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INTERCONVERSION OF TETRAHYDROFOLATE COFACTORS TO DIHYDROFOLATE INDUCED BY TRIMETREXATE AFTER SUPPRESSION OF THYMIDYLATE SYNTHASE BY FLUORODEOXYURIDINE IN L1210 LEUKEMIA CELLS

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Abstract—Previous studies from this laboratory demonstrated that marked suppression of thymidylate synthase activity is required to slow the rate of interconversion of tetrahydrofolate cofactors to dihydrofolate when dihydrofolate reductase is blocked by an antifolate. This finding is due to the high catalytic activity of thymidylate synthase within cells in comparison to the tetrahydrofolate cofactor pool size. In the present study, we assessed the rate of *resumption* of thymidylate synthase catalytic activity in terms of [^{3}H]deoxyuridine incorporation into DNA and dihydrofolate generation from tetrahydrofolate cofactors following exposure of cells to fluorodeoxyuridine. Log phase L1210 leukemia cells, incubated with fluorodeoxyuridine to abolish thymidylate synthase catalytic activity, were suspended into drug-free medium. Resumption of [^{3}H]deoxyuridine incorporation into DNA was negligible; by 4 hr enzyme activity was still inhibited by ~98%. However, this was sufficient to interconvert all available tetrahydrofolate cofactors to dihydrofolate ($T_{\frac{1}{2}} \sim 2$ hr) when dihydrofolate reductase was inhibited by the lipophilic antifolate trimetrexate. Interconversion of tetrahydrofolate cofactors to dihydrofolate correlated with a decline, then cessation, of purine synthesis as measured by the incorporation of [^{14}C]formate into purine bases. These data suggest that an earlier than previously expected depletion of tetrahydrofolate cofactors with consequent inhibition of purine and other folate-dependent synthetic processes is likely to occur when antifolates are administered after a fluoropyrimidine.

Antifolates in combination with fluoropyrimidines can result in synergistic, additive, or antagonistic effects on tumor growth inhibition and cytotoxicity based on the sequence and timing of drug exposure [1, 2]. Methotrexate followed by 5-fluorouracil has shown synergistic effects in a number of tumor cells *in vitro* and *in vivo* [3–7]. Inhibition of *de novo* purine biosynthesis by methotrexate results in an increase in phosphoribosylpyrophosphate (PRPP†); this augments conversion of fluorouracil to fluorouridine nucleotides that are metabolized to 5′-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) which inhibits thymidylate synthase [6]. Inhibition of thymidylate synthase by FdUMP is dependent upon adequate levels of 5,10-methylenetetrahydrofolate to stabilize the enzyme–inhibitor complex [8–10], and the activity of fluoropyrimidines is enhanced by increasing the reduced folate content of tumor cells [11, 12]. While antifolates that inhibit dihydrofolate reductase interconvert all *available* tetrahydrofolate cofactors to dihydrofolate polyglutamates rapidly ($T_{\frac{1}{2}} \sim 1.5$ min) [13–17], this oxidized folate also produces a potent stabilization of the FdUMP–thymidylate synthase complex [18] in the absence of 5,10-methylenetetrahydrofolate.

Fluoropyrimidines inhibit the depletion of tetrahydrofolate cofactors that occurs with subsequent exposure of cells to dihydrofolate reductase inhibitors by blocking their oxidation to dihydrofolate, and, generally, the combination of these agents when given in this sequence is antagonistic [8, 19, 20]. However, previous studies from this laboratory demonstrated that only a very small fraction of residual thymidylate synthase catalytic activity after inhibition at the 5,10-methylenetetrahydrofolate or 2′-deoxyuridine 5′-monophosphate (dUMP) site is sufficient to result in interconversion of available tetrahydrofolate cofactor pools to dihydrofolate after inhibition of dihydrofolate reductase. This is due to the enormous activity of thymidylate synthase in comparison to the levels of tetrahydrofolate cofactors in L1210 leukemia cells [14–17]. Hence, even when thymidylate synthase activity is inhibited in excess of 95% by 5′-fluoro-2′-deoxyuridine (FdUrd), interconversion of *available* tetrahydrofolate cofactors to dihydrofolate in L1210 leukemia cells is more than 96% complete within 90 min [14, 15].

