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The Mechanism of Action of Methotrexate

III. Requirement of Free Intracellular Methotrexate for Maximal Suppression of [^{14}C]Formate Incorporation into Nucleic Acids and Protein

J. COURTLAND WHITE,[1] SHARON LOFTFIELD, AND I. DAVID GOLDMAN[2]

Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298, and Departments of Medicine and Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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SUMMARY

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Recent studies from this laboratory suggest that association of methotrexate with high-affinity intracellular binding sites has little effect upon the incorporation of [^{3}H]deoxyuridine into DNA, while relatively high intracellular levels of methotrexate in excess of the tightly bound fraction are required for inhibition. In this report the effects of different components of intracellular methotrexate on [^{14}C]formate uptake and incorporation into nucleic acids and proteins in cultured L-cell mouse fibroblasts are evaluated. [^{14}C]Formate is rapidly taken up by these cells and metabolized to molecular forms which do not penetrate the cell membrane. After 30 min about 60% of the ^{14}C remains in the acid-soluble fraction. Of the radiolabel in the acid precipitate approximately 55% is associated with RNA, 11% with DNA, and 33% with protein. Intracellular methotrexate sufficient only to saturate high-affinity sites causes only slight inhibition of [^{14}C]formate uptake by intact cells or of [^{14}C]formate incorporation into RNA, DNA, and protein. However, intracellular methotrexate in excess of this fraction markedly inhibits these processes, with 50% inhibition of [^{14}C]formate incorporation into DNA, RNA, and protein at intracellular methotrexate levels of 0.3, 1.0, and 3 μM, respectively. These data suggest that intracellular methotrexate in excess of that necessary to saturate high-affinity sites is required to suppress nucleic acid and protein synthesis—an effect that may be related to an interaction between methotrexate and a dihydrofolate reductase form with a low affinity for this agent.

INTRODUCTION

Recent studies from this laboratory which explored the characteristics of MTX[3] inhibition of DNA synthesis in intact L-cell mouse fibroblasts and Ehrlich

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

ascites tumor cells (1, 2) suggested that association of MTX with more than 99% of the high-affinity intracellular binding sites has little effect on the incorporation of deoxyuridine into DNA. Rather, maximum suppression of this process required intracellular MTX in excess of the tightly bound fraction, with 50% inhibition occurring at an exchangeable[4] intracellular MTX level of 0.2–0.4 μM. The experiments were designed to minimize the possibility that the apparent inhibitory effect of intracellular MTX in excess of the tightly bound fraction on deoxyuridine incorporation into DNA was related to (a) penetration of intracellular compartments containing additional high-affinity sites, (b) competition or exchange phenomena with endogenous tetrahydrofolate cofactors at the level of their common membrane carrier, or (c) displacement of folates from binding sites within the cell (1, 2). These data suggested that inhibition of DNA synthesis by MTX in the intact L-cell requires association of MTX with a "low"-affinity site (or sites) in addition to high-affinity dihydrofolate reductase sites—an interaction which would account for the synergistic effect of vincristine on the chemotherapeutic efficacy of MTX (3), based upon the ability of this agent to increase the intracellular MTX level in excess of the usual tightly bound fraction (2, 4).

The experiments to be reported here employ similar techniques to assess the roles of tightly bound MTX and intracellular MTX in excess of this fraction on the folate-dependent incorporation of [^{14}C]formate into RNA, DNA, and protein in intact L-cell mouse fibroblasts. The results suggest that appreciable inhibition of [^{14}C]formate incorporation into these macromolecules requires MTX in excess of the tightly bound fraction and that the inhibitory effect of this component of intracellular MTX is not related to an interaction between this agent and a target site unique to the synthesis of DNA alone. These studies challenge the classical view that the mechanism of action of MTX is related to a "single" interaction with a high-affinity dihydrofolate reductase binding site and raise the possibility that intracellular dihydrofolate-reducing activity may not be blocked by an interaction between MTX and a single class of high-affinity binding sites.

[4] "Exchangeable" MTX refers to intracellular MTX in excess of the tightly bound fraction. This includes MTX which is osmotically active, or "free", and MTX which is loosely bound within the cell; both components rapidly leave the cell when cells loaded with MTX are suspended into an MTX-free medium.

