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# Depletion of 5,10-Methylenetetrahydrofolate and 10-Formyltetrahydrofolate by Methotrexate in Cultured Hepatoma Cells

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## SUMMARY

The effect of the inhibition of dihydrofolate reductase by methotrexate on the cellular folates involved in *de novo* purine and thymidylate biosynthesis has been measured in H35 hepatoma cells grown in 4 μM folic acid or 20 nM folinic acid. The major cellular folate species in cells from medium with folate or folinate is 10-formyltetrahydrofolate (approximately 5 μM), with lesser amounts of 5,10-methylenetetrahydrofolate and tetrahydrofolate. Cultures were exposed to a pulse dose of methotrexate, resulting in the accumulation of nearly exclusively methotrexate polyglutamates (predominately $Glu_3$, $Glu_4$, and $Glu_5$), or a continuous exposure to the poorly glutamylated analog *threo*-4-fluoromethotrexate, resulting in 93% intracellular monoglutamate. At 4 hr and 18 hr after exposure to either compound there was extensive depletion of the reduced folate coenzymes, which generally corresponded to the extent of inhibition of glycine and deoxyuridine incorporation. This was accompanied by an increase of the cellular dihydrofolate and 10-formyldihydrofolate. In the H35 cells the effect of methotrexate polyglutamates on the reduced folate coenzyme pools was restricted to dividing cultures, because the reduced folate coenzymes were not depleted in confluent cultures. The results demonstrate that the methotrexate and methotrexate polyglutamates that initially accumulate within dividing H35 cells readily inhibit dihydrofolate reductase but are not adequate to inhibit thymidylate synthase and prevent the depletion of reduced folate coenzymes. Thus, inhibition of *de novo* glycine and deoxyuridine incorporation into DNA as a result of dihydrofolate reductase inhibitors appears to be closely related to a reduction in the intracellular concentration of 10-formyltetrahydrofolate and 5,10-methylenetetrahydrofolate, the respective folate coenzymes for *de novo* purine and thymidylate synthesis.

Until recently, the development of inhibitors of folate metabolism has been directed primarily at tight-binding inhibitors of DHFR (EC 1.5.1.3) (1–6). The most widely utilized and studied of these is MTX, which is currently being evaluated in considerable detail with regard to the role of cellular folate metabolism in its activity (7–17). Earlier assessments of MTX activity based upon experimental and theoretical considerations resulted in the hypothesis that the activity was due to blockage of DHFR and subsequent depletion of reduced folate coenzymes, thereby disallowing *de novo* purine and thymidylate biosynthesis (1–3). This hypothesis would predict that the major, if not only, target of MTX in cells is DHFR, a postulate that is reinforced by the high affinity of the inhibitor for the enzyme (1–3) and by the common occurrence of MTX resistance based upon selective DHFR amplification (18).

Several experimental observations complicate this hypothetical construct. It was found that the poly-γ-glutamyl derivatives are probably the active intracellular species of MTX under most cytotoxic conditions (6, 19–23). These derivatives can also inhibit thymidylate synthase (EC 2.1.1.45) at a concentration ($K_i$ = 50 nM) (17, 24, 25) that is readily exceeded intracellularly, a result that suggests that the direct effects of MTX polyglutamates on the synthase may play a role in the activity of MTX. However, it must be kept in mind that the affinity of MTX polyglutamates for its primary target, DHFR, has been estimated as being 2–4 orders of magnitude greater than that for thymidylate synthase (26). MTX polyglutamates have also

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**ABBREVIATIONS:** DHFR, dihydrofolate reductase; MTX, methotrexate (4-amino-10-methylpteroylglutamate); tFMTX, 4-amino-10-methylpteroyl-DL-*threo*-4-fluoroglutamate, with the concentration expressed as the total of both isomers (for an explanation of the stereochemistry, see Ref. 31); $H_4PteGlu_n$, (6*S*)-5,6,7,8-tetrahydropteroylpolyglutamate; 5,10-$CH_2$-$H_4PteGlu_n$, (6*R*)-5,10-methylenetetrahydropteroylpolyglutamate; $H_2PteGlu_n$, 7,8-dihydropteroylpolyglutamate; 10-HCO-$H_4PteGlu_n$, (6*R*)-10-formyltetrahydropteroylpolyglutamate; 10-HCO-$H_2PteGlu_n$, 10-formlydihydropteroylpolyglutamate; 5-HCO-$H_4$PteGlu, (6*S*)-5-formyltetrahydrofolate (also known as folinic acid and leucovorin); PteGlu, pteroylglutamic acid (folic acid); 5-$CH_3$-$H_4$pte $Glu_n$, 5-methyltetrahydropteroylpolyglutamate; GAR, glycinamideribonucleotide; FdUMP, fluorodeoxyuridylate; HPLC, high performance liquid chromatography.

been shown to inhibit GAR and 5-amino-4-imidazolecarboxamide ribotide transformylase (27, 28), but the relatively weak inhibitory properties make it uncertain whether these sites play an important role in MTX-caused inhibition.

