Folate analogs that inhibit dihydrofolate reductase result in only partial interconversion of tetrahydrofolate cofactors to dihydrofolate with preservation of the major portion of reduced cellular folate cofactors in L1210 leukemia cells. One possible explanation for this phenomenon is that low levels of dihydrofolate polyglutamates that accumulate in the presence of antifolates block thymidylate synthase to prevent depletion of reduced folate pools. This paper correlates biochemical analyses of rapid interconversions of radiolabeled folates and changes in purine and pyrimidine biosynthesis in L1210 murine leukemia cells exposed to antifolates with network thermodynamic computer modeling to assess this hypothesis.

When cells are exposed to 1 μM trimetrexate there is an almost instantaneous inhibition of [3H]deoxyuridine or [14C]formate incorporation into nucleotides which is maximal within 5 min. This is associated with a rapid rise in cellular dihydrofolate (tₗ ≈ 1.5 min), which reaches a steady state that represents only 27.9% of the total folate pool. Pretreatment of cells with fluorodeoxyuridine, to inhibit thymidylate synthase by about 95% followed by trimetrexate only slows the rate of folate interconversion (tₗ ≈ 25 min) but not the final dihydrofolate level achieved. This is consistent with computer simulations which predict that direct inhibition of thymidylate synthase by 97, 98, and 99% should increase the half-time of dihydrofolate rise after trimetrexate to 40, 60, and 124 min, respectively, but the final level achieved is always the same as in cells with normal thymidylate synthase activity.

The data reflect the high degree of catalytic activity of thymidylate synthase relative to tetrahydrofolate cofactor pools in the cells and the enormous extent of inhibition of this enzyme that is necessary to slow the rate of folate interconversions after addition of antifolates. The model predicts, and the data demonstrate, that virtually any residual thymidylate synthase activity will permit the interconversion of all tetrahydrofolate cofactors available for oxidation to dihydrofolate when dihydrofolate reductase activity is abolished, but the rate of interconversion will be slowed. Additional simulations indicate that the time course of cessation of tetrahydrofolate-dependent purine and pyrimidine biosynthesis after antifolates in these cells can be accounted for solely on the basis of tetrahydrofolate cofactor depletion alone.

These data exclude the possibility that direct inhibition of thymidylate synthase by dihydrofolate polyglutamates, or any other intracellular folates that accumulate in cells after antifolates, can account for the rapid but partial interconversion of reduced folate cofactors to dihydrofolate. Experimental observations and computer simulations suggest that a fraction of cellular tetrahydrofolates are in a biochemical form, physical compartment, or bound to cellular constituents which make them unavailable for oxidation to dihydrofolate.

Antifolates that inhibit dihydrofolate reductase traditionally have been assumed to block purine and pyrimidine synthesis through depletion of tetrahydrofolate cofactors consumed by oxidation to dihydrofolate during thymidylate biosynthesis. However, more recent studies (1-5) demonstrate that antifolate suppression of these biosynthesis processes occurs in MCF-7 human breast cancer and L1210 murine leukemia cell populations despite the preservation of the major portion of cellular tetrahydrofolate cofactors (6, 7) raising the possibility of direct inhibitory effects of antifolates, and/or dihydrofolate polyglutamates that accumulate in their presence, on purine and pyrimidine synthesis.

It is clear that accumulation and retention of polyglutamyl derivatives of MTX in tumor cells inhibits reactivation of dihydrofolate reductase caused by the addition of exogenous tetrahydrofolate cofactors (8-11) and blocks tetrahydrofolate cofactor utilization at the levels of both thymidylate and purine biosynthesis (6). Likewise, higher polyglutamyl derivatives of MTX and dihydrofolate inhibit thymidylate synthase (12-14), aminoimidazolecarboxamidomethylribofuranoside (15-17), and glycaminamide ribotide (17) transformylase in cell-free systems. A critical issue, however, is whether early inhibitory effects of antifolates (18-20) that occur before the buildup of antifolate polyglutamates (21, 22) are related to tetrahydrofolate cofactor depletion or possible feedback inhibitory effects of dihydrofolate polyglutamates on thymidylate and purine biosynthesis. While dihydrofolate polyglutamates in

