MOLECULAR PHARMACOLOGY, 12, 711-719

Mechanism of Action of Methotrexate

IV. Free Intracellular Methotrexate Required to Suppress Dihydrofolate Reduction to Tetrahydrofolate by Ehrlich Ascites Tumor Cells *in Vitro*

J. COURTLAND WHITE[1] AND I. DAVID GOLDMAN

Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298

(Received December 18, 1975)

SUMMARY

WHITE, J. COURTLAND & GOLDMAN, I. DAVID (1976) Mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells *in vitro*. *Mol. Pharmacol.*, 12, 711-719.

Incubation of Ehrlich ascites tumor cells *in vitro* with [^{3}H]dihydrofolate results in its rapid conversion to [^{3}H]tetrahydrofolate and other radiolabeled compounds, so that dihydrofolate cannot be detected in the cell. When cells are exposed to 10 μM methotrexate for 30 min, a condition sufficient to generate intracellular methotrexate in excess of the tightly bound fraction within the cell (exchangeable methotrexate), following which exchangeable intracellular methotrexate is eliminated, conversion of dihydrofolate to tetrahydrofolate is negligibly affected. Even when cells are maintained in a medium containing 0.05 μM methotrexate, following exposure to 10 μM methotrexate, there is virtually no inhibition of dihydrofolate metabolism. However, as the extracellular methotrexate level is increased to 0.1, 1.0, and 10 μM in the second incubation, there is progressive inhibition of tetrahydrofolate synthesis along with a progressive increase in the level of dihydrofolate within the cells, complete at the highest methotrexate concentration employed. Fifty percent inhibition of the cell tetrahydrofolate level requires an extracellular methotrexate concentration of approximately 0.2 μM, comparable to an exchangeable intracellular level of about 0.15 μM. In contrast to the results with dihydrofolate, after treatment of cells with 10 μM methotrexate for 30 min followed by removal of exchangeable drug, there is marked (approximately 85%) inhibition of folic acid reduction to tetrahydrofolate. Residual reduction of folic acid is completely eliminated when the cells are continuously exposed to 10 μM methotrexate. The requirement for free intracellular methotrexate to abolish tetrahydrofolate synthesis appears to be the basis for the observations from this laboratory that free intracellular methotrexate is necessary to suppress [^{3}H]deoxyuridine incorporation into DNA and [^{14}C]formate incorporation into RNA, DNA, and protein. The demonstration in other studies that mammalian cells have a limited capacity to accumulate free intracellular methotrexate supports the concept that this intracellular component of the drug may be an important determinant of cytotoxicity.

This research was supported by Grant CA 16906 from the National Cancer Institute.

[1] Recipient of United States Public Health Service Postdoctoral Fellowship CA 05067.

INTRODUCTION

Studies from this laboratory utilizing a cellular pharmacokinetic analysis of the

consequences of the interaction between MTX[2] and the intact mammalian cell *in vitro* indicated the importance of free intracellular MTX in the inhibition of THF-dependent nucleic acid and amino acid synthesis by this agent (1–4). These studies clarified the crucial role of free intracellular MTX in this phenomenon, in contrast to possible competitive interactions between extracellular MTX and THF cofactors at the level of a common transport carrier at the cell membrane or at a binding site within the cell (1, 3). It was shown that when cells are loaded with MTX to a level which should achieve more than 99.9% saturation of high-affinity dihydrofolate reductase sites, and then suspended in MTX-free medium, there is a component of intracellular drug that rapidly leaves the cells (exchangeable intracellular MTX) and a portion which remains essentially irreversibly bound within the cell. However, this treatment minimally affects the incorporation of [^{3}H]UdR into DNA or of [^{14}C]formate into DNA, RNA, or protein. Only when the cellular MTX content was raised to appreciable levels in excess of the tightly bound fraction could these processes be inhibited. Because exchangeable MTX appeared to be the critical factor in the inhibition of THF-dependent pyrimidine, purine, and amino acid synthesis, it was suggested that this intracellular MTX component inhibits a site common to these three processes. This raised the possibility that THF synthesis may not be abolished unless substantial levels of exchangeable MTX are present within the cell. In this report we examine directly the conversion of [^{3}H]DHF and [^{3}H]folic acid to [^{3}H]THF by Ehrlich ascites tumor cells *in vitro* and contrast the effects of tightly bound MTX alone with the effects of exchangeable intracellular MTX on these processes. The data indicate that while folic acid reduction is highly sensitive to association of MTX with high-affinity binding sites alone, there is little inhibition of THF synthesis from DHF unless appreciable levels of exchangeable MTX are present within the cell.