A second series of observations questioned the depletion model of MTX activity. With nearly all *in vitro* cell systems, inhibition of DHFR was observed that was not accompanied by depletion of all reduced folylpolyglutamate coenzymes. In L1210 and MCF7 cells *in vitro* only partial depletion of 5,10-$CH_2$-$H_4$PteGlu$_7$ was observed (9, 12, 10, 15). The preservation of 5,10-$CH_2$-$H_4$PteGlu$_n$ and 10-HCO-$H_4$PteGlu$_n$ was attributed to inhibition of thymidylate synthase by the polyglutamates of MTX and possibly by $H_2$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$, which accumulate during inhibition by MTX (7, 8, 13, 15). Under these conditions, thymidylate synthase would be inhibited in addition to DHFR, the former at least partially preventing the conversion of the reduced folate coenzymes to $H_2$PteGlu$_n$ (Fig. 1). Evidence for the inhibition of thymidylate synthase by $H_2$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$ has arisen from several studies using isolated enzyme preparations (8, 17, 24, 25, 27–29). However, this theory remains controversial because maintenance of reduced folate coenzymes in the face of MTX exposure is not a universal effect. For instance, it has been shown in L1210 cells *in vivo* and hepatoma cells *in vitro* (17) that there is significant depletion of 5,10-$CH_2$-$H_4$PteGlu$_n$ and 10-HCO-$H_4$PteGlu$_n$ (10, 14). In addition, recent studies have suggested that the amount of $H_2$PteGlu$_n$ accumulated within certain cells during DHFR inhibition is insufficient to have a profound inhibitory effect on the folate-dependent biosynthetic pathways (12, 17). Definitive evidence against the role of $H_2$PteGlu$_n$-dependent inhibition of thymidylate synthase in cells comes from the fact that far more potent folate and pyrimidine analog inhibitors of thymidylate synthase do not block conversion of reduced folates to dihydrofolate in L1210 cells (12, 30).

In earlier studies the H35 hepatoma cell line was used to evaluate the effects of the DHFR inhibitor trimetrexate on thymidylate biosynthesis (11, 17). These studies showed that a noninhibitory or marginally inhibitory concentration of trimetrexate caused a 50–70% depletion in 5,10-$CH_2$-$H_4$PteGlu$_n$ and 10-HCO-$H_4$PteGlu$_n$ over 72 hr with no accumulation of $H_2$PteGlu$_n$. It was further demonstrated that at higher concentrations of trimetrexate a more severe depletion of 5,10-$CH_2$-$H_4$PteGlu$_n$ occurred, which was accompanied by $H_2$PteGlu$_n$ accumulation. The resulting loss of 5,10-$CH_2$PteGlu$_n$ was in the same range as the concentration needed to support thymidylate biosynthesis in folate-starved cells and as the substrate requirement of isolated thymidylate synthase. Thus, substrate depletion appeared to be a determinant of the inhibition of thymidylate synthase caused by trimetrexate. The $H_2$PteGlu$_n$ that accumulated as a result of DHFR inhibition did not appear to make a major contribution to the inhibitory process. Trimetrexate, unlike MTX, is not converted to polyglutamates. The present study is directed at extending those studies to understand the direct inhibitory effects that MTX polyglutamates may have on enzymes other than DHFR, particularly thymidylate synthase, and to evaluate the capacity of MTX-inhibited hepatoma cells to deplete or sustain 10-HCO-$H_4$PteGlu$_n$ and 5,10-$CH_2$$H_4$PteGlu$_n$. To do this we have examined the alterations in folate pools caused by MTX and tFMTX, a weakly glutamylated MTX analog (22, 31), during inhibition of purine and thymidylate biosynthesis. This allows a comparison of the effects of intracellular MTX polyglutamates with those of a molecule that has a similar inhibitory activity against DHFR but exists primarily as the monoglutamate within cells. The results of this study demonstrate that the perturbations in cellular tetrahydrofolates caused by the addition of MTX and tFMTX are indistinguishable and lead to a depletion of reduced folate coenzymes accompanied by an increase in dihydrofolates.

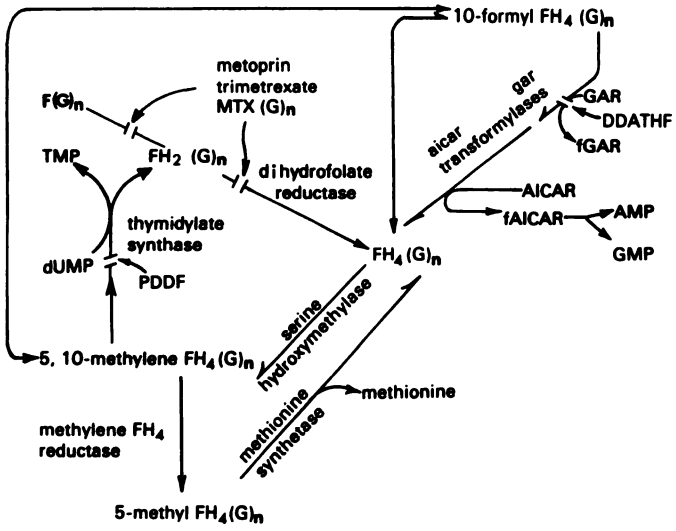


**Fig. 1.** Folate metabolism. *F*, folic acid or folate; *$FH_2$*, dihydrofolic acid; *$FH_4$*, tetrahydrofolic acid; *$(G)_n$*, poly-γ-glutamate derivatives; *PDDF*, 10-propargyl-5,8-dideaza folate; *DDATHF*, 5,10-dideazatetrahydrofolic acid; *AICAR*, 5-amino-4-imidazole-carboxamide ribotide. fAICAR, formyl-AICAR; fGAR, formyl-GAR.

## Materials and Methods

All tissue culture media, fetal calf serum, and horse serum were purchased from GIBCO (Grand Island, NY). Folic acid was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by DEAE-cellulose (Schleicher & Schuell, Keene, NH) column chromatography before use (32). (6*S*)-5-HCO-$H_4$PteGlu (folinic acid) was generously supplied by Lederle Laboratories (Pearl River, NY). [6-$^3$H]Deoxyuridine (23 Ci/mmol) and [6-$^3$H]FdUMP (18 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA), and [2-$^{14}$C]glycine (53 mCi/mmol) from NEN products, DuPont (Boston, MA). MTX was a gift of Lederle Laboratories and [3′5′,7-$^3$H]MTX was purchased from Moravek Biochemicals. Both were purified by DEAE-cellulose column chromatography before use and were at least 99% pure (32). tFMTX and [3′5′,7-$^3$H]tFMTX were synthesized and purified by the method of McGuire *et al.* (31). MTX polyglutamate standards were generously provided by the National Cancer Institute, National Institutes of Health. Sephadex G-25 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and Ready/Solv from Beckman (Fullerton, CA). GAR and pure GAR transformylase (chicken) were supplied by Dr. J. Whiteley of the Research Institute of Scripps Clinic (La Jolla, CA), purified human DHFR by Dr. J. Freisheim of the Medical College of Ohio, and thymidylate synthase (*Lactobacillus casei*) by Dr. G. Maley from the Wadsworth Center for Laboratories and Research. All reduced folate coenzymes were synthesized as the biologically active isomers by previously described methods (17, 29).