MATERIALS AND METHODS

Cells, Media, and Incubation Techniques

L-cell mouse fibroblasts were grown in YLE medium supplemented with 5% calf serum (4). For experimentation cells in the logarithmic growth phase were washed twice in a bicarbonate-buffered electrolyte solution, then suspended in a modified Eagle's medium (1) without folates or serum to a final cytocrit of less than 4%. The pH was maintained at 7.0–7.4 over a 1.5-hr incubation by passing warmed, humidified 95% O_2–5% CO_2 over the mechanically stirred suspension. The incubation temperature was 37°.

Experimental Techniques

Measurement of cellular uptake of [^{14}C]formate. After exposure of cells to [^{14}C]formate, portions of the cell suspensions were dispersed into 10 volumes of 0.85% NaCl solution at 0°; the cell fractions were separated by centrifugation, then washed two additional times in the same solution. The washed cell pellets were then aspirated into the tip of a Pasteur pipette, extruded onto polyethylene tares, and dried overnight at 70°. The dried cells were peeled off the tares and weighed directly on a Beckman LM-800 microbalance, then transferred to liquid scintillation vials. After digestion in 0.1 ml of 1 N KOH for 1 hr at 70°, 10 ml of a methanol-toluene fluor solution (5) were added and radioactivity was determined on a liquid scintillation spectrometer.

Determination of [^{14}C]formate incorporation into RNA, DNA, and protein. Cells were incubated with [^{14}C]formate, washed as described above, then suspended in 0.5

ml of 0.85% NaCl at 0°; protein and nucleic acids were precipitated by the addition of 0.5 ml of 0.4 N $HClO_4$ at 0°. After 10 min at 0° the precipitate was separated by centrifugation and the $HClO_4$-soluble fraction was sampled for radioactivity. The precipitate was washed twice with 0.2 N $HClO_4$ at 0°, then dissolved in 0.5 ml of 0.3 N NaOH, and RNA was digested by incubation at 37° for 1 hr. DNA and protein were then precipitated after chilling to 0° by the addition of 0.05 ml of 5.2 N $HClO_4$ and separated by centrifugation. The supernatant containing the digested RNA was sampled and radioactivity was determined. The precipitate was washed twice with 0.2 N $HClO_4$ at 0°, then suspended in 0.5 ml of 0.5 N $HClO_4$ and incubated at 70° for 25 min to digest DNA. After cooling, the precipitate was separated by centrifugation, the supernatant was sampled, radioactivity in the DNA digest was determined, and the remaining precipitate was washed twice with 0.5 N $HClO_4$ at 0°. Finally, the protein-containing precipitate was digested in 0.5 ml of 0.5 N KOH for 1 hr at 70° and sampled for radioactivity. Final counting efficiencies established with [^{14}C]toluene internal standards were 80–90%. Data are expressed as means ± standard errors.

Chemicals

MTX, obtained from Lederle Laboratories, was purified by DEAE-cellulose chromatography as described previously (5). [^{14}C]Formate (sodium salt), 3 Ci/mole, was obtained from New England Nuclear Corporation and used at a final concentration of 100 μM.

RESULTS

Effect of MTX on Cellular Uptake and Exchangeability of [^{14}C]Formate

Figure 1 illustrates an experiment designed to contrast the effects of tightly bound and exchangeable intracellular MTX on [^{14}C]formate uptake into L-cells.

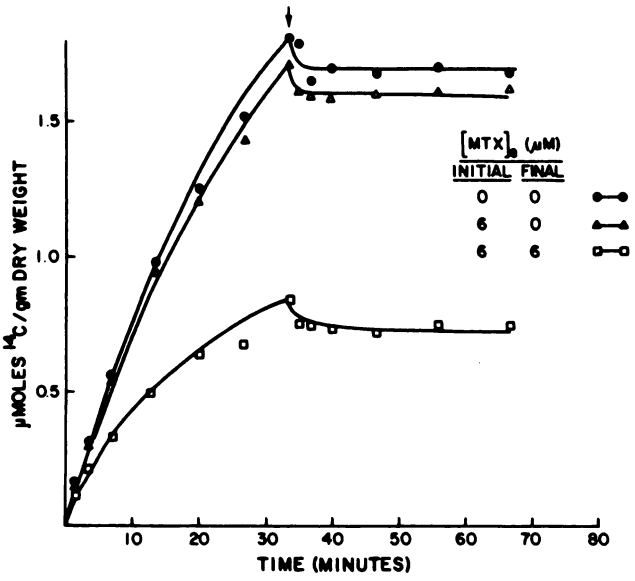

FIG. 1. *Effect of MTX on uptake and efflux of ^{14}C after exposure of cells to* [^{14}C]formate