[2] The abbreviations used are: MTX, methotrexate; THF, tetrahydrofolate; DHF, dihydrofolate.

These observations relate to other studies from this laboratory, which suggest that energy-dependent processes in mammalian cells markedly limit the accumulation of free intracellular MTX (5–9), and other data, which suggest that the level of free intracellular drug may be an important element in MTX inhibition of DNA synthesis and cytotoxicity *in vivo* (10–12). These studies raise the possibility that the efficacy of high-dose MTX protocols may be based, in part, upon the ability of these regimens to generate and sustain high levels of free intracellular drug that are necessary to achieve cessation of THF synthesis and THF-dependent biosynthetic processes.

METHODS

Cells, media, and incubation techniques. Ehrlich ascites tumor cells were obtained from CF_1 mice (Carworth Farms, Boston) 6–12 days after intraperitoneal injection of 0.1 ml of undiluted ascitic fluid. Contaminating erythrocytes were removed by a minimum of two washes at 0°, following which the cells were suspended in a modified Eagle's medium (1) without folates or serum to a final cytocrit of 5–8%. The pH was maintained at 7.0–7.4 by passing warmed, humidified 95% O_2–5% CO_2 over the mechanically stirred suspension at 37°. All incubations employing DHF were performed in the dark.

Chemicals. Unlabeled folic acid, THF, and *p*-aminobenzoylglutamate were purchased from Sigma Chemical Company. DEAE-cellulose was obtained from Bio-Rad, and DEAE-Sephadex A-25, from Pharmacia. MTX (Lederle Laboratories) was purified on DEAE-cellulose as previously described (13). [3′,5′,9-^{3}H]Folic acid, 500 Ci/mole, was similarly purified on a DEAE-cellulose ion-exchange column eluted with a linear gradient of ammonium bicarbonate buffer, pH 8.3 (0.3–1.0 M). Following lyophilization of [^{3}H]folic acid-containing fractions, the purified material was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.3, and stored at −20°. The concentration of folic acid was determined spectrophotometrically at pH 7, using a millimolar extinction coefficient

of 27.6 at 280 nm (14). Dihydrofolic acid was prepared from folic acid by dithionite reduction according to Blakely (15). [^{3}H]DHF was prepared from purified [^{3}H]folic acid by the same procedure on a microscale after reducing the specific activity of the [^{3}H]folic acid to 23 Ci/mole. Small portions of labeled or unlabeled DHF, suspended in a solution of 5 mM HCl and 50 mM 2-mercaptoethanol, were stored at −20°. The DHF concentration was determined at pH 7, using a millimolar extinction coefficient of 28.4 at 282 nm (15). The radiochemical purity of freshly prepared or freshly thawed [^{3}H]DHF after storage for 2 months exceeded 90%. The major labeled contaminant (about 6%) was identified as *p*-aminobenzoylglutamate by chromatography with the unlabeled reference compound [λ_{max} = 273 nm (16)]. DHF was stored at −20° until just before use, and unused portions were discarded.

Chromatographic techniques. After incubation with radiolabeled folate or DHF, cells (0.5–0.8 ml, packed volume) were washed twice with 0.85% NaCl at 0° and then suspended in 4 ml of a 5 mM Tris-HCl buffer (pH 7.2) at 4°, containing 0.2 M 2-mercaptoethanol. The samples were then subjected to sonic oscillation twice for 30 sec on a Heat Systems Ultrasonic sonic oscillator. After centrifugation for 1 hr at 40,000 × *g*, the supernatant fraction plus nonlabeled reference compounds were chromatographed on a 0.9 × 15 cm column of DEAE-cellulose. After equilibration with starting buffer, folates were eluted with a linear gradient of 100 ml of 5 mM Tris (pH 7.2 at 4°) containing 0.2 M 2-mercaptoethanol and 100 ml of buffer containing 1 N NaCl. Up to four such columns could be fractionated simultaneously on two LKB collectors by drawing elution buffer from the same mixing chamber at the same speed using a Buchler four-channel peristaltic pump at a flow rate of 40 ml/hr. Fifty 3.3-ml fractions were collected, and 2-ml samples were counted from each fraction for radioactivity in 10 ml of scintillation fluor solution containing 67% toluene and 33% Triton X-100. The positions of nonlabeled reference compounds were determined for each column, and their identity was confirmed from their characteristic ultraviolet absorption spectra. Chromatography on DEAE-Sephadex A-25, as described by Nixon and Bertino (17), was used to verify further the identity of radiolabeled products derived from [^{3}H]folate or [^{3}H]DHF.

