Elevation of Dihydrofolate Reductase, Thymidylate Synthetase, and Thymidine Kinase in Cultured Mammalian Cells after Exposure to Folate Antagonists

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SUMMARY

Roswell Park Memorial Institute 4265 human lymphoblasts were grown with three dihydrofolate reductase inhibitors: a 2,4-diaminopteridine, methotrexate; a 2,4-diaminoquinazoline, chlorasquin; and a 2,4-diaminotriazine, triazinate. In the absence of inhibitor, dihydrofolate reductase activity increased to a peak at mid-log growth and then declined during the later growth stages. When cells were grown with 10^{-6} M antifolate, cell growth was not affected, but dihydrofolate reductase activity (assayed at pH 7.0) remained at approximately initial levels throughout the growth cycle. This represented 60 to 70% less activity at the mid-log stage of growth, as compared to control cells. Dihydrofolate reductase activity in cells grown with 10^{-8} M methotrexate, when assayed at pH 8.5, reached levels twice those in control cells. Enzyme activity in cells grown with 10^{-8} M chlorasquin, when assayed at pH 8.5, was also higher than at pH 7.0, but it was not as high as that observed in methotrexate-treated cells. Activity in cells grown with 10^{-8} M triazinate was approximately the same when assayed at either pH 7.0 or 8.5.

At 10^{-8} M, the three antifolates had no effect on the activities of thymidylate synthetase, thymidine kinase, serine trans-hydroxymethylase, 5,10-methylentetrahydrofolate dehydrogenase, 10-formyltetrahydrofolate synthetase, and thymidylate kinase. However, when concentrations were used which completely inhibited growth (10^{-7} to 10^{-5} M methotrexate or chlorasquin; 10^{-6} to 10^{-8} M triazinate), dihydrofolate reductase was progressively inhibited, and there was a two- and a threefold elevation of thymidylate synthetase activity. Dihydrofolate reductase activity in cells grown with 10^{-8} M methotrexate, when assayed at pH 8.5, reached levels twice those in control cells. Activity in cells grown with 10^{-8} M triazinate was approximately the same when assayed at either pH 7.0 or 8.5.

INTRODUCTION

Dihydrofolate reductase (2-4), thymidylate synthetase (37), and thymidine kinase (43) activity was found to be elevated in the leukocytes of patients treated with methotrexate. Since these enzymes are responsible for thymidine nucleotide formation, the observations have been pursued in relation to mechanisms of resistance (8, 13, 16, 17, 24, 25, 29, 31, 38, 39). However, each of these studies left the fundamental question of whether the 3 enzymes were elevated within the same cell largely unanswered. Since Hillcoat et al. (24) established that methotrexate elevated dihydrofolate reductase in cultured RPMI 4265 human lymphoblasts, we used these cells to monitor the antifolate effect on dihydrofolate reductase, thymidylate synthetase, and thymidine kinase. The activities of serine trans-hydroxymethylase, 10-formyltetrahydrofolate synthetase, 5,10-methylentetrahydrofolate dehydrogenase, and thymidylate kinase were also examined. Use of folate antagonists such as chlorasquin, a 2,4-diaminoquinazoline (18, 26), and triazinate, a 2,4-diaminotriazine (41) provided an opportunity to test the uniqueness of the methotrexate effect. Comparative studies of these 3 antagonists on the enzymes in cultured mammalian cells are presented herein. Preliminary reports of this work have appeared (10, 11).

MATERIALS AND METHODS

RPMI 4265, a line of immature leukocytes established in culture (33) from a patient with chronic myelocytic leukemia.
mia, was obtained from Dr. George E. Moore of the Roswell Park Memorial Institute. The cells were grown in suspension in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N.Y.) containing 15% fetal calf serum (Flow Laboratories, Rockville, Md.). The medium, purchased as a powder concentrate, was reconstituted with resindistilled water and supplemented with sodium bicarbonate, 2.5 g/liter, penicillin G, 10^8 units/liter (E. R. Squibb & Sons, Inc., New York, N.Y.), and streptomycin sulfate, USP, 0.1 g/liter, (General Biochemicals, Chagrin Falls, Ohio). The pH was adjusted to 7.2 and the medium was sterilized by pressure filtration through a 0.22-μm Millipore filter.

Methotrexate (N-[(2,4-diamino-6-p-tertidinyl)methyl]-methylamino][benzoyl]-L-glutamic acid) was purchased from Lederle Laboratories, Pearl River, N.Y. Chlorosquin, CCNSC 529861 ([L-aspatic acid, N-[(2,4-diamino-5-chloro-6-quinozolynyl)]methyl]amino][benzoyl] dihydrate), and triazinate, CCNSC 139105 (1-[3-chloro-4-(meta-N,N-dimethyl carboxamido)benzoyl]ox2,2-dimethyl, 4,6-diamino, 1,2-dihydro-S-triazine), were supplied by Dr. Florence White, Cancer Chemotherapy National Service Center, National Cancer Institute. All solutions were prepared in resindistilled water and filtered through a 0.22-μm Swinnex (Millipore) filters.