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† To whom correspondence should be addressed: MCV Box 230, Richmond, VA 23298. Tel.: 804-786-9723.
hibit tetrahydrofolate-dependent enzymes in cell-free systems, it is not clear if the degree of this inhibition and the levels of dihydrofolate polyglutamates that accumulate are of sufficient magnitude to produce important effects in cells beyond those attributed to antifolate-induced tetrahydrofolate cofactor depletion. In particular, it is uncertain whether direct inhibition of thymidylate synthase by dihydrofolate polyglutamates is sufficient to result in the preservation of tetrahydrofolate cofactor pools after inhibition of dihydrofolate reductase.

Most studies have examined antifolate effects on cellular folate pools and folate-dependent reactions over long intervals of drug exposure (6, 7, 23). Yet, it is well known that antifolates inhibit biosynthetic processes very rapidly (18–20). In this report, the effects of antifolates on cellular dihydrofolate reductase are correlated with drug effects on tetrahydrofolate-dependent purine and pyrimidine biosynthesis over very brief intervals. The effects of two antifolates are studied: (i) MTX, a classical antifolate transported into cells by a carrier mechanism (1, 2) with subsequent polyglutamation (21), and (ii) trimetrexate, a nonclassical, more lipid-soluble antifolate that enters cells rapidly (24, 25) and does not undergo glutamylations.

The results of these experiments are correlated with computer simulations based upon the network thermodynamic modeling concept (26, 27) and broadened in scope from the model reported earlier from this laboratory (4, 22, 28, 29) to encompass antifolate effects on tetrahydrofolate cofactor interconversions and tetrahydrofolate cofactor-dependent biosynthetic reactions. Previous studies from this (4, 22, 28–31) and another laboratory (3, 5, 31) have demonstrated the value of computer modeling in understanding the mechanism of action of antifolates: in particular, the limitations in the intuitive approach to the extrapolation of biochemical data obtained in cell-free systems to the more complex dynamic processes in the intact cell. These analyses, described in detail in the Miniprint Supplement, specifically address the basis for antifolate effects on tetrahydrofolate cofactor-dependent biosynthetic processes and, in particular, the pharmacologic ramifications of the cellular accumulation of dihydrofolate after brief intervals of exposure of cells to these agents.

MATERIALS AND METHODS2

**Chemicals—**[3',5',7-3H]Methotrexate was obtained from American Corp. and purified by HPLC prior to use (21). Unlabeled MTX, obtained from the National Cancer Institute, was purified by DEAB-cellulose column chromatography (32). [3',5',7-3H]Folic acid was purchased from Moravek Biochemicals (Brea, CA) and purified by HPLC (6). [6-3H]dUrd was purchased from Du Pont-New England Nuclear and purified by HPLC (6). [14C]Formic acid was purchased from ICN. Trimetrexate glucuronate was the gift of Dr. David Fry (Warner-Lambert, Ann Arbor, MI).

**Cell Culture Conditions—**The murine leukemia cell line, L1210, was maintained in continuous culture in RPMI 1640 medium containing 10% supplemented bovine serum (HyClone Laboratories, Logan, UT), 2 mM l-glutamine, penicillin (100 units/ml), streptomycin (100 mg/ml), and 2-mercaptoethanol (20 μM). Cells were confirmed to be free of mycoplasma contamination following a 2-week period of incubation in medium without antibiotics.

Cells were harvested from spinner flasks at a density of 1.0–1.2 × 10^7 cells/ml, washed, and resuspended in complete RPMI 1640 medium at a density of 1–5 × 10^7 cells/ml in incubation flasks. In particular, it is uncertain whether direct inhibition of thymidylate synthase by dihydrofolate polyglutamates is sufficient to result in the preservation of tetrahydrofolate cofactor pools after inhibition of dihydrofolate reductase.

