into DNA. Figure 7 illustrates the effects of exposing cells to MTX first and FU second on the rate of [$^{14}$C]-formate incorporation into RNA, DNA and protein. In comparison to

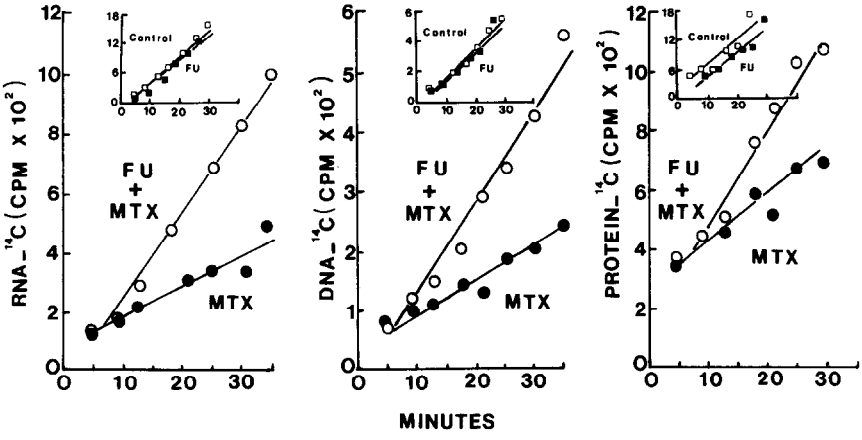


*Fig. 5. The effect of simultaneous administration of* 1 μM *FU and* 50 μM *MTX on* [$^{14}$C]*-formate incorporation into RNA, DNA and protein. Ehrlich tumor cells were incubated with FU, FUdR and/or MTX for* 30 min, *following which* [$^{14}$C]*-formate was added to the cell medium. The incorporation of* $^{14}$C *into RNA, DNA and protein was determined by measuring* $^{14}$C *into a dilute acid extract of a perchlorate precipitate.*

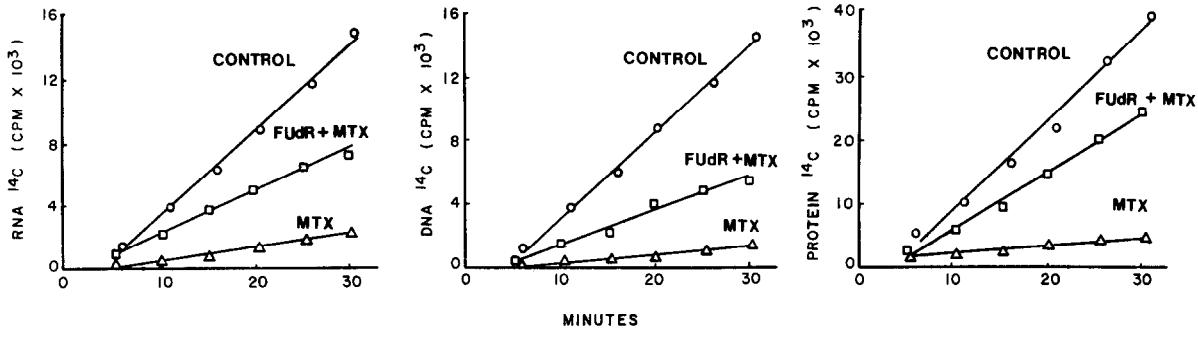


*Fig. 6. The effect of simultaneous administration of FUdR and MTX on* [$^{14}$C]*-formate incorporation into RNA, DNA and protein.*

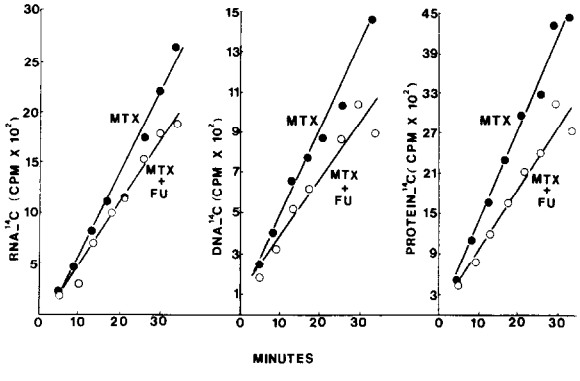


*Fig. 7. The effect of exposing Ehrlich cells first to MTX for* 10 min *and then to FU for* 1 min *on* [$^{14}$C]*-formate incorporation into RNA, DNA and protein.*

MTX alone (4 experiments), MTX and FU decreased the rate of $^{14}$C incorporation into RNA, DNA and protein by 21.55 ± 5.62%, 29.48 ± 4.87% and 37.99 ± 10.20% respectively.