RESULTS

Effects of MTX on metabolism of [^{3}H]folic acid. Cells were incubated in the presence of 10 μM MTX for 30 min to generate intracellular MTX in excess of the high-affinity binding capacity, then separated from the medium and washed twice with MTX-free buffer at 0°. The cells were then divided into two portions and incubated with or without 10 μM MTX at 37° for an additional 30 min. During this second incubation, exchangeable MTX leaves the cells resuspended in MTX-free medium, and intracellular MTX achieves a steady state in those cells incubated in MTX-containing medium. The cells were then separated by centrifugation and resuspended in fresh medium of the same composition, and [^{3}H]folic acid was added to attain a final concentration of 10 μM in these cells as well as control cells processed similarly except for the omission of MTX in any of the incubations. After 1 hr the cells were removed from the medium and washed twice with 0.85 NaCl at 0°, sonic extracts were prepared, and the distribution of radiolabel was determined by DEAE-cellulose chromatography as described in METHODS. Control cells converted [^{3}H]folic acid to other molecular forms, although about 45% of the total ^{3}H in the soluble extract remained as unchanged folic acid (Fig. 1A). The major radiolabeled product derived from [^{3}H]folic acid co-chromatographed with unlabeled THF. To verify the identity of this product, an extract from cells incubated under similar conditions was chromatographed on DEAE-Sephadex A-25. Again the major ^{3}H peak coincided with the reference THF. A small peak corresponding to *p*-aminobenzoylglutamate was identified, but other minor radiolabeled compounds shown in Fig. 1A have not as yet been identified and may represent other folate coenzyme forms or degradation products. Figure 1B indicates the distribution of radiolabel in

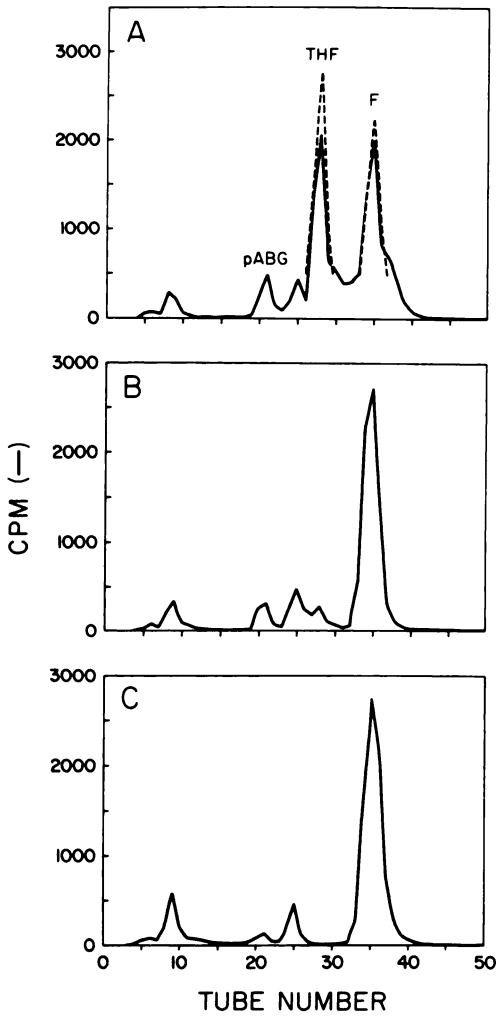

FIG. 1. *Effect of tightly bound MTX and exchangeable intracellular MTX on metabolism of [³H]folic acid*

Cells were divided into two portions; one was exposed to medium containing 10 μM MTX for 30 min at 37°, while the other (control cells) was incubated without MTX. The cells were collected by centrifugation and washed twice with buffer at 0°. Half the MTX-treated cells were incubated again with 10 μM MTX (C) for 30 min, while control cells (A) and the other half of the MTX-treated cells (B) were incubated without MTX. Finally the cells were collected, washed twice, and resuspended in fresh medium of the same composition, and [³H]folic acid was added to achieve a final concentration of 10 μM. After incubation with [³H]folic acid for 60 min, sonic extracts of the cells plus nonlabeled reference compounds were chromatographed on DEAE-cellulose as described in METHODS. - - -, chromatographic distribution of nonlabeled folic acid (F, A_{282}) and THF (A_{295}). The position of nonlabeled *p*-aminobenzoylglutamic acid is indicated as pABG.

cells which were first incubated with 10 μM MTX, following which exchangeable intracellular MTX was removed. Although reduction of [³H]folic acid was markedly reduced in these cells and the level of folic acid was increased, a low level of THF was detected within the cells. In five such experiments, radioactivity in the THF peak was 15.3 ± 2.4% (SE) of that found in control cells. However, when MTX in excess of the tightly bound fraction was maintained within the cells (Fig. 1C), the small residual synthesis of THF was eliminated.