**Cell culture.** *Mycoplasma*-free H35 hepatoma cells were grown in monolayer culture (17, 32) and were depleted of folates by culturing for 1 week in folate-free Swims medium containing 10% fetal calf serum. These cultures were then subcultured in 4 μM PteGlu or 20 nM (6*S*)-5-HCO-$H_4$PteGlu for 72 hr and were utilized for all experiments, unless

otherwise indicated. The concentrations of folic acid (4 μM) and (6S)-folinic acid (20 nM) were maintained during drug exposure.

**Thymidylate and purine biosynthesis.** [6-$^3$H]Deoxyuridine incorporation into DNA was used as a measure of *de novo* thymidylate biosynthesis (33). *De novo* purine biosynthesis was measured by the incorporation of [$^{14}$C]glycine into DNA according to the method of Cadman *et al.* (34). The results were expressed as dpm/mg of cellular protein. Cell protein was measured by the method of Lowry *et al.* (35).

**Cellular reduced folates, MTX, and tFMTX.** Cellular metabolites of [$^3$H]MTX or [$^3$H]tFMTX were analyzed by HPLC as previously described (22, 31). 5,10-$CH_2H_4$PteGlu$_n$, $H_4$PteGlu$_n$, and $H_2$PteGlu$_n$ in the H35 cell extracts were measured by the ternary complex formation of [$^3$H]FdUMP-thymidylate synthase-5,10-$CH_2$-$H_4$PteGlu$_n$, as previously described (17). 10-HCO-$H_4$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$ were measured after conversion to $H_4$PteGlu$_n$ in the presence of GAR transformylase or in the presence of DHFR and GAR transformylase, respectively (29). Individual reduced folate species were calculated by the appropriate subtractions based on each enzymatic conversion (10, 14, 17, 29, 36). The standard deviations of triplicate measurements of each of the folate species from a single cell preparation are as follows: 5,10-$CH_2$-$H_4$PteGlu$_n$, ±2.3%; $H_4$PteGlu$_n$, ±3.7%; 10-HCO-$H_4$PteGlu$_n$, ±4.8%; $H_2$PteGlu$_n$, ±7.4%; and 10-HCO-$H_2$PteGlu$_n$ ±14%.

Recoveries of the folate coenzymes were assessed by adding known amounts (1–4 pmol) of the individual folylpolyglutamate (five or seven glutamate residues) coenzymes to each of the following culture systems: (a) H35 cells grown in the absence of folates, (b) control cultures grown with either folate or folinic acid, and (c) control cultures grown with either folate or folinate and treated with MTX. In cultures b and c the amount of reduced folate coenzymes within the cell was subtracted from the amount found in cells with added standard. Under all conditions recoveries for known amounts of added standards were 75% or greater. The data presented were not corrected for recovery.

The recovery of total folates by the linked enzyme assay in MTX- and tFMTX-treated cells is 76 ± 11%, compared with untreated controls. Part of the apparent loss is due to the 15–25% reduction of total cellular folates due to antifolate treatment, compared to nontreated controls, as measured by total [$^3$H]- or [$^{14}$C]folate incorporation. In addition, the recoveries (75%) of $H_2$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$ are the lowest of the folates measured, whereas the recoveries of all the tetrahydro derivatives are in excess of 90%. Inclusion of mercaptoethanol in the extraction buffer increased the amounts of dihydro derivatives in cells by approximately 40%, but it could not be routinely used because it caused 5,10-$CH_2H_4$PteGlu$_n$ to be degraded to $H_4$PteGlu$_n$ (17). Thus, when the antifolate-treated cells are corrected for the overall loss in folates caused by MTX and tFMTX and the reduced recovery of $H_2$PteGlu$_n$, the total amount of folates measured in antifolate-treated cultures is between 80 and 100% of that measured in untreated controls.

## Results

The present study was conducted to evaluate the capacity of the polyglutamate derivatives of MTX to deplete cultured hepatoma cells of the reduced folate coenzymes that serve as substrates for purine and thymidylate biosynthesis. These are 10-HCO-$H_4$PteGlu$_n$ and 5,10-$CH_2$-$H_4$PteGlu$_n$, respectively. In doing this we have compared MTX with its analog tFMTX, which has properties similar to those of MTX with regard to transport and DHFR inhibition but is very poorly glutamylated (22, 31). By using this experimental approach we propose to analyze the effects of intracellular MTX, which exists nearly exclusively as polyglutamates, and those of an analog (tFMTX) that exists within the cell nearly exclusively as the monoglutamate. The intent is to determine whether the presence of the MTX polyglutamates cause the depletion of the reduced folate coenzymes by virtue of inhibition of DHFR or whether depletion is impaired due to an additional direct inhibitory effect of MTX polyglutamates on thymidylate synthase. If the latter event occurs it should not be observed with tFMTX. To examine this, exposure to MTX and tFMTX must be different because of the rapid loss of monoglutamate (tFMTX) and retention of polyglutamates. Therefore, the cultures were exposed to tFMTX continuously to ensure adequate inhibitory levels of the antifolate. In contrast, MTX was present for only a 2-hr pulse to allow adequate formation of polyglutamates and subsequent loss of the monoglutamates.