This report characterizes in L1210 leukemia cells the *resumption* of thymidylate synthase catalytic activity following exposure to FdUrd based upon the oxidation of tetrahydrofolate cofactors to

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† Abbreviations: PRPP, phosphoribosylpyrophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; dUrd, 2′-deoxyuridine; FdUrd, 5′-fluoro-2′-deoxyuridine; and FdUMP, 5′-fluoro-2′-deoxyuridine 5′-monophosphate.

dihydrofolate, the most sensitive indicator of low levels of this enzyme activity. The data indicate that resumption of less than 2% of thymidylate synthase activity as assessed by the incorporation of [^{3}H]dUrd into DNA was sufficient to deplete all available tetrahydrofolate cofactors within 4 hr. Hence, when inhibitors of dihydrofolate reductase are administered following fluoropyrimidines, tetrahydrofolate cofactor depletion can occur much more rapidly than has heretofore been appreciated.

METHODS

Chemicals. [6-^{3}H]Deoxyuridine and [3′,5′,7,9-^{3}H]folic acid were purchased from Moravek Biochemicals (Brea, CA). Radioisotopes were purified by HPLC as necessary prior to use [13]. Trimetrexate glucuronate was provided by Warner–Lambert (Ann Arbor, MI). Calcium leucovorin (racemic) was obtained from the National Cancer Institute. All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell culture. The murine leukemia line L1210 was maintained in suspension culture in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% supplemented bovine serum (HyClone Laboratories, UT), L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and 2-mercaptoethanol (20 μM). For experiments, cells in log phase growth were harvested from suspension cultures at a density of $\sim 1 \times 10^6$ cells/mL and resuspended in culture medium at a density of 1×10^6 or 1×10^7 cells/mL in teflon stirred incubation flasks at 37° in a gas phase of 95% air : 5% CO_2.

Measurement of cellular folate pools. L1210 leukemia cells (12 hr doubling time) were equilibrated with [^{3}H]folic acid (2 μM, 1000 dpm/pmol) for 72 hr prior to drug exposures in medium with dialyzed serum that lacks nonlabeled folates. More than 98.5% of the total intracellular folate pools are uniformly labeled under these conditions with a mean intracellular concentration of 42 ± 6 (SEM) nmol/g dry wt. Cells were harvested, washed twice, and then resuspended in fresh medium free of [^{3}H]-folic acid. Following drug exposures, cells were washed and then divided into two pellets. One pellet was analyzed for total folate, based upon the total level of tritium. This pellet was aspirated into the tip of a Pasteur pipet, extruded onto a plastic tare, dried overnight at 70° and weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, CA). The weighed pellet was transferred to a glass scintillation vial and digested in 0.2 mL KOH (1 M) for 60 min at 70°. The sample was neutralized with 0.25 mL HCl (1 N), and total radioactivity was measured in a liquid scintillation spectrometer.

The other cell pellet was analyzed for endogenous folate pools by a procedure described previously [13, 14]. Briefly, the pellet was resuspended into nitrogen-saturated maleate buffer (0.1 M, pH 6.0) with 2-mercaptoethanol (20 μM) in foil-covered tubes with serum stoppers. Folylpolyglutamates were cleaved to their respective monoglutamates with hog kidney conjugase for 60 min at 37° [21]. Enzyme activity was terminated and proteins precipitated by heating for 90 sec in boiling water. The folate monoglutamates were analyzed immediately by HPLC using a C-18 reverse phase column (4.6 × 250 mm, IBM Instruments).

Extraction efficiency of tetrahydrofolate cofactors determined with radiolabeled standards were: 5-formyltetrahydrofolate (99%); 5-methyltetrahydrofolate (> 90%); 10-formyltetrahydrofolate (80%); 5,10-methenyltetrahydrofolate (64%); and 5,10-methylenetetrahydrofolate (< 5%). Negligible amounts of *p*-aminobenzoylglutamate are detected in chromatograms in the absence of antifolate. However, as cellular dihydrofolate levels increase in the presence of antifolates, there is a proportional increase in *p*-aminobenzoylglutamate presumed to arise from oxidative cleavage of dihydrofolate. Therefore, cellular dihydrofolate levels reported are corrected for the portion oxidized to *p*-aminobenzoylglutamate [14].