Effects of MTX on metabolism of [³H]DHF. The effects of MTX on the reduction and metabolism of [³H]DHF were evaluated similarly, except that cells were incubated for only 15 min with radiolabel to minimize nonenzymatic degradation of DHF. Control cells converted the substrate to [³H]THF, as indicated by co-chromatography of radiolabel with nonlabeled THF on DEAE-cellulose (Fig. 2A) and DEAE-Sephadex A-25. DHF could not be identified within the cell. As may be seen, a large portion of the total ³H accumulated by the cells was not adsorbed to the ion-exchange resin and may represent degradation products, unidentified folate compounds, or folate adsorbed to protein. Radioactivity found in a peak coincident with *p*-aminobenzoylglutamate, although relatively low in the experiment illustrated in Fig. 2, was highly variable from day to day and is presumed to be a result of variable extracellular oxidative breakdown of DHF. Cells exposed to MTX to generate appreciable levels in excess of the tightly bound fraction, following which exchangeable drug was removed, showed almost no alteration in the distribution of radiolabel (Fig. 2B), in contrast to the observation with [³H]folic acid. There was only a very small ³H-labeled peak coincident with unlabeled DHF. Even when cells, after the treatment with 10 μM MTX, were maintained in a medium containing 0.05 μM

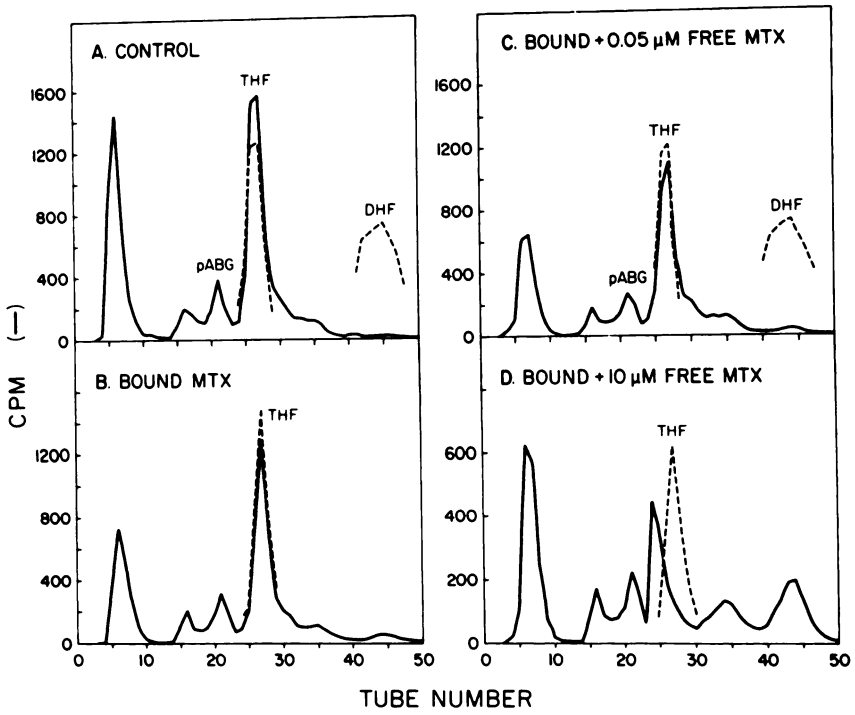

FIG. 2. *Effect of tightly bound MTX and exchangeable intracellular MTX on metabolism of [³H]DHF*
Cells were treated with or without (A) 10 μM MTX, then incubated in MTX-free medium (B) or medium containing 0.05 μM (C) or 10 μM (D) MTX. After incubation as described in Fig. 1, the cells were exposed to 10 μM [³H]DHF for 15 min. Sonic extracts plus nonlabeled reference compounds were then chromatographed on DEAE-cellulose as described in METHODS. - - -, chromatographic distribution of nonlabeled DHF (A_{282}) and THF (A_{295}). The position of nonlabeled *p*-aminobenzoylglutamic acid is indicated as pABG.