Most studies have examined antifolate effects on cellular folate pools and folate-dependent reactions over long intervals of drug exposure (6, 7, 23). Yet, it is well known that antifolates inhibit biosynthetic processes very rapidly (18–20). In this report, the effects of antifolates on cellular dihydrofolate reductase are correlated with drug effects on tetrahydrofolate-dependent purine and pyrimidine biosynthesis over very brief intervals. The effects of two antifolates are studied: (i) MTX, a classical antifolate transported into cells by a carrier mechanism (1, 2) with subsequent polyglutamation (21), and (ii) trimetrexate, a nonclassical, more lipid-soluble antifolate that enters cells rapidly (24, 25) and does not undergo glutamylations.

The results of these experiments are correlated with computer simulations based upon the network thermodynamic modeling concept (26, 27) and broadened in scope from the model reported earlier from this laboratory (4, 22, 28, 29) to encompass antifolate effects on tetrahydrofolate cofactor interconversions and tetrahydrofolate cofactor-dependent biosynthetic reactions. Previous studies from this (4, 22, 28–31) and another laboratory (3, 5, 31) have demonstrated the value of computer modeling in understanding the mechanism of action of antifolates: in particular, the limitations in the intuitive approach to the extrapolation of biochemical data obtained in cell-free systems to the more complex dynamic processes in the intact cell. These analyses, described in detail in the Miniprint Supplement, specifically address the basis for antifolate effects on tetrahydrofolate cofactor-dependent biosynthetic processes and, in particular, the pharmacologic ramifications of the cellular accumulation of dihydrofolate after brief intervals of exposure of cells to these agents.

**MATERIALS AND METHODS**

2 Portions of this paper (including part of "Materials and Methods," part of "Results," Figs. 6-11, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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RESULTS

Analysis of Early Effects of Trimetrexate or MTX on Total Cell Tritium or Thymine in Acid-soluble and -insoluble Fractions after Exposure of L1210 Leukemia Cells to [3H]dUrd—Cells incubated with 3 μM [3H]dUrd maintain a constant rate of uptake of radiolabel for at least 15 min. There was negligible accumulation of [3H]dUMP in the cells during this interval and under these conditions. Rather, the rates of formation of thymidylate and the incorporation of dTTP into DNA were so great that 93 ± 5% of cellular radiolabel in control cells at zero time. at 400-s portions of the cell suspension were added to other flasks containing the indicated antifolate. Data are expressed as the ratio of inhibition of total 3H uptake by trimetrexate was essentially instantaneous with a decline to negligible rates over the next 5 min (Fig. 1A). Monitoring total [3H]thymine derived from [3H]thymidine and its phosphorylated derivatives in the cells after exposure to [3H]dUrd by HPLC showed a similar rapid cessation of the synthesis of thymidine after exposure of cells to this antifolate (data not shown). These effects are not due to direct inhibition of thymidylate synthase by trimetrexate since this agent has no effect on enzyme activity in a thymidylate release assay (i) using a homogenate of these L1210 leukemia cells or (ii) using thymidylate synthase derived from Lactobacillus casei. Nor is thymidylate synthesis retarded by inhibition of precursor transport or metabolism since dUrd and dUMP accumulate in cells only after antifolate is added (data not shown).

Trimetrexate is a nonclassical antifolate that traverses the cell membrane rapidly, consistent with the imperceptible lag in onset of expression of pharmacologic activity. On the other hand, MTX is transported into cells more slowly by a carrier mechanism (32, 35). Since expression of antifolate effects require that the intracellular drug level exceed molar equivalence with dihydrofolate reductase (4, 18, 19, 36), the onset of the suppression of [3H] accumulation in cells by MTX after exposure to [3H]dUrd is delayed. It can be seen in Fig. 2 that essentially all MTX transported into cells over 60–80 s is bound to dihydrofolate reductase. Following this, continued rapid incorporation into DNA are much slower (Fig. 1B). Since the data described above and in Fig. 1A indicate that the onset of retardation of thymidylate synthesis is virtually instantaneous after addition of trimetrexate, continued rapid incorporation of radiolabel into DNA must be due to consumption of a pool of radiolabeled thymidine nucleotides formed over the interval of exposure to [3H]dUrd prior to addition of antifolate. This is confirmed in Fig. 1C which demonstrates the fall in thymidine nucleotides when measured as total thymine in the acid-soluble fraction of cells following exposure to antifolates associated with inhibition of thymidylate synthesis and continued consumption of dTTP as it is incorporated into DNA.