Incorporation of [^{3}H]*dUrd into DNA or* [^{14}C]-*formate into purines.* Following resuspension of cells into FdUrd-free medium, portions of the cell suspension were exposed to [^{3}H]dUrd (3 μM, 450 dpm/pmol) or [^{14}C]formate (100 μM, 20 dpm/pmol) in separate flasks. To approximate initial rates of [^{3}H]dUrd incorporation into DNA, cells were sampled over a 10-min interval during which the rate of tritium incorporation was constant. Alternatively, cells were resuspended into medium containing [^{3}H]dUrd or [^{14}C]formate, and the time-course of incorporation was monitored up to 4 hr. At the times indicated, portions of the cell suspensions were transferred to tubes containing 10 vol. of 0.85% saline at 0°, and the cell fraction was separated by centrifugation and washed two times with the saline solution. Cell pellets were divided into two portions to assess total cellular radiolabel (described above) and incorporation of radiolabel into acid-soluble and -insoluble fractions.

For analysis of [^{3}H]dUrd incorporated into DNA, pellets were lysed and nucleic acids precipitated in 2 mL trichloroacetic acid (0°, 10%). After 10 min on ice, the acid precipitate was separated by centrifugation and then washed two times with the 0° trichloroacetic acid solution. The precipitate was dried overnight at 70°, then weighed, and total tritium along with tritium in the acid precipitate was quantified as described above. [^{14}C]Formate incorporation into purines was quantified by treatment of the acid precipitate with 0.5 N perchloric acid for 1 hr at 70° to hydrolyze purine and pyrimidine nucleosides and nucleotides to their respective bases. After cooling, the precipitated proteins were removed by centrifugation. Supernatants were concentrated by lyophilization and neutralized with KOH, and the potassium perchlorate precipitate was removed by centrifugation. The free bases were analyzed by HPLC as described previously [14].

Computer simulations of folate interconversions and one-carbon metabolism. Computer simulations of folate interconversions and one-carbon metabolism in *de novo* purine and thymidylate biosynthesis were based on a network thermodynamic model which utilizes the circuit simulating program SPICE-2. A detailed explanation of network thermodynamics, the SPICE-2 program and previous

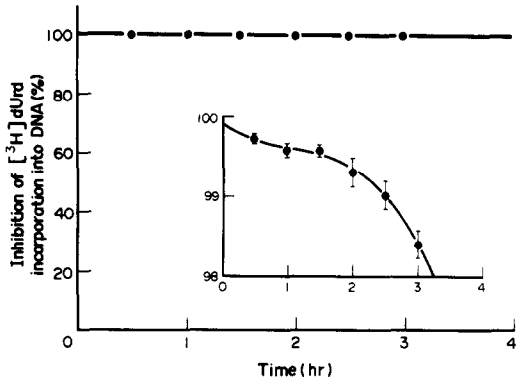

Fig. 1. Resumption of thymidylate synthase catalytic activity after exposure of cells to FdUrd followed by resuspension of cells into drug-free medium. At 30-min intervals, portions of the cell suspensions were removed and incubated with [^{3}H]dUrd (3 μM) for 15 min. The rate of [^{3}H]dUrd incorporation into DNA was determined by linear regression analysis using at least three data points. Inset: y-axis scale is expanded to increase the sensitivity by a factor of 50. Data are the means ± SEM of five experiments performed on different days.

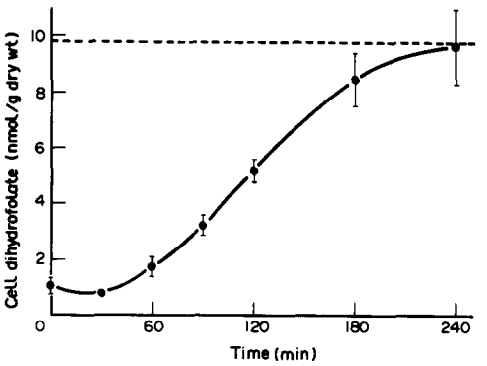

Fig. 2. Oxidation of tetrahydrofolate cofactors to dihydrofolate as an indicator of resumption of thymidylate synthase catalytic activity after FdUrd. Cellular folate pools were radiolabeled as described in Methods. Cells were incubated with 3 μM FdUrd for 15 min and then resuspended into FdUrd-free medium containing 10 μM trimetrexate; intracellular levels of [^{3}H]dihydrofolate were assessed by liquid chromatography. The interrupted line represents the steady-state dihydrofolate level achieved by 10 μM trimetrexate in control cells not exposed to FdUrd. Data are the means ± SEM of seven experiments performed on different days.

applications reported from this laboratory have been published [14–16, 22–25].