**Cellular concentration of folate coenzymes in H35 cells.** The concentrations of the cellular folate coenzymes that are the substrates or products of purine and thymidylate biosynthesis in H35 hepatoma cells are shown in Table 1. 10-HCO-$H_4$PteGlu$_n$ is the major form and is present at approximately twice the concentration of 5,10-$CH_2$-$H_4$PteGlu$_n$. Small amounts of $H_2$PteGlu$_n$ are observed and no 10-HCO-$H_2$PteGlu$_n$ can be detected in control cultures. Although not reported here, 5-$CH_3$-$H_4$PteGlu$_n$ and 5-HCO-$H_4$PteGlu$_n$ are minor components in this system,[1] ranging from being undetectable to being at most 10% of the total folates in control cultures. Verification of the values shown in Table 1 has been accomplished by analyzing parallel samples of H35 cells for folate pools by the ternary complex assay described here and by HPLC (36). It is noteworthy that the folate coenzyme concentration of cells grown in 4μM folic acid is nearly the same as that of cells, grown in 20 nM folinic acid. Analogous results have been observed in L1210 cells (37). The inhibition studies reported below were conducted with both folates as the growth support. Folic acid is the usual form of folate in culture medium, but folinic acid differs in that it does not require reduction before coenzyme function, which more closely mimics *in vivo* conditions. 5-$CH_3$-$H_4$PteGlu, the circulatory form of folic acid in mammals (38), could not be used for this purpose because it is unstable ($t_{1/2}$ ≅ 24 hr by HPLC) in the medium used to conduct these experiments.

**Effects of MTX and tFMTX on precursor incorporation and cellular folate pools.** The inhibitory effects of MTX and tFMTX were examined after 4 and 18 hr of exposure. The 4-hr experimental cultures were incubated with 1.6 or 10 μM MTX for 2 hr followed by removal of MTX or with 8 or 16 μM tFMTX continuously. At 4 hr the higher concentration of each inhibitor used was adequate to approximate or exceed 80% blockade of deoxyuridine and glycine incorporation (Figs. 2 and 3, *upper*). At both concentrations in either folate or folinate the cellular MTX was not less than 98% polyglutamates, predominantly $Glu_4$ and $Glu_5$, whereas the cellular tFMTX was at least 95% unmetabolized with only small amount (<5%) of $Glu_3$ and $Glu_4$. These metabolic profiles of MTX and tFMTX are similar to previous results (22, 31) and those observed after a 18-hr incubation (see below). The inhibition by MTX causes a >75% reduction in 5,10-$CH_2$-$H_4$PteGlu$_n$ and 10-HCO-$H_4$PteGlu$_n$ when the cells are grown in folic acid (Fig. 2A). This is accompanied by approximately an 8-fold increase in the combined $H_2$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$ pool. Similar results are observed with cells grown in 20 nM 5-HCO-$H_4$PteGlu except that the reduction in 5,10-$CH_2$-$H_4$PteGlu$_n$, $H_4$PteGlu$_n$, and 10-HCO-$H_4$PteGlu$_n$ is less dramatic (Fig. 2B). The depletion in reduced folates with tFMTX after a 4-hr incubation follows a similar pattern (Fig. 3). With the higher

[1] M. S. Rhee and J. Galivan, unpublished observations.

TABLE 1

**Cellular folates in H35 cells grown in 4 μM folic acid or 20 nM folinic acid**

Folate coenzymes were measured in extracts of H35 cells after 72 hr in culture, as described in Materials and Methods. Values are mean ± standard deviation of three independent experiments assayed in duplicate.

| Medium folate | Cellular folates | | | |
|---|---|---|---|---|
| | 5,10-$CH_2H_4F$[a] | $H_4F$ | 10-HCO-$H_4F$ | $H_2F$ |
| | μM | | | |
| None | 0.04 ± 0.009 | 0.03 ± 0.005 | 0.06 ± 0.01 | 0.1 ± 0.01 |
| Folic acid, 4 μM | 2.5 ± 0.28 | 1.4 ± 0.06 | 4.6 ± 0.20 | 0.8 ± 0.08 |
| Folinic acid, 20 nM | 2.8 ± 0.16 | 1.4 ± 0.12 | 5.7 ± 0.27 | 0.6 ± 0.01 |

[a] F, $PteGlu_n$.