The Effects of Antifolates on [14C]Formate Incorporation into Thymine and Purines—Trimetrexate and MTX inhibit...
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Folate pools were radiolabeled by exposure of cells to 2 μM [3H]folic acid for 48 h. Following this, intracellular levels of tetrahydrofolate cofactors and dihydrofolate were monitored by HPLC before and after exposure to trimetrexate or MTX. Fig. 4 illustrates alterations in dihydrofolate levels after exposure of cells to 1 μM trimetrexate at zero time. There is a rapid rise in dihydrofolate which becomes maximal within 5 min. There was no further increase in cellular dihydrofolate when cellular folate levels were monitored over intervals of up to 90 min (Fig. 5). Fig. 4 also illustrates changes in cellular dihydrofolate after exposure of cells to MTX. There is a slow rise in dihydrofolate for about 60-80 s (to the point at which antifolate reaches molar equivalence with dihydrofolate reductase; see Fig. 2), following which cellular dihydrofolate rapidly rises to levels comparable to those observed in the presence of trimetrexate. Hence, effects of antifolates on dihydrofolate levels in cells are very rapid, essentially complete within 5 min, and correlate temporally with the cessation of tetrahydrofolate cofactor-dependent purine and pyrimidine biosynthesis.

The Effects of Direct Inhibition of Thymidylate Synthase on the Rate and Extent of Folate Pool Interconversions to Dihydrofolate in Cells: Correlation with Computer Simulations—While intracellular dihydrofolate rises rapidly to a steady state in cells after trimetrexate, the interconversion of reduced to oxidized folate is incomplete. There is a 24.2 ± 5.2% fall in dihydrofolate to a level 27.9 ± 2.4% that of the total folate pool. One possible basis for the incomplete interconversion of folates is that dihydrofolate polyglutamates inhibit thymidylate synthase to a sufficient magnitude to block tetrahydrofolate cofactor oxidation resulting in the preservation of reduced folate cofactor pools and a stable intracellular dihydrofolate level.

To assess the effects of inhibition of thymidylate synthase on tetrahydrofolate cofactor pool interconversions to dihydrofolate in cells, thymidylate synthase activity was progressively reduced by decreasing the $V_{\text{max}}$ of the enzyme as would occur with FdUrd. The time course and extent of dihydrofolate rise was then simulated after suppression of dihydrofolate reductase with trimetrexate. It can be seen from the simulations in

FIG. 4. Analysis of the rise in cellular dihydrofolate after exposure to 10 μM MTX or 1 μM trimetrexate (TMTX) at zero time. Folate pools in cells were equilibrated with [3H]folic acid for 48 h prior to experimentation. Data are from a representative experiment.

FIG. 5. A quantitative analysis of the rise in cellular dihydrofolate produced by 1 μM trimetrexate in control L1210 leukemia cells (A) and cells in which thymidylate synthase activity was reduced by prior incubation with 10 nM FdUrd for 15 min (B). Cellular folate pools were equilibrated with [3H]folic acid for 2 days prior to experimentation. The SPICE2 simulations are indicated by the interrupted lines. The experimental data are the mean ± S.E. from five experiments performed on different days.