Two portions of cells were incubated with 6 μM MTX at 37° for 15 min while a third portion (●) was incubated as a control. The cells were separated by centrifugation, washed twice in 0° buffer, then resuspended in medium with (□) or without (△) 6 μM MTX. After 20 min the cells were separated, washed, and resuspended in fresh medium of the same composition. Ten minutes later [^{14}C]formate was added to achieve a final concentration of 100 μM. At the arrow, cells were separated from their media by centrifugation, washed in 0° buffer, then resuspended in a large volume of 37° formate-free medium to measure efflux of intracellular radiolabel.

Two portions of cells were incubated with 6 μM MTX for 15 min, a concentration of MTX and interval of exposure well in excess of those required to saturate high-affinity sites. The cells were then separated from the medium by centrifugation, washed twice with 0° buffer, then incubated in fresh medium with or without 6 μM MTX for 20 min. The cells were again separated by centrifugation and suspended into fresh medium of the same composition. During the latter incubations MTX in excess of the tightly bound fraction exited from cells suspended in MTX-free medium, and intracellular MTX achieved a steady state in those cells resuspended in medium with MTX. Control cells were incubated in the same way but without exposure to MTX in the initial or final incubations. [^{14}C]Formate was then added to achieve a final concentration of 100 μM, following which ^{14}C associated with the cells was determined as described in MATERIALS AND METHODS. In cells in which only high-affinity sites are saturated with MTX there is only a slight alteration in the total cell uptake of ^{14}C. In five such experiments the suppression of ^{14}C uptake at 30 min is only 12.6 ± 4.2%. However, when MTX in excess of the high-affinity sites is provided, there is a marked inhibition of ^{14}C uptake (63.3 ± 6.9% after 30 min). At the arrow (Fig.1) cells were separated from their media by centrifugation and resuspended in a large volume of formate-free medium to observe the undirectional efflux characteristics of the intracellular radiolabel. It can be seen that the fraction of radiolabel free to leave the cell is minute compared to the total cell radiolabel, suggesting that formate is rapidly metabolized to molecular forms which do not penetrate the cell membrane. In addition, exposure of cells to MTX does not alter the level of the small component of intracellular radiolabel which is free to leave the cell.

MTX Inhibition of ^{14}C Incorporation into RNA, DNA, and Protein

Figure 2 illustrates the effect of a high level of MTX (30 μM) on ^{14}C incorporation into the perchloric acid-soluble fraction and into RNA, DNA, and protein. L-cells were incubated with and without MTX for 1 hr before addition of [^{14}C]formate. In three experiments the bulk of intracellular ^{14}C, 60.6 ± 5.1%, is found in the perchloric acid-soluble fraction after 30 min. Of the radiolabel in the perchloric acid precipitate, 55.2 ± 5.3% is associated with RNA, 11.4 ± 1.5% with DNA, and 33.3 ± 5.6% with protein. After an initial lag period, the rate of incorporation of ^{14}C into these fractions is constant over the interval of observation. In the presence of 30 μM MTX, formate incorporation into DNA is inhibited by 93.6 ± 1.4%, incorporation of formate into RNA is inhibited by 89.3 ± 3.4%, and incorporation into protein, although less sensitive, is inhibited by 78.6 ± 4.4%.