Cells in late logarithmic growth were resuspended in RPMI Medium 1640, 15% with respect to fetal calf serum, at 1.25 to 2.0 x 10^5 cells/ml. After addition of drug, cells were dispensed into 150-ml serum bottles (Grand Island Biologic Co.), 50 ml/bottle, and placed in a CO₂ incubator at 37°C. At 12- and 24-hr intervals, cells were counted with a Coulter Counter and harvested by centrifugation at 4°C. Cell counts of bottles from the same experimental group differed no more than 10%. Sufficient cells were harvested from each experimental group to yield duplicate samples containing 30 to 45 x 10^6 cells. The cell packs were stored at -68°C for later determination of enzymatic activity. Enzymes to be used were stored for periods of at least 3 months.

Crude enzyme extracts were prepared by thawing the cell packs in 0.05 M Tris-HCl buffer, pH 7.5, at a density of 25 to 30 x 10^6 cells/ml. The cells were disrupted by sonic oscillation, and the sonic extract was centrifuged at 37,000 x g for 20 min in a Sorval RC-2 centrifuge to remove cell debris. All procedures were carried out at 0-4°C. The supernatant was assayed for enzyme activity. As a control, the activity of thymidylate synthetase and/or dihydrofolate reductase was always determined. When thymidine kinase activity was measured, cells were sonically extracted with 0.05 M Tris-HCl buffer, pH 7.5, 0.163 mM with respect to dihydrofolate, to stabilize the enzyme. This did not affect the activities of the other enzymes. Protein content was determined by a microbiuret method (23).

**Enzyme Assays.** Dihydrofolate reductase activity was determined at 37°C by measuring the combined decrease in absorbance at 340 nm from the conversion of NADPH (ε = 6200) and dihydrofolate (ε = 5800) to NADP and tetrahydrofolate, respectively (5). NADPH was purchased from Sigma Chemical Co., St. Louis, Mo., dihydrofolate was synthesized by Blakley's modification (7) of the method of Futterman (22). The decrease in absorbance was recorded at 5-sec intervals with a Gilford Model 2000 attachment to a Beckman DU spectrophotometer. Dihydrofolate reductase activity was expressed as μmoles dihydrofolate reduced per hr per mg protein.

Thymidylate synthetase activity was measured by the method of Roberts (36). Components were purchased from the following sources: dL-tetrahydrofolate from Sigma; dUMP from Calbiochem, San Diego, Calif.; and [H]dUMP (16 Ci/m mole) from Schwarz/Mann, Orangeburg, N.Y. The reaction, 0.1 mM with respect to [H]dUMP (152.5 μCi/m mole), was initiated by the addition of enzyme (10 to 35 μg protein), and proceeded for 60 min at 37°C with shaking. Enzymatic activity was linear for 80 min over a protein range of 8 to 40 μg. Radioactivity was measured in Aquasol (New England Nuclear, Boston, Mass.) with a Packard Tri-Carb Model 3320 scintillation counter. Activity was expressed as μmoles TMP formed per hr per mg protein.

Thymidine kinase activity was measured by the method of Chello and Jaffe (9). ATP was purchased from P-L Biochemicals, Milwaukee, Wis.; creatine phosphokinase was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio (35 units/mg); creatine phosphate and unlabeled thymidine from Calbiochem, and [H]thymidine (54.1 μCi/m mole) was from New England Nuclear. The reaction, 1 mM with respect to [H]thymidine (10 μCi/m mole), was initiated by the addition of enzyme (0.1 to 0.25 mg protein), and proceeded at 37°C for 20 min with shaking. Enzymatic activity was linear for 30 min over a protein range of 0.05 to 0.25 mg protein. The reaction was terminated by heating, followed by centrifugation to precipitate denatured protein. Twenty μl of supernatant were spotted on 1-cm squares of Whatman DE81 DEAE-cellulose paper (W. & R. Balston Ltd., England) (21). The squares were washed for two 15-min periods in 95% ethanol, dried, and counted in Liquifluor (New England Nuclear). The washing procedure removed 99% of the [H]thymidine without affecting the retention of [H]TMP. Enzyme activity was expressed as μmoles TMP formed per hr per mg protein.