MTX (Fig. 2C), the cellular THF level was minimally reduced. However, when cells were continuously exposed to 10 μM MTX, so that appreciable levels of exchangeable MTX were sustained within the cells (Fig. 2D), the ³H peak representing THF was abolished and DHF reached its maximum level in the cells. The major peak (tube 24), aside from the portion of radiolabel not adsorbed to the column, was distinct from THF, N^5-formyl-THF, and N^5-methyl-THF. This compound, presently unidentified, is formed from [³H]DHF in media without cells and is under further study. Figure 3 illustrates the relationship between the extracellular MTX level after association of MTX with high-affinity sites, and THF and DHF levels within the cell. Following exposure to 10 μM MTX for 30 min, cells were washed, divided into four portions, and resuspended in fresh medium for an additional 45 min with 0.01, 0.1, 1.0, or 10 μM MTX before addition of [³H]DHF. THF levels began to decline and DHF accumulated when cells were incubated with 0.1 μM MTX, but THF production was still evident during exposure to 1 μM MTX (Fig. 3). Figure 4 is a composite of three such experiments which quantitates the relationship between the exchangeable intracellular MTX levels and percentage changes in the levels of intracellular DHF and THF. Since 0.01 μM MTX did not significantly alter the cell THF level (Fig. 2), cell THF in the presence of this concentration of MTX is considered equal to 100% of the uninhibited level. DHF levels in the cell are presented as a percentage of the maximum intracellular DHF level when THF synthesis was completely blocked by 10 μM MTX, The data indicate that to achieve 50% depresssion of the cell THF

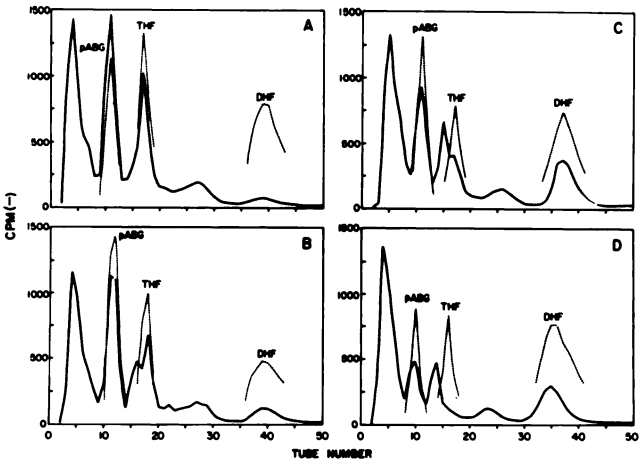

FIG. 3. *Effect of intracellular MTX in excess of tightly bound fraction on metabolism of [³H]DHF*

Cells were exposed to 10 μM MTX for 30 min, then incubated with 0.01 μM (A), 0.1 μM (B), 1.0 μM (C), or 10 μM (D) MTX for 45 min. The cells were then incubated in fresh medium of the same composition containing 10 μM [³H]DHF for 15 min, and sonic extracts were chromatographed. ····, chromatographic distribution of nonlabeled *p*-aminobenzoylglutamic acid (pABG, A_{273}), THF (A_{295}), and DHF (A_{282}).

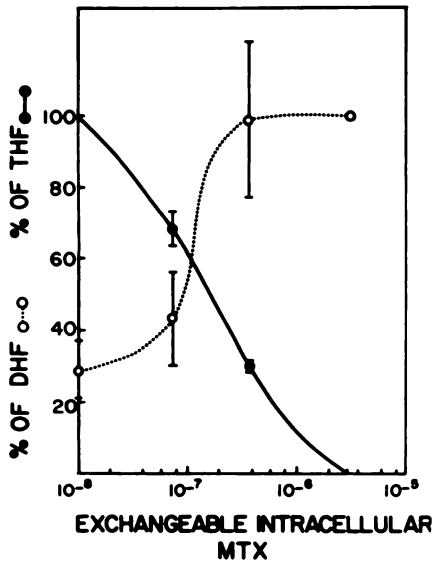

FIG. 4. *Effect of intracellular MTX in excess of tightly bound fraction on [³H]THF and [³H]DHF levels in the cell after exposure to [³H]DHF*

The data from three experiments as deseribed in Fig. 3 were used to describe the cell THF and DHF levels (mean ± standard error) as a function of the exchangeable intracellular MTX concentration. ●——●, data plotted as a percentage of the THF level when exchangeable intracellular MTX was 0.01 μM (a concentration which negligibly affects cell THF); O····O, data plotted as a percentage of the DHF level when exchangeable intracellular MTX was 5 μM (a concentration at which the cell DHF level is maximum).

level and an increase of cellular DHF to 50% of the maximum level required maintenance of an exchangeable intracellular MTX level in the range of approximately 0.15 μM. To achieve this intracellular MTX concentration requires an extracellular MTX level of about 0.2 μM.