To assess the inhibitory role of exchangeable intracellular MTX, a portion of cells was suspended in 6 μM MTX to saturate high-affinity sites, then divided into five portions. These portions were incubated in the absence of MTX or in the presence of 0.06, 0.12, 0.6, or 6.0 μM MTX. After intracellular MTX levels reached a steady state in cells suspended in MTX-containing media and exchangeable MTX left the cells suspended in the MTX-free medium, the cells were separated by centrifugation and suspended in fresh media of the same composition. [^{14}C]Formate was then added, and radioactivity in the RNA, DNA, and protein fractions was determined. When high-affinity binding sites are saturated with MTX but intracellular MTX in excess of this level is not present, ^{14}C incorporation into RNA is only slightly decreased as compared with control cells not treated with MTX; increasing levels of MTX in excess of this fraction are required to inhibit this process substantially (Fig. 3). The same general pattern is observed for incorporation of ^{14}C into DNA (Fig. 4). Inhibition by tightly bound MTX alone is minor, but when exchangeable intracellular MTX is present marked inhibition is observed. Similarly, inhibition of ^{14}C incorporation into protein requires intracellular MTX in excess of the high-affinity sites (Fig. 5); however, as also indicated in Fig. 2, this process is less sensitive to MTX than is ^{14}C incorporation into RNA or DNA.

Relationship between Percentage Inhibition of ^{14}C Incorporation into Nucleic Acids and Protein and Intracellular MTX Level in Excess of Tightly Bound Fraction

The data of Fig. 6 are a composite of five experiments similar to those of Figs. 3–5. Fifty per cent inhibition of ^{14}C incorporation into DNA occurs at 0.3 μM MTX, 50% inhibition of incorporation into RNA requires about 1 μM MTX, and 50% inhibition of formate incorporation into protein requires an exchangeable intracellular MTX level near 3 μM.

CONCLUSION

It was previously observed (1) that when L-cell mouse fibroblasts are loaded with MTX to a level which exceeds the capacity of the high-affinity binding sites, following which the cells are suspended in MTX-free medium, a portion of the MTX rapidly leaves the cells while another fraction remains tightly bound to intracellular sites such that over the interval of these experiments these sites should remain more than 99% saturated with MTX. This component of intracellular MTX must represent, at least in part, drug associated with high-affinity dihydrofolate reductase binding sites; DEAE-cellulose chromatography of [^3H]MTX accumulated by L-cells excluded the possibility that the retained radiolabel represents metabolites of MTX (1). Saturation of high-affinity sites with MTX only minimally inhibited incorporation of [^3H]deoxyuridine into DNA, whereas MTX in excess of the tightly

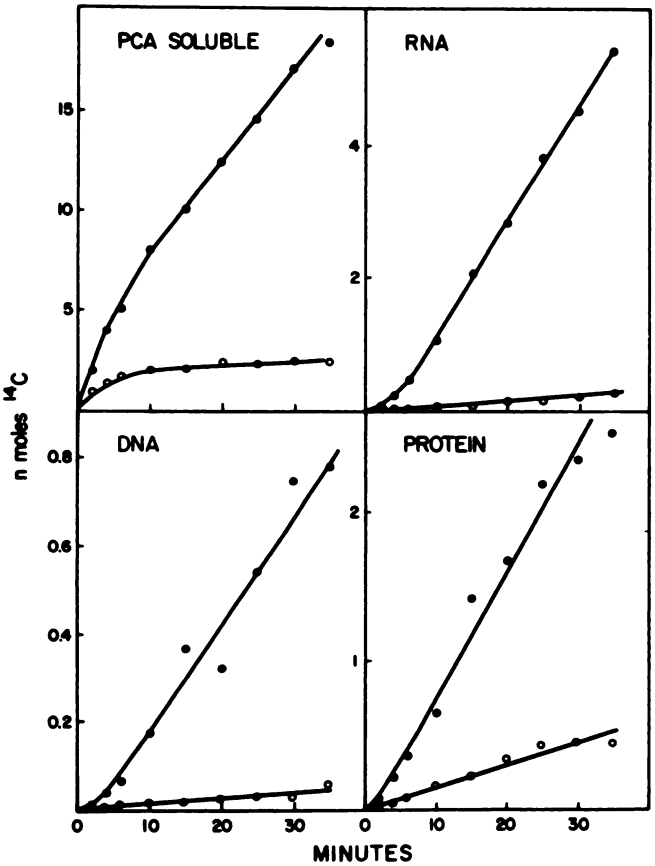

FIG. 2. *Effect of 30 μM MTX on incorporation of ^{14}C into perchloric acid-soluble fraction and into RNA, DNA, and protein after exposure of cells to [^{14}C]formate*

Cells were incubated for 1 hr in medium with (○) or without (●) 30 μM MTX before addition of [^{14}C]formate to a final concentration of 100 μM . The rate of incorporation of radiolabel into the perchloric acid (PCA) supernatant and into RNA, DNA, and protein was monitored as described in MATERIALS AND METHODS.