RESULTS

Resumption of [^{3}H]*dUrd incorporation into DNA following resuspension of FdUrd-treated cells into FdUrd-free medium.* The initial rate of [^{3}H]dUrd incorporation into DNA in L1210 leukemia cells in logarithmic growth was 18.5 ± 1.0 nmol/g dry wt/min. After cells were exposed to 3 μM FdUrd for 15 min, [^{3}H]dUrd incorporation was not detectable. To assess the rate of thymidylate synthase reactivation following exposure to FdUrd, cells were resuspended into FdUrd-free medium and pulses of [^{3}H]dUrd incorporation into DNA were monitored in portions of the cell suspension. As shown in Fig. 1, [^{3}H]dUrd incorporation appeared to be inhibited completely for at least 3 hr. However, when these data were subjected to a more sensitive analysis by expanding the ordinate scale by a factor of 50 (inset, Fig. 1), a time-dependent reactivation of thymidylate synthase catalytic activity was evident. But even after 3 hr in FdUrd-free medium, [^{3}H]dUrd incorporation into DNA was still inhibited by ~98%.

To assess the effect of isotopic dilution resulting from expansion of the endogenous dUMP pool, [^{3}H]dUrd incorporation was measured following a similar exposure to FdUrd when the extracellular concentration of [^{3}H]dUrd was increased 10-fold from 3 to 30 μM. This resulted in a 4-fold increase in the measured rate of increase of [^{3}H]dUrd incorporation over the subsequent 1.5 to 2 hr but even after 2 hr [^{3}H]dUrd incorporation into DNA remained suppressed in excess of 98%. The observed resumption of cellular thymidylate synthase catalytic activity was not altered by the addition of cycloheximide, excluding the possibility that reactivation is related, in part, to new enzyme synthesis.

Resumption of oxidation of reduced folates to dihydrofolate following resuspension of FdUrd-treated cells into FdUrd-free medium containing trimetrexate. Figure 2 illustrates the time-course of build-up of dihydrofolate following a 15-min exposure of 3 μM FdUrd and then resuspension of cells into FdUrd-free medium containing 10 μM trimetrexate, a measure of the net oxidation of tetrahydrofolate cofactors through thymidylate synthase. Within 30–60 min measurable tetrahydrofolate cofactor oxidation resumed even though [^{3}H]dUrd incorporation was suppressed in excess of 99% (Fig. 1). By 1.5 hr, interconversion of tetrahydrofolate cofactors to dihydrofolate reached its maximal rate (~3.7 nmol/g dry wt/hr) when [^{3}H]dUrd incorporation was still less than 2% of the velocity observed in control cells. Within 4 hr, interconversion to dihydrofolate had reached a maximum level of ~10 nmol/g dry wt. The level of total tetrahydrofolate cofactors in these cells was 42 nmol/g dry wt so that only 23% of reduced folates had interconverted to dihydrofolate, consistent with previous observations from this laboratory [13–17]. This limited interconversion is related, at least in part, to sequestration of reduced folates within mitochondria [26], and a component of non-S phase cells [17] in which thymidylate synthase catalytic activity must be essentially absent and oxidation of folates does not occur after antifolates.

Time-course of suppression of purine synthesis following resuspension of FdUrd-treated cells into FdUrd-free medium containing trimetrexate. As illustrated in Fig. 3, following resuspension of FdUrd-treated cells into FdUrd-free medium containing trimetrexate, purine synthesis as measured by the