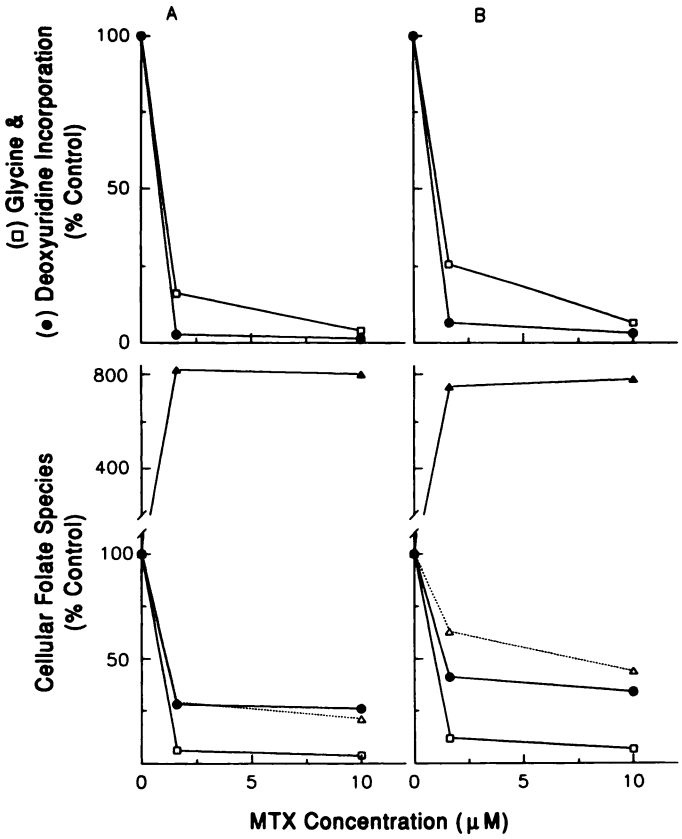


**Fig. 2.** Effect of 4-hr exposure to MTX on thymidylate and purine biosynthesis and folate coenzymes in H35 cells. H35 cells were grown in 4 μM folic acid (A) or 20 nM folinic acid (B) for 72 hr and incubated with MTX for the last 2 hr, followed by 2 hr in the absence of drug. Thymidylate and purine biosynthesis and folate coenzymes were measured as described in Materials and Methods and expressed as percentage of control. ▲, $H_2PteGlu_n$ and 10-HCO-$H_2PteGlu_n$; ●, 5,10-$CH_2$-$H_4PteGlu_n$; △, $H_4PteGlu_n$; □, 10-HCO-$H_4PteGlu_n$. Each point is the average of two independent experiments; the difference between the two values for glycine incorporation is 14% and for deoxyuridine incorporation is 5%.

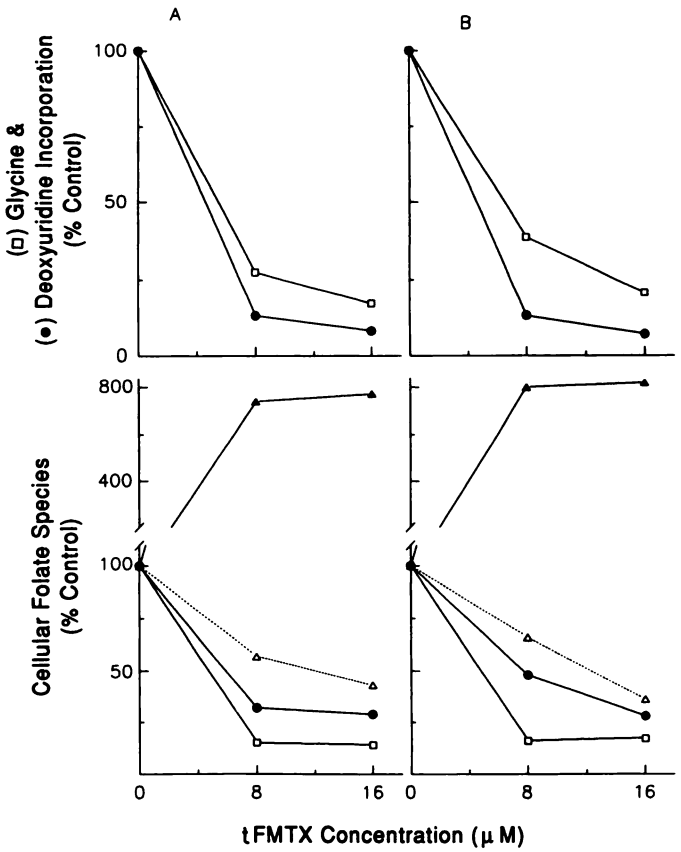


**Fig. 3.** Effect of 4-hr exposure to tFMTX on thymidylate and purine biosynthesis and folate coenzymes in H35 cells. The experiments and symbols were identical to those described for Fig. 2 except that tFMTX was continuously present for 4 hr.

concentration of tFMTX in either folate- or folinate-supplemented medium there is a greater inhibition of glycine and deoxyuridine incorporation, which is accompanied by an increased depletion of 10-HCO-$H_4PteGlu_n$ and 5,10-$CH_2H_4PteGlu_n$.

Extension of the incubation with inhibitors to longer time periods causes greater inhibition of precursor incorporation to occur. Use of an 18-hr exposure results in greater inhibition of glycine and deoxyuridine incorporation (Table 2), compared with a 4-hr exposure. The effects of continued exposure to 16 μM tFMTX cause approximately the same level of inhibition of purine and thymidylate biosynthesis as 10 μM MTX (2-hr pulse with 16 hr in the absence of MTX). An additional higher concentration of MTX (25 μM) was employed to ensure a higher level of intracellular polyglutamates after 16 hr in the absence of MTX. Under all conditions there is >90% inhibition of purine and thymidylate biosynthesis. At 25 μM MTX the intracellular concentration of all MTX species is approximately 14 μM of which 95% are polyglutamates with $Glu_4$ and $Glu_5$ predominating. Similar results are obtained with cells grown in folate or folinate. The cells treated with 16 μM tFMTX contain approximately 7 μM intracellular tFMTX, of which only 6% (0.4–0.5 μM) are polyglutamates when cells are grown in either 4 μM folic acid or 20 nM folinic acid (Table 2). Thus, under these experimental conditions MTX and its poorly glutamy-

TABLE 2

**Inhibition of glycine and deoxyuridine incorporation in H35 cells by MTX or tFMTX and their polyglutamate derivatives**

Cells grown in 4 μM folinic acid or 20 nM folinic acid for 54 hr were exposed to MTX or tFMTX. MTX was added for 2 hr followed by the removal of drug for 16 hr and tFMTX was present continuously for 18 hr. Glycine and deoxyuridine incorporation was measured as described in Material and Methods. MTX and tFMTX polyglutamate derivatives were analyzed after the cells were incubated with [$^3$H]MTX or [$^3$H]tFMTX. Values are averages of two or three independent experiments.