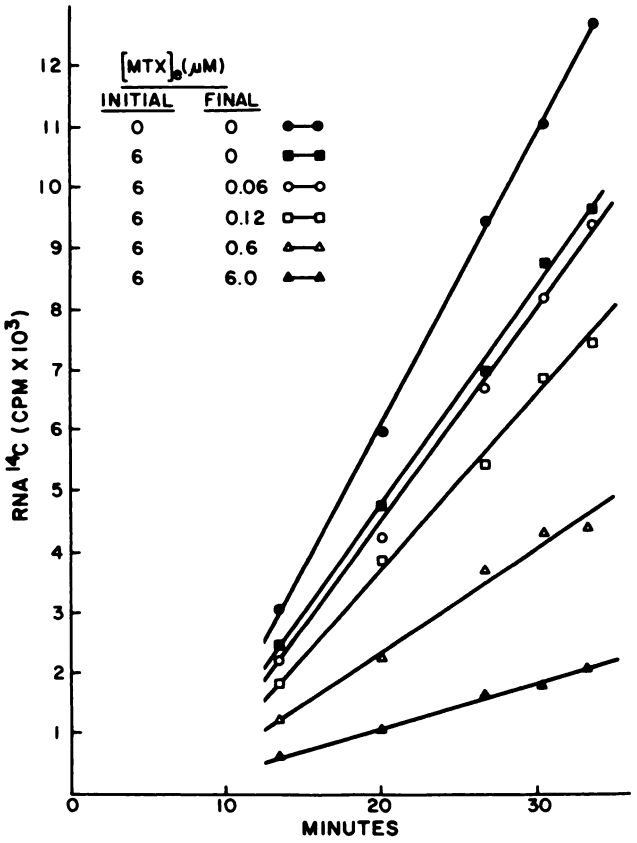

FIG. 3. *Effect of tightly bound MTX and exchangeable intracellular MTX on ^{14}C incorporation into RNA after exposure of cells to* [^{14}C]formate

Cells were incubated with or without 6 μM MTX for 15 min. The MTX-treated cells were then divided into five portions and incubated without MTX or in the presence of 0.06, 0.12, 0.6, or 6.0 μM MTX, while cells treated similarly but not exposed to MTX were incubated as a control. The cells were incubated for 20 min, then separated, washed with 0° buffer, and suspended into fresh medium of the same composition. Ten minutes later [^{14}C]formate was added to a final concentration of 100 μM. Cell samples were processed as described in MATERIALS AND METHODS to determine ^{14}C associated with RNA.

bound fraction was required for maximum inhibition of this process. While the K_i for the MTX interaction with L-cell dihydrofolate reductase is less than 1 nM,[5] 50% inhibition of deoxyuridine incorporation into DNA by exchangeable intracellular MTX required a level of 0.2–0.4 μM. This effect could not be accounted for on the basis of (a) heteroexchange phenomena between MTX and tetrahydrofolate cofactors at their common membrane carrier, (b) penetration of the added MTX into an intracellular compartment containing additional high-affinity sites, or (c) inhibition of newly synthesized enzyme (1).

[5] M.J. Poe, personal communication.