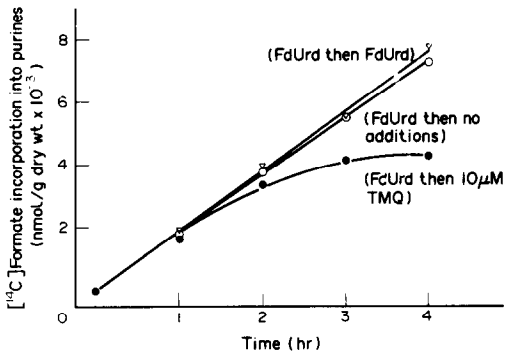

Fig. 3. Time-course of suppression of purine synthesis following resuspension of FdUrd-treated cells into FdUrd-free medium containing trimetrexate (TMQ). Cells were incubated with 3 μM FdUrd for 15 min and then resuspended at 1×10^6 cells/mL into FdUrd-free medium containing 10 μM trimetrexate (●), 3 μM FdUrd (▽), or no additions (○). At time zero, 100 μM [^{14}C]formate was added and incorporation into purines was monitored over a 4-hr period, as described in Methods. The data are from a representative experiment.

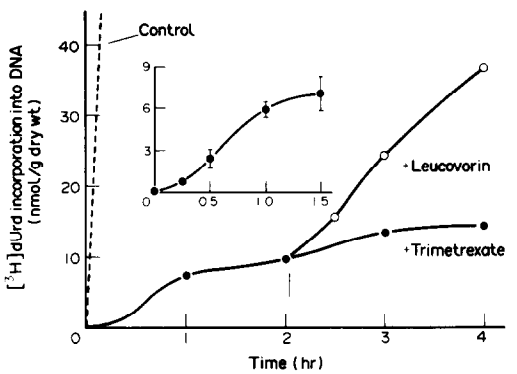

Fig. 4. Effect of sequential exposure to FdUrd and then trimetrexate followed by leucovorin on the resumption of [^{3}H]dUrd incorporation into DNA. Cells were incubated with 3 μM FdUrd for 15 min and then resuspended at 1×10^6 cells/mL into FdUrd-free medium containing 10 μM trimetrexate and 3 μM [^{3}H]dUrd (●). After 2 hr a portion of the cells was transferred to another incubation flask containing 10 μM leucovorin (○). The interrupted line (far left) represents control cells not exposed to FdUrd with normal thymidylate synthase activity. The data are from a representative experiment. Inset: Analysis of [^{3}H]dUrd incorporation into DNA over 1.5 hr. Data are the means ± SEM of four experiments performed on different days.

incorporation of [^{14}C]formate into purine bases was unchanged as compared to control cells until between hr 1 and 2 when the rate began to fall, reaching a negligible level within 4 hr. Hence, purine synthesis declined as interconversion of reduced folates to dihydrofolate began and ceased when interconversion ceased. Continuous exposure to FdUrd, alone, did not affect the incorporation of [^{14}C]formate into purines.

Analysis of the time-course of [^{3}H]*dUrd incorporation into DNA when FdUrd-treated cells were suspended into FdUrd-free medium containing trimetrexate; effect of leucovorin.* When the time-course of [^{3}H]dUrd incorporation into DNA was monitored continuously after resuspension of FdUrd-treated cells in FdUrd-free medium containing trimetrexate, a biphasic pattern emerged (Fig. 4). There was a slow onset of incorporation of [^{3}H]dUrd into DNA over the first hour that accounted for 6.4 ± 0.5 nmol/of tritium/g dry wt after which the rate of incorporation decreased to a much slower velocity that continued over the next 3 hr of observation. The mean net increase in [^{3}H]dUrd incorporation over the 1- to 4-hr interval was 4.2 ± 1.4 nmol/g dry wt. Addition of leucovorin at any point beyond ~60 min resulted in the augmentation of [^{3}H]dUrd incorporation into DNA but the maximum rate achieved was less than 2% of the control velocity. Interestingly, when the concentration of trimetrexate was increased 10-fold to 100 μM, the stimulatory effect of leucovorin was increased by a factor of two (not shown).

Effect of leucovorin on the resumption of [^{3}H]*dUrd incorporation into DNA in cells treated with FdUrd.* When cells pretreated with FdUrd were resuspended in the presence of FdUrd, incorporation of [^{3}H]dUrd, monitored continuously, was abolished completely, indicating that the appearance of radiolabel was dependent entirely upon its conversion