| Inhibitor | Glycine incorporation | Deoxyuridine incorporation | Polyglutamates[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Total | $Glu_1$ | $Glu_2$ | $Glu_3$ | $Glu_4$ | $Glu_5$ | $Glu_6$ |
| | % | % | μM | | | | | | |
| 4 μM Folic acid | | | | | | | | | |
| MTX, 10 μM | 3.2 | 0.5 | 10.7 | 0.05 | 0.2 | 0.2 | 3.1 | 5.2 | 2.0 |
| MTX, 25 μM | 2.0 | 0.2 | 14.2 | 0.7 | 0.6 | 0.6 | 5.5 | 5.8 | 1.0 |
| tFMTX, 16 μM | 3.5 | 1.5 | 7.3 | 6.8 | | 0.2 | 0.3 | | |
| 20 nM Folinic acid | | | | | | | | | |
| MTX, 10 μM | 7.0 | 1.5 | 9.6 | 0.1 | 0.1 | 0.2 | 3.3 | 3.8 | 2.2 |
| MTX, 25 μM | 3.1 | 0.4 | 14.4 | 0.2 | 0.1 | 0.3 | 4.5 | 5.2 | 4.1 |
| tFMTX, 16 μM | 3.2 | 2.0 | 7.0 | 6.6 | | 0.3 | 0.1 | | |

[a] $Glu_n$ is the total number of glutamate residues in the species described. Hence MTX is $Glu_1$.

lated analog tFMTX can achieve comparable levels of metabolic inhibition in dividing H35 cells despite the dramatic difference in the intracellular polyglutamate population.

The cellular folates were measured in cells exposed to MTX and tFMTX for 18 hr (Table 3). 5,10-$CH_2$-$H_4$PteGlu$_n$ is further depleted to 85–90% of control in folate-grown cells by either MTX or tFMTX, and 10-HCO-$H_4$PteGlu$_n$ reaches approximately 95% depletion. Corroboration of the depletion of 10-HCO-$H_4$PteGlu$_n$ was verified by assessing its concentration by HPLC, according to the method of Allegra *et al.* (7, 8). Incubation of the cultures with 4 μM [$^3$H]- or [$^{14}$C]folate in independent experiments results in an intracellular 10-HCO-$H_4$PteGlu$_n$ concentration of 5.0 and 7.0 μM, respectively. Treatment with 25 μM MTX under the conditions described in Table 3 results in an 80% reduction in the 10-formyl pool when the cells are labeled with [$^3$H]folate and an 87% reduction when cells are labeled with [$^{14}$C]folate.[1] The elevated concentrations of $H_2$PteGlu$_n$ and 10-HCO-$H_2$PteGlu$_n$ observed at 4 hr (Figs. 2 and 3) are maintained over the 18-hr period (Table 3). Some variance in the amount of 10-HCO-$H_2$PteGlu$_n$ occurs as a result of the drug exposure, and this is not yet fully understood. It may in part be due to the poorer recovery and greater variability in the measurement of this derivative (see Materials and Methods). During this period of time there is no loss in total cell number as a result of the presence of the antifolates. Cells

TABLE 3

**Effect of 18-hr exposure to MTX and tFMTX on folate coenzymes in H35 cells grown in 4 μM folic acid or 20 nM folinic acid**

Reduced folate coenzymes in the cell extracts were measured as described in Materials and Methods, after the cells were treated with MTX or tFMTX as in Table 2. Results for untreated cultures are in Table 2. Values are averages of two experiments assayed in duplicate.

| Inhibitor | Cellular folates | | | | |
|---|---|---|---|---|---|
| | 5,10-$CH_2$-$H_4$F[a] | $H_4$F | 10-HCO-$H_4$F | $H_2$F | 10-HCO-$H_2$F |
| | μM | | | | |
| 4 μM Folic acid | | | | | |
| MTX, 10 μM | 0.3 | 0.1 | 0.2 | 3.6 | 0.3 |
| MTX, 25 μM | 0.3 | 0.2 | 0.2 | 3.8 | 3.0 |
| tFMTX, 16 μM | 0.4 | 0.4 | 0.3 | 4.7 | 0.8 |
| 20 nM Folinic acid | | | | | |
| MTX, 10 μM | 0.6 | 0.4 | 0.3 | 4.2 | 1.2 |
| MTX, 25 μM | 0.6 | 0.6 | 0.3 | 4.2 | 1.2 |
| tFMTX, 16 μM | 0.5 | 0.3 | 0.3 | 7.1 | 0.6 |

[a] F, PteGlu$_n$.

grown in folinic acid with antifolates present for 18 hr experience a similar depletion in reduced folate coenzymes and enhancement in the dihydro species. Although not shown here, identical changes in folate coenzyme pools occur after 4- or 18-hr exposure when the MTX concentration is 50 μM. Under these conditions the cellular polyglutamates reach 30–40 μM, predominately $Glu_4$ and $Glu_5$. Throughout all these studies there appears to be no significant difference in the depletion of the reduced folate coenzymes 5,10-$CH_2H_4$PteGlu$_n$ and 10-HCO-$H_4$PteGlu$_n$ or in the increase in the dihydro components, in spite of there being vastly different intracellular polyglutamate pools of MTX, compared with the monoglutamate tFMTX.

Considering the capacity of MTX to extensively deplete the reduced folate coenzymes in dividing H35 cultures, it was informative to determine whether depletion also occurred in confluent cultures. The activity of thymidylate synthase in extracts of confluent culture (as measured with [5-$^3$H]deoxyuridylate) and in intact cells (as measured by tritium release from [5-$^3$H]deoxyuridine) is approximately 15% of that in dividing cultures.[1] Untreated confluent cultures have a folate profile similar to that of dividing cultures, i.e., a total concentration of approximately 10 μM tetrahydro derivatives, predominately 10-HCO-$H_4$PteGlu$_n$, 5,10-$CH_2H_4$PteGlu$_n$, and $H_4$PteGlu$_n$. The dividing cultures lose the majority of the reduced species within 30 min (Fig. 4). The rapid, apparently selective, loss of 10-HCO-$H_4$PteGlu$_n$ is interesting and somewhat surprising. In contrast to the dividing cultures there is only a modest loss of reduced folates in H35 confluent cultures. This agrees with the L1210 system *in vitro*, where depletion of reduced folate coenzymes does not completely occur in stationary-phase cultures (39). Consistent with this alteration is the fact that $H_2$PteGlu$_n$ does not accumulate in the confluent H35 cultures (data not shown). L5178Y cells *in vivo* lose 63% of the 5,10-$CH_2H_4$PteGlu$_n$ as a result of MTX treatment when dividing rapidly, but no change occurs in slowly dividing cells (40). Thus, whereas H35 cells differ quantitatively from several other cell lines by exhibiting extensive depletion of the reduced folate coenzymes in dividing cultures after MTX exposure, they are similar in that the reduced folate coenzymes are not readily depleted during confluence.