To determine whether exchangeable intracellular MTX specifically inhibited a step in deoxyuridine metabolism (e.g., inhibition of thymidylate synthetase) or was related to a more generalized disruption of tetrahydrofolate-dependent pathways, the effect of MTX on the incorporation of [^{14}C]formate into RNA, DNA, and protein was studied. Formate carbon is inserted via tetrahydrofolates into purine nucleotides at C-2 and C-8 as well as into thymidine, serine, and methionine, following which these units are polymerized into macromolecules (6). These studies suggest that when intracellular MTX was limited to an amount sufficient only to saturate high-affinity sites, there was little inhibition of

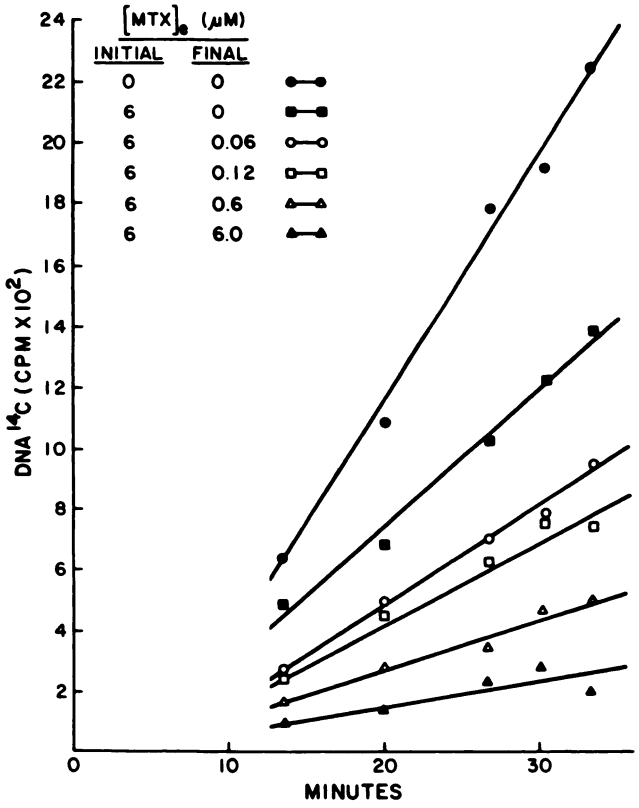

FIG. 4. *Effect of tightly bound MTX and exchangeable intracellular MTX on ^{14}C incorporation into DNA after exposure of cells to [^{14}C]formate*

After digestion of RNA in the experiment illustrated in Fig. 3, radiolabel associated with DNA was determined as described in MATERIALS AND METHODS.

total cell uptake of [^{14}C]formate or of ^{14}C incorporation into RNA, DNA, and protein. Indeed, the small depression in the incorporation rate of radiolabel under these conditions could have been related to suppression of the metabolism of endogenous nonlabeled 1-carbon units during the initial incubation with MTX, resulting in a reduced specific activity of the 1-carbon pool after addition of [^{14}C]formate in comparison to the control cells, which were not exposed to MTX during the preliminary incubations. The observation that intracellular MTX in excess of the tightly bound fraction was required to inhibit formate metabolism substantially by these three pathways suggests that this fraction of intracellular MTX acts at an inhibitory site which influences these pathways through a common mechanism, and raises the possibility that saturation of high-affinity sites within the cell by MTX is inadequate to inhibit the synthesis of tetrahydrofolate from dihydrofolate. This is supported by initial studies from this laboratory, which suggest that dihydrofolate reduction to tetrahydrofolate continues in the absence of exchangeable intracellular MTX,[6] and is compatible with previous observations which suggested that the inhibitory effects of MTX can be reversed by the administration of dihydrofolate (7). Assuming sustained reduction of dihydrofolate under the experimental conditions designed to saturate high-affinity sites, the observed effect of the added intracellular MTX in excess of this amount might be due to the following. (a) The excess MTX inhibits the few remaining high-affinity

[6] J. C. White and I. D. Goldman (1975). Free intracellular MTX is a critical factor in the inhibition of dihydrofolate reduction in Ehrlich ascites tumor cells *in vitro*. Proc. Amer. Assoc. Canc. Res. (in press).