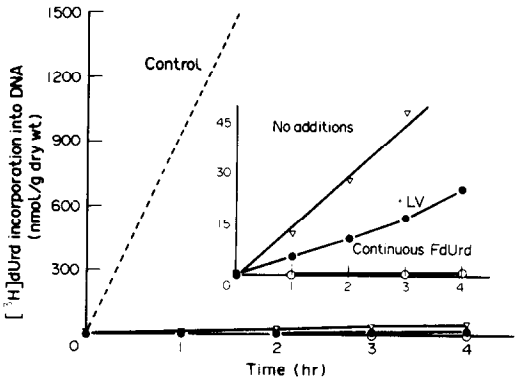

Fig. 5. Effect of leucovorin on the resumption of [^{3}H]dUrd incorporation into DNA. Cells were incubated with 3 μM FdUrd for 15 min and then resuspended at 1×10^6 cells/mL into FdUrd-free medium containing 3 μM FdUrd (○) or no additions (▽); (●) indicates cells that were exposed to 10 μM leucovorin 30 min prior to, during, and then after the exposure to 3 μM FdUrd. At time zero, 3 μM [^{3}H]dUrd was added and incorporation into DNA was monitored continuously for 4 hr. The interrupted line represents control cells not exposed to FdUrd with normal thymidylate synthase activity. Inset: y-axis scale is expanded to increase the sensitivity by a factor of 30. The data are from a representative experiment.

to thymidylate through thymidylate synthase (Fig. 5). In cells exposed to 3 μM FdUrd for 15 min prior to resuspension into fluoropyrimidine-free medium, reactivation was negligible in comparison to the rate

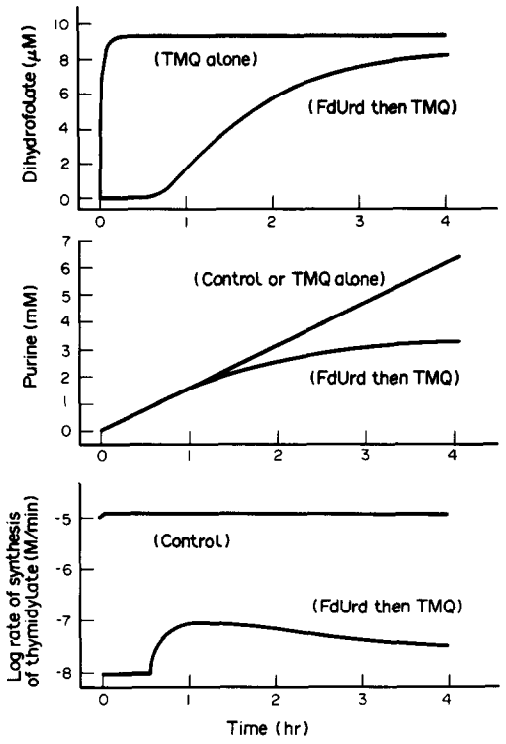

Fig. 6. Network thermodynamic computer simulations of the effect of sequential exposure of cells to FdUrd and then trimetrexate, according to the experimental design in Figs. 2 and 3, on the rate and extent of oxidation of cellular tetrahydrofolate cofactors to dihydrofolate (upper panel), *de novo* purine biosynthesis (middle panel), and the velocity of thymidylate synthesis (lower panel).

of [^{3}H]dUrd incorporation into DNA in control cells. However, when compared to cells continuously exposed to FdUrd, a low level of reactivation was detected rapidly (Fig. 5, inset). Pretreatment of cells with this concentration of FdUrd for a period five times longer (75 min) caused only a small additional slowing of the time-course of reactivation (not shown). When cells were incubated with 10 μM leucovorin for 30 min prior to, during, and then after exposure to FdUrd, the resumption of incorporation was slowed. This is consistent with stabilization of the thymidylate synthase–fluoropyrimidine complex by 5,10-methylenetetrahydrofolate [8–10]. However, even in the presence of leucovorin, catalytic activity resumed and increased within the 4-hr interval of observation, indicating that stabilization was not complete under these conditions.