DISCUSSION

The previous observations from this laboratory that suppression of THF-dependent purine, pyrimidine, and amino acid synthesis requires free intracellular MTX raised the possibility that reduction of DHF to THF continues unless appreciable levels of free MTX are present within the cell (1–4). The studies which we now report support this formulation. These data may account for the observations *in vivo* that (a) MTX toxicity can be reversed by DHF (18) and (b) high doses of MTX are required to suppress THF synthesis from DHF in mouse intestine and liver (19). The apparent role for free intracellular MTX in the inhibition of THF synthesis from DHF may be accounted for on the basis of an interaction between MTX and a single high-affinity form of dihydrofolate reductase. Clearly, the interaction between MTX and dihydrofolate reductase is not stoichiometric and follows saturation kinetics at physiological pH (20). Hence some free drug would be required to satu-

rate the enzyme on this basis. While it is apparent that the MTX loading and washout procedure must result in association of the major portion of high-affinity dihydrofolate reductase sites with MTX, only a small percentage of total enzyme activity may be necessary to meet cellular requirements for THF synthesis. Jackson and Harrap (21) estimated that only 5% of the total dihydrofolate reductase activity would be necessary to sustain logarithmic growth of L1210 leukemia cells in culture. In the present studies experimental conditions did not permit cellular replication, so that even a smaller fraction of dihydrofolate reductase activity might sustain THF cofactor levels. As pointed out by Jackson and Harrap (21), when cellular DHF levels are very low in comparison to the K_m for dihydrofolate reductase, as enzyme is inactivated by MTX, transient inhibition of DHF reduction results in an increase in the cellular DHF level and restoration of DHF reduction as the net rate of interaction between the higher level of DHF and enzyme sites unassociated with MTX increases in proportion to the rise in cell DHF. Also, as cell DHF rises to the level of its K_m, at a time when the free intracellular MTX level is very low, it competes with MTX for the remaining few dihydrofolate reductase sites. This sequence of events maintains THF production at nearly normal rates until the cellular DHF level approaches its K_m and a major portion of the enzyme is associated with drug. The critical fraction of the enzyme is so small that complete suppression of THF synthesis can only be achieved when high levels of free MTX are present within the cell. Conversely, when MTX-loaded cells are transferred to MTX-free medium, as the free intracellular MTX level falls the initially high DHF levels compete with drug for dihydrofolate reductase, displacing some MTX from enzyme sites until THF synthesis resumes and DHF levels again fall well below the K_m. At this point there is no significant competition by DHF for MTX binding sites, so that a large portion of the total intracellular MTX remains bound within the cell, but represents a value less than the total dihydrofolate reductase capacity. The very high capacity of the enzyme for DHF reduction is suggested by the observation that intracellular DHF could not be detected in control cells in the absence of MTX. We would estimate on this basis that the cellular DHF level must be less than 10 nM. Since the DHF K_m is about 0.86 μM,[3] the enzyme must be at a very low state of saturation with respect to DHF under the conditions of these experiments. Based upon the observation that 0.01 μM MTX negligibly affects the cell THF level, the percentage of total dihydrofolate reductase activity required to sustain THF synthesis at this MTX level was estimated from the measured MTX K_i (1.6×10^{-11} M at pH 7.2)[3] and DHF K_m (see above), assuming (a) that these constants are equivalent to the binding constants and (b) that cell DHF could rise to 10 μM when the MTX level is 0.01 μM. According to this, only about 2% of the dihydrofolate reductase activity would be required to sustain basal rates of THF synthesis in these cells.

In addition, the requirement for free intracellular MTX might be related to enzyme heterogeneity within the cell. Since only a small fraction of total dihydrofolate reductase activity sustains nearly normal rates of THF synthesis, a small amount of an enzyme species with an affinity for MTX 1–2 orders of magnitude less than the "high-affinity" form would have a profound effect on the level of free drug required to disrupt THF synthesis, even though the affinity of this enzyme for MTX would nonetheless be quite high. Multiple forms of dihydrofolate reductase have been identified in bacteria and mammalian cells, which differ in their electrophoretic behavior depending upon the presence or absence of an associated NADPH molecule (22–24). In the absence of NADPH, the affinity of MTX and other antifols is reduced (25–29). The possible role these enzymes forms may play in maintaining DHF reduction in the absence of exchangeable intracellular MTX requires further evaluation.