## Discussion

This study has been directed at understanding the perturbations in the folate coenzyme pools in H35 hepatoma cells

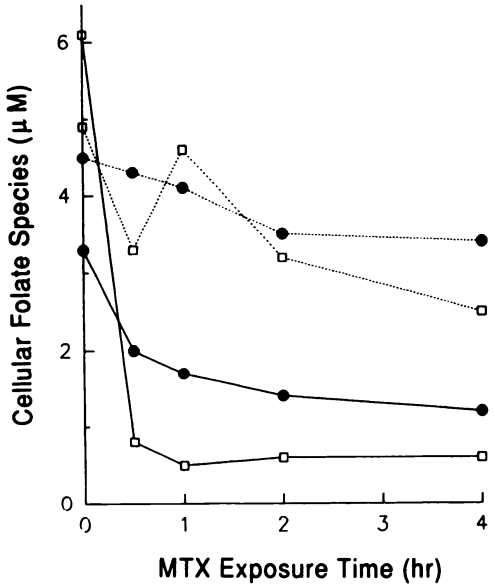


**Fig. 4.** Effect of MTX on the depletion of cellular reduced folates in midlogarithmic phase or confluent H35 cultures. H35 cells were grown in 20 nM folinic acid for 72 hr (——) or 120 hr (. . . . .), after which cells were continuously exposed to 25 μM MTX for the indicated time. 10-HCHO-$H_4PteGlu_n$ (□) and 5,10-$CH_2$-$H_4PteGlu_n$ and $H_4PteGlu_n$ (●) were measured as described in Materials and Methods. Each point is a single experiment run in duplicate. The standard deviation of triplicate assays of selected points is no greater than 10% for 10-HCO-$H_4PteGlu_n$ or 5,10-$CH_2$-$H_4PteGlu_n$ plus $H_4PteGlu_n$.

caused by MTX and its polyglutamates. To do this we have compared the effects of MTX and tFMTX. Under the conditions of the experiments the MTX that is present in the cells is nearly exclusively polyglutamates, whereas tFMTX, because of its altered glutamate structure, exists almost entirely as the monoglutamate. Other properties of MTX and tFMTX are similar (31), which allows analysis of the specific contributions of the polyglutamates to the activity of MTX by comparison with tFMTX. The chief objective was to determine whether tetrahydrofolate derivatives that are substrates for *de novo* purine and thymidylate biosynthesis were depleted by inhibition of dihydrofolate reductase by MTX. A possibility was that the MTX polyglutamates inhibit secondary enzymatic sites, especially thymidylate synthase, and prevent depletion of these coenzymes.

The media utilized in these studies contained either folic acid or folinic acid. Other studies in cell culture systems on this topic have utilized primarily folic acid (10–12, 15, 17, 30, 39). However, this creates additional complexity, because folic acid has an additional reductive step, which is also inhibited by MTX, before entering the tetrahydrofolate pool (Fig. 1). This is not the case *in vivo*, where the circulatory folate is a fully reduced folate (38). In the present study folinic acid was used as the tetrahydrofolate due to its chemical stability. With folinic acid only a single reaction is catalyzed by DHFR, and this occurs after the conversion of 5,10-$CH_2H_4PteGlu_n$ to $H_2PteGlu_n$ that is catalyzed by thymidylate synthase. The utilization of this system has shown that the inhibition of DHFR results in a severe depletion of the tetrahydrofolate coenzymes, and this occurs with either form of folic acid in the medium and with either MTX or tFMTX as the DHFR inhibitor.

A comparison of H35 cells with other cell systems reveals a wide variety of responses when the effects of DHFR inhibitors on the reduced folate coenzymes are evaluated. L1210 cells *in vivo* show extensive depletion, accompanied by $H_2PteGlu_n$ expansion (14). However, L1210 cells assayed *in vitro* either by an enzyme entrapment assay similar to that used here (10) or by HPLC (9, 12) show limited depletion. MCF-7 cells *in vitro* show selective depletion, with loss of $H_4PteGlu_n$ and 5-$CH_3$-$H_4PteGlu_n$ but not 10-HCO-$H_4PteGlu_n$ (7, 8). With H35 cells all major tetrahydrofolate coenzymes are substantially depleted after exposure to MTX as polyglutamates (using MTX) or monoglutamate (using tFMTX) (Figs. 2 and 3; Tables 2 and 3). The most extensively depleted is 10-HCO-$H_4PteGlu_n$, which is also the predominant species in control cultures. Thus, the presence of MTX polyglutamates, which are potent inhibitors of thymidylate synthase, does not appear to impair the oxidation of the tetrahydro derivatives by this enzyme. If there is a difference, the decrease in tetrahydro derivatives is greater in the presence of MTX polyglutamates. These results suggest that the high affinity binding of MTX polyglutamates to DHFR (2–4 orders of magnitude greater than with thymidylate synthase) (5, 17, 25, 26) favors rapid inactivation of DHFR and buildup of $H_2PteGlu_n$ at the expense of the tetrahydrofolate pools. When compared with enzyme kinetic data (17, 29), the data in Table 2 suggest that adequate MTX polyglutamates are accumulated in H35 cells to severely inhibit thymidylate synthase and prevent folate depletion. With higher extracellular MTX (50 μM), the intracellular polyglutamates (~40 μM) exceed the $K_i$ for thymidylate synthase by 3 orders of magnitude. The results shown in Table 3 and Figs. 2 and 3 imply that inhibition of DHFR occurs much more rapidly and causes depletion before the potential blockage of thymidylate synthase. However, evidence for direct inhibition of thymidylate synthase by MTX polyglutamates in L1210 cells exists, provided that the MTX is added several hours before measurement of the incorporation of 5-[$^{14}$C]HCO-$H_4PteGlu_n$ into thymidylate (9). Preliminary results indicate similar results in the H35 cell system. When increased concentrations (10–50 μM) of folinic acid are added after a 2-hr exposure to MTX, inhibition of *de novo* thymidylate biosynthesis occurs in spite of 5,10-$CH_2H_4PteGlu_n$ being present at the concentration observed in control cultures.[1] It has been pointed out that this may be part of the basis for the selectivity of leucovorin rescue (9).