**FIG. 3.** The effects of 10 μM MTX or 1 μM trimetrexate (TMTX) on total cellular uptake of [14C]formate (A), incorporation of [14C]formate into total cell guanine and adenine nucleotides (B), or incorporation of [14C]formate into total cell thymine nucleotides (C) assessed by HPLC and described under “Materials and Methods.” Cells were exposed to [14C]formate at zero time, following which portions of the cell suspensions were transferred to flasks containing antifolate at 10 min. The data are from a representative experiment.
Fig. 8 of the Miniprint Supplement that marked suppression of enzyme activity is necessary to slow the rise in cellular dihydrofolate when dihydrofolate reductase is blocked. For instance, 90, 95, 97, 98, and 99% inhibition of thymidylate synthase decreases the half-time for the dihydrofolate rise from ~1.5 min when enzyme activity is normal to 12, 25, 40, 60, and 124 min, respectively. For all these conditions, and irrespective of whether inhibition is simulated as noncompetitive or competitive, the final level of dihydrofolate achieved is the same when dihydrofolate reductase activity is completely abolished by an antifolate.

This model was confirmed in cells as illustrated in Fig. 5, a composite of five separate experiments in which the rise in dihydrofolate was monitored over 90 min after addition of trimetrexate to cells in which thymidylate synthase activity was normal (closed triangles) or to cells pretreated with 10 nM FdUrd for 10 min (closed circles) to reduce enzyme activity by 93–97% (differences in these degrees of inhibition cannot be discriminated accurately on the basis of [3H]FdUrd incorporation into DNA). The rise in the cellular dihydrofolate level is slowed in cells in which thymidylate synthase is inhibited, but the final level achieved is the same as in control cells. The interrupted lines are the computer simulations based on control cells (through the closed triangles) or based on 95% inhibition of thymidylate synthase (through the closed circles) the value most consistent with the data. These findings are the same when inhibition of thymidylate synthase activity is achieved by folate analogs that interact with the 5,10-methylene tetrahydrofolate site on the enzyme (to be the subject of a separate forthcoming report). These data indicate clearly that thymidylate synthase activity, or the capacity of cells to oxidize 5,10-methylene tetrahydrofolate, is so great relative to the level of cellular folates that an enormous degree of inhibition of this enzyme is necessary to suppress the interconversion of tetrahydrofolates to dihydrofolate when dihydrofolate reductase is blocked in cells.

The network model was employed to estimate whether any degree of feedback inhibition of thymidylate synthase by dihydrofolate could account for the rapid (t<sub>n</sub> ~ 1.5 min) but incomplete interconversion of tetrahydrofolates observed experimentally. These simulations were generated by decreasing the dihydrofolate K<sub>i</sub> for thymidylate synthase. Fig. 9 in the Miniprint Supplement illustrates that dihydrofolate at the published K<sub>i</sub> value of 3.9 μM (37) has a trivial effect on the rate of rise of dihydrofolate in cells after trimetrexate and no effect on the final level achieved. If the dihydrofolate K<sub>i</sub> is reduced by a factor of about 250 to 16 nM, the cellular level of dihydrofolate achieved at 90 min is similar to what is observed experimentally. However, this reduction in the extent of interconversion is accompanied by a marked decline in the rate of dihydrofolate rise; further, the final level achieved is ultimately the same as in control cells (see the Miniprint Supplement). This pattern is predicted irrespective of whether inhibition by dihydrofolate polyglutamates is simulated as competitive or noncompetitive (Fig. 9, Miniprint Supplement) when dihydrofolate reductase activity is abolished by an antifolate. Hence, feedback inhibition by dihydrofolate at the level of thymidylate synthase cannot account for the observed rapid rate but limited extent of dihydrofolate accumulation in cells and cannot be a basis for the incomplete interconversion of reduced to oxidized folates observed in the presence of antifolates.