[3] M. J. Poe, Merck Institute for Therapeutic Research, Rahway, N. J., personal communication. Determinations were performed on purified dihydrofolate reductase from Ehrlich ascites tumor cells in 0.05 M Tris–0.05 M NaCl at pH 7.2.

While the major intracellular folate detected after incubation of cells with [^{3}H]folic acid was also THF, the sensitivity of this metabolic pathway to MTX is considerably different from that of DHF. Association of MTX with high-affinity sites alone markedly inhibits (approximately 85%) folic acid reduction. This difference in the effects of MTX on DHF and folic acid reduction may reflect the large differences in the rate of reduction of the two substrates at neutral pH in cell-free systems (20). The usual rate of folic acid reduction is considerably slower than that of DHF, so that, unlike DHF, folic acid accumulates in the cell in the absence of MTX and there is no striking increase in this intracellular component in the presence of MTX. Hence, as dihydrofolate reductase activity is reduced by MTX in the absence of a rise in the cellular folic acid level, increased folic acid reduction by the remaining enzyme sites is not possible.

The critical role of free drug in the suppression of THF synthesis is of considerable interest in view of the limited capacity of mammlian cells to accumulate this intracellular component—a phenomenon which is related to (a) the anionic nature of the MTX molecule and (b) an exit pump which may suppress accumulation of free drug within the cell (5–8, 30). Indeed, accumulation of free intracellular MTX may be so limited in some cells that this may be an important factor in tumor resistance (9, 11). Hence the enhanced chemotherapeutic efficacy of high-dose MTX protocols (31–34) may be related, in part, to the development of extracellular MTX concentrations which are so high that the critical levels of free intracellular drug necessary to suppress THF synthesis are achieved. In this context, the concurrent administration of vincristine, which augments net accumulation of intracellular MTX (35–37) and potentiates MTX inhibition of UdR incorporation into DNA (2), may offer the opportunity to generate critical intracellular MTX concentrations at lower extracellular drug levels (5).

What is particularly intriguing about the success (albeit limited) of MTX–5-formyl-THF acid rescue protocols is that these regimens are usually directed against slowly growing tumors, which should be inherently insensitive to an agent which achieves its lethal effect by attacking cells in S-phase. This raises the possibility that the cytotoxic effects of MTX in these drug regimens may be related, in part, to inhibition of THF-dependent RNA synthesis, and consequent inhibition of protein synthesis in the non-S-phase cell (38). The inhibitory effect of MTX on purine synthesis has been demonstrated in rapidly proliferating cell populations (39, 40). In fact, a requirement for high levels of free intracellular MTX in the inhibition of THF synthesis in non-S-phase cells would be expected due to a very low requirement for DHF reduction as THF is consumed at a slow rate in nuclear (DNA repair) and mitochondrial deoxythymidylate synthesis (38). This may be a basis, in part, for the observation that very high levels of MTX are required to achieve cytotoxicity and inhibit UdR incorporation into DNA in stationary compared with logarithmic phase L5178Y cells (41).

REFERENCES

1. Goldman, I. D. (1974) *Mol. Pharmacol.*, **10**, 257–274.
2. Goldman, I. D. & Fyfe, M. J. (1974) *Mol. Pharmacol.*, **10**, 275–282.
3. White, J. C., Loftfield, S. & Goldman, I. D. (1975) *Mol. Pharmacol.*, **11**, 287–297.
4. Goldman, I. D. (1975) *Cancer Chemother. Rep.*, **6**, 51–61.
5. Goldman, I. D., Gupta, V., White, J. C. & Loftfield, S. (1976) *Cancer Res.*, **36**, 276–279.
6. Goldman, I. D. (1969) *J. Biol. Chem.*, **244**, 3779–3785.
7. Goldman, I. D. (1971) *Ann. N. Y. Acad. Sci.*, **186**, 400–422.
8. Goldman, I. D. (1975) *Cancer Chemother. Rep.*, **6**, 63–72.
9. Goldman, I. D. (1973) in *Drug Resistance and Selectivity: Biochemical and Cellular Basis* (Mihich, E., ed.), Vol. 1, pp. 299–358, Academic Press, New York.
10. Sirotnak, F. M. & Donsbach, R. C. (1975) *Cancer Res.*, **35**, 1737–1744.
11. Sirotnak, F. M. & Donsbach, R. C. (1973) *Cancer Res.*, **33**, 1290–1294.
12. Chabner, B. A. & Young, R. C. (1973) *J. Clin. Invest.*, **52**, 1804–1811.
13. Goldman, I. D., Lichtenstein, N. S. & Oliverio,