The reason for the variation in depletion of reduced folate coenzymes among several other cell lines *in vitro* is not understood. The primary mechanism for depleting tetrahydrofolates would appear to be the continued function of thymidylate synthase during the blockade of DHFR. It appears that the initial inhibitory effects of MTX polyglutamates are not sufficient in H35 cells to prevent the conversion of tetrahydro derivatives to dihydro derivatives. The H35 cell thymidylate synthase appears kinetically similar to the enzyme from other sources (17) and is probably not the cause of the differential effects of the MTX polyglutamate inhibition on synthase from different sources. It is possible that the enzyme is present in differing amounts in different tissues, causing inhibition in certain cells to be less complete than in others. H35 cells have relatively high concentrations of the enzyme (0.2 μM at midlogarithmic phase)[1] but the catalytic turnover of purified thymidylate synthase appears similar to that of other mammalian preparations (17). In terms of enzyme activity, this means that H35 cells have the capacity to convert 150 nmol of reduced

folate to $H_2PteGlu_n$ per g of cell protein per min, based upon the molecular weight and specific activity of the purified enzyme (17). As Goldman and co-workers (30, 39) have pointed out, most dividing cells have an overwhelming activity of thymidylate synthase, relative to total reduced folate coenzyme pools. In the case of H35 cells the content of reduced folates is approximately 40 nmol/g of cell extract under the growth conditions described here. Thus, if DHFR is completely blocked, all the reduced folate coenzymes can be converted to $H_2PteGlu_n$ in 0.25 min, providing that the entire pool is in rapid equilibrium. It is interesting to note that it theoretically would take only 1.5% of the enzyme that is present to achieve the depletion observed in Fig. 4 for dividing cultures. Although the H35 cells deplete tetrahydrofolates more than other cells, they are similar in that there is little depletion in nondividing cultures.

Another possibility that may cause dividing cultures of H35 cells to differ from other lines involves the intracellular folate dynamics. It must also be remembered that cells of hepatic origin may behave differently than other cells because the liver serves as a reservoir of folates for other tissues in the body. Thus, different mechanisms may be present with regard to folate regulation. One study on the effects of MTX on folate coenzymes in dividing human HepG2 hepatoma cells has shown essentially a total depletion of all reduced folate coenzymes, which is accompanied by $H_2PteGlu_n$ expansion (41). Thus, these two hepatic carcinoma cell lines may provide unique models to study the depletion of reduced folate coenzymes by antifolates. In both cases 10-HCO-$H_4PteGlu_n$ is the major cellular species and is essentially lost during DHFR inhibition. Moreover, in H35 cells 10-HCO-$H_4PteGlu_n$ is the folate species that expands the most in response to increases in medium folate and can achieve intracellular concentration as high as 20 $\mu$M.[1] Studies are underway in this laboratory to elucidate the factors that control the intracellular accumulation and depletion of this critical folate coenzyme.

In summary, these studies show that the response of reduced folate coenzymes to DHFR inhibition by MTX and other antifolates varies when different cell lines are compared (10–12, 15, 17, 30, 39). H35 cells are among the most severely depleted of tetrahydrofolate, whereas L1210 and MCF-7 *in vitro* cells retain significant proportions of various $H_4PteGlu_n$ derivatives. It remains possible that the wide variation seen in the response of cellular reduced folate coenzymes to DHFR inhibition is not as closely related to the mechanism of antifolate activity as it is to variations in cellular folate regulation in different cell types. Our present studies with H35 cells do not clearly distinguish the role of the inhibition of secondary enzymatic sites by MTX polyglutamates and how these interactions contribute to the overall inhibitory activity of MTX. As before in this system (Fig. 3; Table 3; Ref. 17) and in the L1210 system (12, 30, 39), dihydrofolylpolyglutamates do not appear to be playing a significant role in preventing depletion of the tetrahydrofolate species. This is underscored by the fact that $H_2PteGlu_n$ is a 100-fold weaker inhibitor of H35 cell thymidylate synthase than are methotrexate polyglutamates (17). Certainly substrate depletion appears to be related to the cessation of precursor incorporation into DNA. However, at the low concentrations of 5,10-$CH_2$-$H_4PteGlu_n$ and 10-HCHO-$H_4PteGlu_n$ present after DHFR inhibition, thymidylate synthase and the transformylases involved in purine biosynthesis could be more sensitive to blockage by the accumulated MTX polyglutamates. It should be noted that limitations exist in the application of isolated enzyme kinetic data to elucidation of the function of enzymes in intact cells. For instance, sufficient 10-HCO-$H_4PteGlu_n$ exists within cells to severely limit thymidylate synthase activity based upon isolated enzyme studies (29). Inhibition does not appear to occur, however, because at cellular concentrations of 10-HCO-$H_4PteGlu_n$ 100-fold in excess of the $K_i$ for thymidylate synthase (Table 1) (17, 29) normal rates of growth and deoxyuridylate incorporation occur.

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