**DISCUSSION**

The application of computer modeling to the analysis of biological systems provides an important approach for interpretation of data and testing of hypotheses. Often questions can be addressed that are unanswerable in the laboratory because of the insensitivity of available methodologies. Computer simulation of antifolate actions was initially applied in the classic studies of Jackson and Harrap (33) which demonstrated, among other things, that only a small fraction of dihydrofolate reductase activity in cells is necessary to sustain tetrahydrofolate cofactor-dependent reactions, that intracellular levels of antifolates above molar equivalence with the enzyme are required before tetrahydrofolate cofactor depletion occurs, and that the interaction among MTX, dihydrofolate reductase, and dihydrofolate is competitive within cells. This model was consistent with experimental data which demonstrated a requirement for cellular MTX levels in excess of the dihydrofolate reductase binding capacity to achieve inhibition of tetrahydrofolate synthesis from dihydrofolate (36) and tetrahydrofolate-dependent purine and pyrimidine biosynthesis (18, 19, 38). Subsequent utilization of a network thermodynamic model in this laboratory (i) characterized the nature of the interaction among methotrexate, dihydrofolate, and dihydrofolate reductase in cell and cell-free systems (4), (ii) analyzed the ramifications of increased levels of dihydrofolate reductase and decreased levels of thymidylate synthase activity in an MTX-resistant cell line (28), and (iii) clarified the roles and relationship among MTX transport, polyglutamylation, and cytotoxicity in normal and malignant cells (22).

With the advent of more sophisticated analytical technology it became possible in recent years to measure more accurately the effects of antifolates on several folate pools within cells (6, 7, 10, 11, 37, 39, 40). These studies have demonstrated that the interconversion of cellular tetrahydrofolate cofactors to dihydrofolate induced by antifolates is incomplete although the extent of interconversion may vary from one cell system to another (6, 7, 41). Further, tetrahydrofolate cofactor-dependent purine biosynthesis has been shown to be inhibited under condition in which the 10-formyltetrahydrofolate level in cell populations is reduced by only 20–30% (6, 7). This has raised the possibility that there may be direct inhibitory consequences of antifolates at the levels of purine and pyrimidine biosynthesis.

Direct inhibition of the purine transformylases and thymidylate synthase by MTX polyglutamates that accumulate to appreciable levels in tumor cells has been demonstrated (6, 11). This, along with the diminished reactivation of dihydrofolate reductase in tumor cells that contain MTX congeners, is a factor in the reduced ability of leucovorin to "rescue" cells that contain these derivatives (6, 11). The lesser build-up of MTX polyglutamates in normal cells of the gut (22, 42) and bone marrow (42–44) allow reactivation of dihydrofolate reductase and utilization of tetrahydrofolate cofactors and is one important basis for the "selectivity" of leucovorin "rescue" (8–11). Beyond this, the demonstration that dihydrofolate polyglutamates are more potent inhibitors of glycaminamide ribotide and aminomimidazolecarboxamide ribotide transformylase (15–17) than the monoglutamate in cell-free systems has raised the possibility that these oxidized folates which accumulate in the presence of antifolates, may play a role in the anti-purine effects of this agent. Finally, the demonstration of direct inhibition of thymidylate synthase by dihydrofolate polyglutamates (12–14) has further raised the further possibility that this inhibition in cells shuts off dihydrofolate generation, preserving the major portion of the tetrahydrofolate cofactor pools.

This paper focuses on the kinetics and extent of change in cellular dihydrofolate after exposure of cells to trimetrexate or MTX when thymidylate synthase activity is normal or
reduced and correlates some of these changes with tetrahydrofolate cofactor-dependent purine and pyrimidine biosynthesis. The former agent does not form polyglutamate derivatives, and the time scale of the experiments is too short to permit accumulation of these MTX congeners. The experimental data demonstrate a rapid onset of inhibition of purine and pyrimidine biosynthesis upon addition of trimetrexate which is associated with a rapid build-up of cellular dihydrofolate. These alterations are essentially complete within a few minutes. Computer simulations indicate that the time course of these changes can be accounted for solely on the basis of depletion of relevant tetrahydrofolate cofactors (Fig. 10, Miniprint Supplement). Indeed, careful analysis of the simulations published earlier by Jackson and Harrap (3) predicts a similar rapid decline in folate-dependent thymidylate synthesis after addition of an antifolate due to folate cofactor depletion. Further, there is little change in the simulations when published inhibitor constants, for higher dihydrofolate polyglutamates are incorporated into the model to allow feedback inhibitory effects at the levels of both purine and pyrimidine biosynthesis (Fig. 10, Miniprint Supplement). Hence, direct inhibitory effects of dihydrofolate polyglutamates at the levels of purine or pyrimidine biosynthesis, while having some minor pharmacologic effect, need not be implicated to account for the rapidity in which these reactions cease following exposure of cells to antifolates.