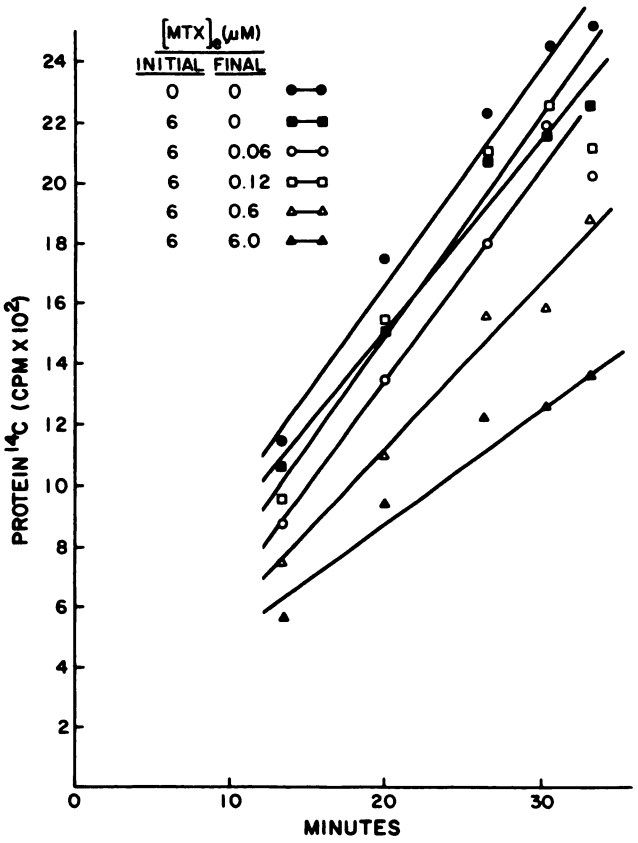

FIG. 5. *Effect of tightly bound MTX and exchangeable intracellular MTX on ^{14}C incorporation into protein after exposure of cells to [^{14}C]formate*

After digestion of RNA and DNA, radiolabel associated with protein was determined as described in MATERIALS AND METHODS.

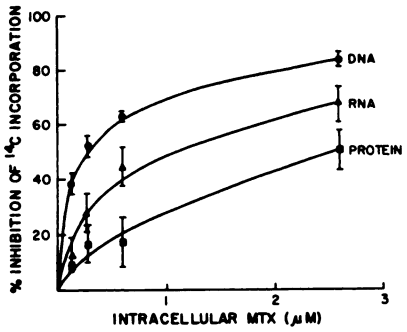

FIG. 6. *Effect of exchangeable intracellular MTX on ^{14}C incorporation into RNA, DNA, and protein*

Inhibition of ^{14}C incorporation into RNA, DNA, and protein by exchangeable intracellular MTX was determined from the difference in incorporation rates between cells containing MTX sufficient only to saturate high-affinity sites and cells containing intracellular MTX in excess of this amount. Exchangeable intracellular MTX was determined as described previously (1).

dihydrofolate reductase molecules. However, during the initial loading with MTX the level achieved (approximately 3 μM) is three to four orders of magnitude above the dihydrofolate reductase-MTX K_i (less than 1 nM),[5] so that the high-affinity sites should be more than 99.9% saturated with MTX. Furthermore, since binding of MTX to high-affinity sites is essentially irreversible during these experiments (1), to account for the inhibitory effect on the basis of an interaction between the added MTX and additional high-affinity sites would require that less than 1% of the usual tetrahydrofolate production is sufficient to sustain tetrahydrofolate-dependent reactions. (b) Alternatively, and more likely, exchangeable intracellular MTX may interact with another dihydrofolate reductase form with a lower affinity for this agent. It is now established that multiple

forms of dihydrofolate reductase may be present in mammalian cells and bacteria (8–16). Two forms of dihydrofolate reductase have been reported, interconvertible by the addition or removal of 1 molecule of NADPH, in bacterial (11, 14) and mammalian systems (12), with alterations in the ratios of the two forms upon development of drug resistance by L1210 cells (15). Otting and Huennekens (16) have reported a dissociation constant in the range of 1 μM for MTX binding to a low-affinity form of the *Lactobacillus casei* enzyme lacking NADPH. Williams *et al.* (10) and Poe *et al.* (13) observed two NADPH binding sites in *Escherichia coli* dihydrofolate reductase, one of which may influence the binding affinity for MTX. Two apparently distinct, nonintercovertible forms of the enzyme have been observed (8, 9); a second form of dihydrofolate reductase from rat liver bound MTX with 1/50 the affinity of the primary form (though still very tightly) and proliferated to a greater degree as the animals were treated with sublethal doses of MTX (8). For the L-cell system under study, however, the nature of the enzyme form which might sustain dihydrofolate reduction in the absence of activity of high-affinity sites requires further clarification.