Network thermodynamic computer simulation of the predicted time-course of oxidation of reduced folates to dihydrofolate and cessation of purine synthesis following resuspension of FdUrd-treated cells into F-dUrd-free medium containing trimetrexate. Computer simulations were employed to address the issue as to whether the low levels of thymidylate synthase activity that resume following exposure of cells to FdUrd can account for the time-course of oxidation of dihydrofolate and the cessation of purine synthesis observed experimentally upon the addition of trimetrexate. In Fig. 6 the reactivation of thymidylate synthase following a 15-min pulse of FdUrd was simulated in the SPICE-2 model using a sinusoidal modulation of enzyme activity. The lower panel illustrates the rate of thymidylate synthesis and indicates that there was sustained inhibition for ~0.5 hr. Then, as thymidylate synthase reactivated, there was a small increase in thymidylate synthesis that reached a maximum velocity of <1% of the control rate at 1–2 hr, corresponding to the maximal rates of dihydrofolate generation (see upper panel). From ~2 to 4 hr, the velocity of thymidylate biosynthesis declined as available tetrahydrofolate cofactor pools were depleted by their oxidation to dihydrofolate in the presence of trimetrexate. It can be seen that the simulations of the rise in dihydrofolate (upper panel) and the decline in purine synthesis (middle panel) closely approximate the experimental data in Figs. 2 and 3, respectively.

DISCUSSION

When fluoropyrimidines abolish thymidylate synthase activity, depletion of tetrahydrofolate cofactors by oxidation to dihydrofolate is not possible and inhibition of *de novo* purine biosynthesis on this basis cannot occur. As long as thymidylate synthase catalytic activity is absent, trimetrexate, which acts solely at the level of dihydrofolate reductase, can have no pharmacologic effect. However, antifolates such as methotrexate which form polyglutamyl derivatives [27–29] could produce pharmacologic activity through the build-up of polyglutamates within cells with the potential for direct inhibition of purine synthesis at AICAR and GAR transformylase [30–32].

The previous demonstration that only trivial levels of thymidylate synthase activity can catalyze the oxidation of all *available* reduced folates [14, 15] raised the question as to how long after fluoropyrimidine administration is inhibition of thymidylate synthase sufficient to prevent tetrahydrofolate cofactor pool interconversion to dihydrofolate after antifolates. These studies indicate that following exposure of L1210 leukemia cells to FdUrd to completely suppress thymidylate synthase activity, tetrahydrofolate cofactor depletion can occur within 4 hr when resumption of thymidylate synthase catalytic activity, as assessed by [^{3}H]dUrd incorporation into thymidylate, is barely detectable.

When the time-course of [^{3}H]dUrd incorporation into DNA was analyzed in cells after prior exposure to FdUrd and upon resuspension into 10 μM trimetrexate, an early phase of resumption of thymidylate synthase activity was detected over the first hour (Fig. 4). This accounted for ~6 nmol of tritium incorporated per g dry wt of cells, a low estimate due to the dilution of radiolabel by nonlabeled cellular pyrimidine nucleotides. Over this interval there was a net increase of only ~1 nmol of dihydrofolate (the total dihydrofolate pool is accurately quantitated by the radiolabeling technique), indicating that there must be sufficient residual dihydrofolate reductase activity to reduce

the dihydrofolate generated during thymidylate synthesis under these conditions. The cellular requirement for one-carbon units is met through the recycling of dihydrofolate to tetrahydrofolate. This is in the range of residual dihydrofolate reductase activity expected in these cells in the presence of 10 μM trimetrexate estimated previously at ~6.5 nmol/g dry wt/hr [15] and consistent with the concept that when thymidylate synthase activity is minimal, dihydrofolate reductase activity required to maintain tetrahydrofolate cofactor pools is minimal as well [15, 24, 33].

One hour after resuspension of the FdUrd-treated cells into trimetrexate the rate of [^{3}H]dUrd incorporation into DNA slowed markedly (Fig. 4), a change that appeared to be due to depletion of 5,10-methylenetetrahydrofolate as intracellular trimetrexate rose to maximum levels reported to occur within 30–45 min [34, 35]. High levels of trimetrexate are required to achieve the extent of suppression of dihydrofolate reductase necessary to abolish the very low levels of enzyme activity that are sufficient to meet cellular needs for tetrahydrofolate production under these conditions of low thymidylate synthase activity. Consistent with the role of reduced folate depletion in this decline in [^{3}H]dUrd incorporation was the observation that leucovorin enhanced incorporation (Fig. 4) despite the fact that it slowed the reactivation of thymidylate synthase (Fig. 5). Over the next 3 hr net [^{3}H]dUrd incorporation increased only ~4 nmol/g dry wt while the build-up of dihydrofolate increased by ~8 nmol/g dry wt (Fig. 2), a difference consistent with dilution of [^{3}H]dUrd by nonlabeled nucleotide pools.