Beyond this, these studies demonstrated that only 25% of cellular tetrahydrofolate cofactors are oxidized to dihydrofolate in the presence of antifolate but indicate clearly that, although limited, this interconversion is complete within a few minutes. Feedback inhibitory effects of dihydrofolate polyglutamates at the level of thymidylate synthase cannot account for the time course of the dihydrofolate rise or the stable but limited levels of this oxidized folate achieved in cells. First, because thymidylate synthase activity is enormous compared to folate pools in cells, only marked inhibition of this enzyme significantly affects the rate of rise of cellular dihydrofolate. Further even with 95% suppression of thymidylate synthase achieved experimentally or 99% inhibition as simulated in the model (Fig. 8, Miniprint Supplement), while the rate of rise of dihydrofolate is slowed, the final level achieved is comparable to that obtained in cells with normal catalytic activity.

If the dihydrofolate pentaglutamate $K_i$ for thymidylate synthase is reduced by 1/250th to 16 nM (Fig. 9, Miniprint Supplement) so as to reproduce the extent of interconversion seen experimentally 90 min after addition of trimetrexate, the rate of rise of dihydrofolate in the cells is markedly reduced and, whether the model simulates competitive or noncompetitive inhibition, the interconversion of tetrahydrofolate cofactors to dihydrofolate will ultimately continue to the level achieved more rapidly in cells with normal thymidylate synthase catalytic activity. This is in sharp contrast to the experimental data where a reduced but stable dihydrofolate level is achieved within 5 min in L1210 leukemia cells after exposure to trimetrexate when there is marked suppression of dihydrofolate reductase. It is important to note that even low residual levels of catalytic activity for dihydrofolate reductase will influence the level of dihydrofolate achieved in cells after antifolates with normal or inhibited thymidylate synthase activity; this will be the subject of a subsequent report from this laboratory.

The experimental data and computer simulations argue against an important role for dihydrofolate polyglutamates as factors in antifolate actions in these L1210 leukemia cells. Dihydrofolate levels in cells are too small and their affinity for thymidylate synthase too low (i) to produce pharmacologically important direct inhibitory effects on thymidylate or purine biosynthesis beyond that achieved by tetrahydrofolate cofactor substrate depletion alone or (ii) to account for the incomplete interconversion of reduced to oxidized folates. Clearly, dihydrofolate polyglutamates can interact with tetrahydrofolate-requiring enzymes in cell-free systems, and clearly conditions can be created in which inhibitory effects on purine synthesis can be expressed within cells. For instance, exposure of MCF-7 breast cancer cells to high levels of dihydrofolate over long intervals, when thymidylate synthase is first blocked with FdUrd and dihydrofolate reductase is blocked with MTX, can result in suppression of transformation (23). However, the data presented here indicate that this direct effect of dihydrofolate polyglutamates is unimportant in the L1210 leukemia at the levels that could build up from existing tetrahydrofolate cofactor pools within cells grown in media with usual folate levels when dihydrofolate reductase is inhibited by antifolates.