Although there were differences in the degree of inhibition by MTX of the individual pathways of formate metabolism, an observation reported by other investigators (17, 18), this is not inconsistent with a single site of action by exchangeable intracellular MTX. The balance in the various cellular coenzyme levels may be altered by the properties and quantities of interconversion enzymes, affecting individual cofactors differently, as the total tetrahydrofolate level falls. Furthermore, the degree of inhibition of tetrahydrofolate-dependent reactions will be determined by the relationship between the individual tetrahydrofolate cofactor levels in the absence of MTX and the reaction K_m: the higher the former in relation to the latter, the smaller the initial decline as the tetrahydrofolate cofactor level is reduced. The very low sensitivity of these cells to MTX inhibition of [^{14}C]formate incorporation into protein is compatible with the observation that 5-methyltetrahydrofolate is the major cofactor within the mammalian cell and that when the cell is starved for folate or regeneration of tetrahydrofolate from dihydrofolate is inhibited, interconversion enzymes apparently sustain this cofactor at the expense of others (19–22). The rapid onset of inhibition and complete cessation of [^{14}C]formate incorporation into DNA and RNA after exposure to high levels of MTX (Fig. 2) suggest that there are low cellular stores of the specific cofactors necessary to sustain these reactions and that both processes are highly dependent upon the sustained regeneration of tetrahydrofolate from dihydrofolate. In the case of thymidylate synthesis, at least, close metabolic if not structural coupling of dihydrofolate reductase and thymidylate synthetase has been proposed (23, 24). However, the data do not exclude the possibility that (a) the effects of exchangeable intracellular MTX are related to interactions with the specific enzymes involved in RNA, DNA, and protein synthesis or (b) intracellular MTX in excess of the tightly bound fraction inhibits the utilization of tetrahydrofolate cofactor stores within the cell.

The observation that suppression of DNA, RNA, and protein synthesis by MTX requires continuous exposure of cells to this agent in order to achieve appreciable levels of exchangeable intracellular MTX may account in part for the enhanced chemotherapeutic efficacy of protocols with MTX which employ sustained high dose infusions of this agent (25–29) for the treatment of resistant tumors. The efficacy of drug regimens which do not achieve this pharmacological end point in the treatment of other highly sensitive tumors may be related to the lethal effect of very brief periods of cessation of nucleic acid and protein synthesis or to the absence of low-affinity sites capable of sustaining nucleic acid and protein synthesis when the high-affinity sites are saturated with MTX. The data suggest that while the cytotoxic effects of MTX have been attributed largely to inhibition of DNA synthesis (30), the sensitivity of RNA synthesis to this drug is only slightly less, so that conditions which achieve inhibition of

thymidylate incorporation into DNA are likely to produce a comparable inhibition of purine synthesis. The concurrent inhibition of purine synthesis with administration of MTX may play an important role in the cytotoxicity of MTX, as suggested by other authors (31, 32).

Since a substantial level of free intracellular MTX would be required to achieve appreciable interaction between this agent and a low-affinity intracellular site, the data support the possibility that the free component of MTX may be a crucial determinant of the cytotoxicity of this agent. This might account for differences in cellular sensitivities to MTX (33–35). The relationship between the elements of the MTX-cell interaction determined by the properties of the membrane transport system and the cytotoxic determinants of MTX have been considered in detail previously (35). Furthermore, since there is evidence to suggest that there may be an energy-dependent process which limits the accumulation of exchangeable intracellular MTX (2, 36), inhibition of this process might be expected to augment the chemotherapeutic efficacy of this agent. Indeed, synergism between vincristine and MTX (3) may be due to the ability of vincristine to increase the intracellular MTX level in excess of the usual tightly bound fraction (2, 4). This phenomenon has been attributed to the inhibition of energy-dependent processes by this agent (2, 4) with the inhibition of an exit pump for MTX (36). In addition, inhibition of energy metabolism by vincristine may increase the cellular NADPH/NADP ratio resulting in the formation of NADPH-MTX-enzyme ternary complexes from dihydrofolate reductase forms unassociated with the coenzyme and with a low-affinity for MTX. This is considered in forthcoming publications (37, 38).

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