## DISCUSSION

The studies reported here support the concept that fluoropyrimidine-induced augmentation of MTX suppression of nucleic acid and protein synthesis is related, in part, to the rate at which deoxyuridylate is converted to dTMP. This was established by examining the effect of FU, FUdR and MTX on [$^{14}$C]-formate incorporation into RNA, DNA and protein. This incorporation of [$^{14}$C]-formate occurs as a result of the insertion of the formate carbon via tetrahydrofolates into purine nucleotides at C-2 and C-8 as well as into methionine and serine [24]. The observation that the rate of formate incorporation into RNA, DNA and protein is increased upon simultaneous exposure of cells to MTX and FU or FUdR is compatible with studies in which pretreatment with fluoropyrimidines antagonized the effects of MTX on THF-dependent processes [6, 7].

Antagonism to MTX-suppression of formate incorporation into nucleic acid and protein when cells are exposed simultaneously to MTX and FU or FUdR points to the importance of the rates of transport, metabolism and interaction of these agents with their target sites. When cells are exposed to MTX and the drug enters the intracellular compartment the following sequence of events would be expected to occur: as MTX binds to DHFR, free drug does not accumulate

within the cell since influx is rate-limiting to binding [14]. Initially, the ratio of free enzyme to intracellular drug is great, and therefore there is a small reduction in the rate of THF synthesis and, consequently, 5, 10-$CH_2$-THF. Ultimately, as MTX binds to the major portion of DHFR the DHF level approaches its $K_m$, and the rate of THF synthesis and the THF cofactor levels fall. Because DHF has risen to high levels and because the interaction between MTX and DHFR is not stoichiometric at physiological pH [25], DHF can compete with MTX for the few remaining DHFR sites. To achieve saturation of DHFR sites and to suppress THF synthesis maximally, free intracellular MTX orders of magnitude above its $K_i$ is required [26]. The consequence of increasing levels of free intracellular MTX on DHF metabolism would be a gradual depletion of the THF pool size from the continued utilization of 5, 10-$CH_2$-THF for dTMP synthesis. Eventually, MTX would decrease THF-dependent RNA, DNA and protein synthesis because of a decrease in THF pools.

In the interaction of the fluoropyrimidines with the cell, transport is considerably faster than metabolism to FdUMP [17], a phosphorylated derivative which is retained in the cell and binds covalently to thymidylate synthetase. Our studies indicate that fluoropyrimidine transport and formation of FdUMP is faster than MTX transport and the maximal accumulation of free intracellular MTX. The consequence of rapid FU and FUdR transport and formation of FdUMP is a reduction in the utilization of 5, 10-$CH_2$-THF and a maintenance of cellular THF pools at a level required to support purine and amino acid synthesis. Hence the alteration of THF pools by MTX could be prevented by the inhibition of the process which converts 5, 10-$CH_2$-THF to the inactive DHF form [12].

Suppression of the rate at which DHF is converted to THF and subsequently to 5, 10-$CH_2$-THF first should decrease the reduced-folate pool for dTMP synthesis. However, even in the presence of MTX, DHF is converted to THF [26] and hence to 5, 10-$CH_2$-THF, which forms in the presence of MTX, to further decrease the availability of reduced folates for amino acid synthesis and purine synthesis. Our studies are consistent with this formulation. These studies demonstrate that cells exposed to MTX first and FU second suppressed the rate of [$^{14}$C]-formate incorporation into RNA, DNA and protein greater than MTX alone. The time of MTX exposure comprised the time for maximal suppression of dTMP synthesis followed by the earliest time period in which FdUMP was identified.

Important therapeutic implications are attached to the fact that the rate of dTMP synthesis is a critical determinant of MTX and fluoropyrimidine cytotoxicity. First, tumors with a slow growth rate would require low levels of THF. Since the fraction of DHFR activity required to maintain THF under this condition would be small, complete inhibition of DHFR activity would require high levels of free intracellular MTX. Also, the slow rate of THF consumption would require a long exposure to MTX. THF deficiency then might produce cytotoxicity due to a purine-deficient impairment of RNA, DNA and protein synthesis. Second, DHF can compete with MTX for enzyme because of reversible binding of MTX [21], thus producing low levels of THF which can be converted to 5, 10-$CH_2$-THF. Fluoropyrimidines administered after MTX may bind these low levels of 5, 10-$CH_2$-THF to further suppress THF-dependent RNA, DNA and protein synthesis.

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