There are compelling reasons to suggest that the limited interconversion of tetrahydrofolate cofactors to dihydrofolate is due to compartmentation of a significant portion of tetrahydrofolate cofactors that are unavailable for oxidation to dihydrofolate in the cell populations studied. This could relate to the heterogeneity of the cells with an appreciable fraction having essentially no thymidylate synthase activity (45, 46). Alternatively, a portion of folates in all cells could be in a biochemical form, or bound to protein sites, or sequestered in subcellular compartments (e.g. mitochondria (47)) making them unavailable as substrates to support purine and/or pyrimidine biosynthesis. Indeed, binding of 10-formyltetrahydrofolate within L1210 leukemia cells has been reported although it is unclear if this phenomenon diminishes the availability of folate substrates for folate requiring enzymes (48). Fig. 11 in the Miniprint Supplement indicates that the actual rise in dihydrofolate to 25% of total cellular folate conforms to the predicted rise in cellular dihydrofolate in a system in which 75% of intracellular folates are unavailable for interconversion. Irrespective of the cause of compartmentation, the effects on interconversions will be the same in the laboratory or by computer simulations: only tetrahydrofolate cofactors available for oxidation will be rapidly and completely interconverted to dihydrofolate within minutes after exposure of cells to antifolates.

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Fig. 1. Computer simulation of the effects of time-course on the accumulation of total thymidine and thymidine kinase to dihydrofolate. Starting with the computer model at standard conditions (see Table I), the addition of 1 mmol of dihydrofolate to the intracellular compartment at t = 1.0 min is simulated.

Fig. 2. Computer simulation of the effects of time-course on the accumulation of dihydrofolate after thymidine kinase activity is added to the model (see Table I). The addition of 1 mmol of thymidine kinase to the intracellular compartment at t = 1.0 min is simulated.

Fig. 3. Computer simulation of the effects of time-course on the accumulation of dihydrofolate after thymidine kinase activity is added to the model (see Table I). The addition of 1 mmol of thymidine kinase to the intracellular compartment at t = 1.0 min is simulated.

Fig. 4. Computer simulation of the expected effects on thymidine incorporation into thymidylate (upper panel) or thymidine kinase activity (lower panel) when the net production of dihydrofolate is reduced to 50% by the addition of 0.1 mmol of dihydrofolate at t = 1.0 min. The model predicts a rapid and sustained decrease in thymidine incorporation and thymidine kinase activity, with a gradual return toward normal activity after 10 min. The improvement in thymidine incorporation and thymidine kinase activity is the result of a reduction in the net production of dihydrofolate by 50%, which affects the intracellular concentration of dihydrofolate in a non-linear manner.

Fig. 5. Computer simulation of the expected effects on thymidine incorporation into thymidylate (upper panel) or thymidine kinase activity (lower panel) when the net production of dihydrofolate is reduced to 50% by the addition of 0.1 mmol of dihydrofolate at t = 1.0 min. The model predicts a rapid and sustained decrease in thymidine incorporation and thymidine kinase activity, with a gradual return toward normal activity after 10 min. The improvement in thymidine incorporation and thymidine kinase activity is the result of a reduction in the net production of dihydrofolate by 50%, which affects the intracellular concentration of dihydrofolate in a non-linear manner.

Fig. 6. Computer simulation of the expected effects on thymidine incorporation into thymidylate (upper panel) or thymidine kinase activity (lower panel) when the net production of dihydrofolate is reduced to 50% by the addition of 0.1 mmol of dihydrofolate at t = 1.0 min. The model predicts a rapid and sustained decrease in thymidine incorporation and thymidine kinase activity, with a gradual return toward normal activity after 10 min. The improvement in thymidine incorporation and thymidine kinase activity is the result of a reduction in the net production of dihydrofolate by 50%, which affects the intracellular concentration of dihydrofolate in a non-linear manner.

Fig. 7. Computer simulation of the expected effects on thymidine incorporation into thymidylate (upper panel) or thymidine kinase activity (lower panel) when the net production of dihydrofolate is reduced to 50% by the addition of 0.1 mmol of dihydrofolate at t = 1.0 min. The model predicts a rapid and sustained decrease in thymidine incorporation and thymidine kinase activity, with a gradual return toward normal activity after 10 min. The improvement in thymidine incorporation and thymidine kinase activity is the result of a reduction in the net production of dihydrofolate by 50%, which affects the intracellular concentration of dihydrofolate in a non-linear manner.