V. T. (1968) *J. Biol. Chem.*, **243**, 5007-5017.
14. Rabinowitz, J. C. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H., & Myrbäck, K., eds.), Vol. 2, p. 185, Academic Press, New York.
15. Blakely, R. L. (1960) *Nature*, **188**, 231-232.
16. Kallen, R. G. & Jencks, W. P. (1966) *J. Biol. Chem.*, **241**, 5845-5851.
17. Nixon, P. F. & Bertino, J. R. (1971) *Methods Enzymol.*, **18**, 661-663.
18. Mead, J. A. R., Venditti, J. M., Schrecker, A. W., Goldin, A. & Keresztesy, J. C. (1963) *Biochem. Pharmacol.*, **12**, 371-383.
19. Mishra, L. C., Parmar, A. S. & Mead, J. A. R. (1972) *Anal. Biochem.*, **48**, 515-523.
20. Bertino, J. R., Booth, V. A., Beiber, A. L., Cashmore, A. & Sartorelli, A. C. (1964) *J. Biol. Chem.*, **239**, 479-485.
21. Jackson, R. C. & Harrap, K. R. (1973) *Arch. Biochem. Biophys.*, **158**, 827-841.
22. Dunlap, R. B., Gunderson, L. E. & Huennekens, F. M. (1971) *Biochem. Biophys. Res. Commun.*, **42**, 772-777.
23. Gunderson, L. E., Dunlap, R. B., Harding, N. G. L., Freisheim, J. H., Otting, F. & Huennekens, F. M. (1972) *Biochemistry*, **11**, 1018-1023.
24. Huennekens, F. M., DiGirolamo, P. M., Fujii, K., Henderson, G. B., Jacobson, D. W., Neef, V. & Rader, J. I. (1974) *Adv. Enzyme Regul.*, **12**, 131-153.
25. Otting, F. & Huennekens, F. M. (1972) *Arch. Biochem. Biophys.*, **152**, 429-431.
26. Freudenthal, R., Lowe, J. & Hebborn, P. (1970) *Mol. Pharmacol.*, **6**, 617-620.
27. Williams, M. N., Greenfield, N. J. & Hoogsteen, K. (1973) *J. Biol. Chem.*, **248**, 6380-6386.
28. Williams, M. N., Poe, M., Greenfield, N. J., Hirshfield, J. M. & Hoogsteen, K. (1973) *J. Biol. Chem.*, **248**, 6375-6379.
29. Perkins, J. P. & Bertino, J. R. (1966) *Biochemistry*, **5**, 1005-1012.
30. Hakala, M. (1965) *Biochim. Biophys. Acta*, **102**, 210-225.
31. Capizzi, R. L., DeConti, R. C., Marsh, J. C. & Bertino, J. R. (1970) *Cancer Res.*, **30**, 1782-1788.
32. Djerassi, I., Rominger, C. J., Kim, J. S. *et al.* (1972) *Cancer*, **30**, 22-50.
33. Pratt, C. B., Roberts, D., Shanks, E. C. *et al.* (1974) *Cancer Res.*, **34**, 3326-3331.
34. Jaffe, N., Frei, E., III, Traggis, D. *et al.* (1974) *N. Engl. J. Med.*, **291**, 994-997.
35. Zager, R. F., Frisby, S. A. & Oliverio, V. T. (1973) *Cancer Res.*, **33**, 1670-1676.
36. Bender, R. A., Bleyer, W. A., Frisby, S. A. & Oliverio, V. T. (1975) *Cancer Res.*, **35**, 1305-1308.
37. Fyfe, M. J. & Goldman, I. D. (1973) *J. Biol. Chem.*, **248**, 5067-5073.
38. Goldman, I. D. (1976) *Cancer Chemother. Rep.*, in press.
39. Hryniuk, W. M. (1975) *Cancer Res.*, **35**, 1085-1092.
40. Hryniuk, W. M., Brox, L. W., Henderson, J. F. & Tamaoki, T. (1975) *Cancer Res.*, **35**, 1427-1432.
41. Hryniuk, W. M., Fischer, G. A. & Bertino, J. R. (1969) *Mol. Pharmacol.*, **5**, 557-564.