As indicated above, when leucovorin was added to cells in which dihydrofolate reductase activity was markedly suppressed by trimetrexate, the rate of [^{3}H]dUrd incorporation into DNA increased. Prior studies indicated that competitive interactions among reduced folate derivatives of leucovorin [36–38] and methotrexate or trimetrexate at the level of dihydrofolate reductase result in the resumption of endogenous dihydrofolate reductase activity (this is distinct from the direct effects of dihydrofolate generated from leucovorin [36]). However, leucovorin augmentation of thymidylate synthesis observed here cannot be attributed to reactivation of dihydrofolate reductase since the effect was not decreased when the trimetrexate level was increased 10-fold as would be expected if there was a competitive interaction at this enzyme site. Rather, we conclude, as indicated above, that in this study this was due to the provision of reduced folate substrate providing 5,10-methylenetetrahydrofolate for thymidylate synthesis. While the increase in [^{3}H]-dUrd incorporation produced by leucovorin was profound in comparison to the rate in cells exposed to FdUrd alone, the velocity achieved was less than 5% of control cells. This was not, however, an insignificant level of activity since normal cellular growth rates can be maintained by 10–20% of the thymidylate synthase activity in log phase L1210 leukemia cells [39].

What is difficult to explain is the *temporal* difference between the fall in [^{3}H]dUrd incorporation and the interconversion of tetrahydrofolate cofactors to dihydrofolate. Hence, by the time [^{3}H]dUrd incorporation had slowed (Fig. 4), the net increase in the cell dihydrofolate level remained very small (Fig. 2). While this was likely due, in part, to dilution of [^{3}H]dUrd as the endogenous dUMP pools in the cell increased [40, 41], it is also possible that there is an initial rapid depletion of 5,10-methylenetetrahydrofolate that precedes the major depletion of total cellular tetrahydrofolate cofactors. Changes in 5,10-methylenetetrahydrofolate and total reduced folate pools have been shown to be discordant upon exposure of L1210 leukemia cells to leucovorin [39]. Computer simulations based upon the model and techniques previously reported [14–16] exclude the possibility that the low levels of dihydrofolate that accumulate during this interval could result in sufficient feed-back inhibition of thymidylate synthase to account for these observations.

The exact quantitation of thymidylate synthase activity in these studies was not possible because the nonlabeled cellular dUMP pool was not measured. However, only very small changes in the dUMP pool have been reported for L1210 leukemia cells over the interval of this study [42] and even when the specific activity of the radiolabeled dUrd was decreased by a factor of ten the results were essentially unchanged. Further, the measured rates of interconversion of reduced folates to dihydrofolate (Fig. 2) and the decline in purine synthesis (Fig. 3) after dihydrofolate reductase was suppressed completely is consistent with reactivation of only very low levels of thymidylate synthase based upon computer predictions (Fig. 6). Hence, while the exact level of thymidylate synthase activity in these cells was not measured experimentally, it is clear that only a trivial fraction of enzyme activity was sufficient to result in interconversion of tetrahydrofolate cofactors to dihydrofolate with concurrent cessation of purine synthesis.

The assumption that the lack of efficacy of regimens in which methotrexate follows fluorouracil is always due to preservation of tetrahydrofolate cofactor pools may not be valid. Rather, depletion of tetrahydrofolate cofactors necessary to sustain reactions other than thymidylate synthesis such as purine biosynthesis may occur much more rapidly than previously appreciated. Diminished cytotoxicity with these regimens may then be due to "balanced" [39, 43, 44] inhibition of both purine and pyrimidine synthesis or other, as yet, undefined mechanisms. Of course, the reversibility of the inactivation of thymidylate synthase in clinical regimens would depend upon the dose of the fluoropyrimidine, the co-administration of leucovorin, and the synthesis of new enzyme which can occur rapidly after this agent [39]. Likewise, the interval between administration of the fluoropyrimidine and antifolate could be critical since if given close together antifolate might be cleared from cells at a time when sufficient residual thymidylate synthase activity had appeared. This is less likely for antifolates such as methotrexate which form polyglutamyl derivatives that persist in cells to sustain inhibition of dihydrofolate reductase long after the monoglutamyl extracellular drug has been cleared.

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