Thymidylate kinase activity was measured by the formation of [H]TMP from [H]dUMP. The assay, in a final volume of 1.0 ml, contained Tris-HCl buffer, pH 7.5 (Sigma), 140 μmoles; ATP (P-L Biochemicals), 7.5 μmoles; MgCl₂, 7.5 μmoles; creatine phosphokinase (35 units/mg; Nutritional Biochemicals), 0.1 mg; creatine phosphate (Calbiochem), 22.5 μmoles; [H]dUMP (labeled from Schwarz/Mann, 48 μCi/m mole as the disodium salt; unlabeled from Schwarz BioResearch as the disodium salt), 0.1 μmoles (4 μCi/m mole); and enzyme, 0.2 ml (0.25 to 0.45 mg). The reaction was initiated by the addition of enzyme and proceeded with shaking for 20 min at 37°C. Enzymatic activity was linear for 20 min over a protein range of 0.1 to 0.8 mg protein. The reaction was terminated by heating, followed by centrifugation to precipitate denatured protein.

For quantification of enzymatic activity, [H]dUMP was separated from [H]dTMP by treating the reaction supernatant with human seminal fluid phosphatase (21). Fresh seminal fluid was centrifuged at 37,000 x g for 20 min at 4°C in a Sorval RC-2 centrifuge. The cloudy supernatant was diluted with equal volumes of 1 mM glycine buffer, pH 8.5, and 0.1 M MgCl₂ and was stored at -20°C. In a 2:1 ratio, reaction supernatant was mixed with seminal phosphatase and incubated for 10 min at 37°C. After cooling, 50 μl of the phosphate-treated assay supernatant were spotted on 1.5-cm
squares of Whatman DE81 paper and washed as in the thymidine kinase assay. The seminai phosphatase lost no activity on freeze-thawing and was capable of dephosphorylating 99% of the TMP remaining in the reaction mix, without affecting the diphosphate product. Enzymatic activity was expressed as μmoles TDP formed per hr per mg protein.

Serine trans-hydroxymethylase was assayed according to the method of Taylor and Weissbach (42). Pyridoxal phosphate and dl-L-tetrahydrofolate were purchased from Sigma; dimedone was from Pfaltz & Bauer, Inc., Flushing, N. Y. The reaction, containing 0.1 ml of enzyme extract (0.1 to 0.35 mg protein), was initiated with [14C]serine (labeled from Amersham-Searle, 48 mCi/mmol; unlabeled from Schwarz/Mann), 0.25 mM (1 mCi/mmol), and incubated for 15 min at 37° with shaking. Enzymatic activity was linear for 30 min over a protein range of 0.05 to 0.40 mg. Radioactivity was measured in Aquasol (New England Nuclear). Activity was expressed as μmoles 5,10-methylenetetrahydrofolate formed per hr per mg protein.

The activity of 10-formyltetrahydrofolate synthetase was determined by the method of Bertino et al. (6). dl-L-Tetrahydrofolate was purchased from Sigma; ATP was from P-L Biochemicals. The reaction was initiated with enzyme (0.1 to 0.16 mg protein) and incubated for 10 min at 37° with shaking. Enzymatic activity was linear for 20 min over a protein range of 0.1 to 0.4 mg protein. The reaction was terminated with 2 N trichloroacetic acid, followed by centrifugation to precipitate denatured protein. The acidification converted the product, 10-formyltetrahydrofolate, to 5,10-methylenetetrahydrofolate, which was measured by light absorption at 355 nm (ε = 22 × 10⁶ sq cm/mole) with a Beckman DU spectrophotometer. A blank containing all components except formate was run for each enzyme sample. Enzyme activity was expressed as μmoles 10-formyltetrahydrofolate formed per hr per mg protein.

The activity of 5,10-methylenetetrahydrofolate dehydrogenase was determined by the method of Bertino et al. (6). NADP and dl-L-tetrahydrofolate were purchased from Sigma. The reaction was initiated by the addition of enzyme (0.08 to 0.16 mg protein) and continued for 10 min at 37° with shaking. Enzymatic activity was linear for 20 min over a protein range of 0.05 to 0.5 mg. The reaction was terminated with 2 N trichloroacetic acid and centrifuged to precipitate denatured protein. The 5,10-methylenetetrahydrofolate formed was determined by light absorption at 355 nm (see above). A blank minus formaldehyde was run for each enzyme sample. Enzyme activity was expressed as μmoles 5,10-methylenetetrahydrofolate formed per hr per mg protein.

Heat Stability Studies. Crude enzyme was prepared from cells harvested in the exponential stage of growth. 5,10-Methylenetetrahydrofolate was prepared by dissolving dl-L-tetrahydrofolate (Sigma) and a 5-fold excess of formaldehyde (Fisher Scientific Co., Pittsburgh, Pa.) in 0.23 M Tris-HCl buffer, pH 7.5, 0.08 M with respect to β-mercaptoethanol (Eastman Kodak, Rochester, N.Y.), and allowing 5 min at room temperature for chemical synthesis (27, 35). Synthesized dihydrofolate (7, 22) was dissolved in 0.23 M Tris-HCl buffer, pH 7.5, 0.08 M with respect to β-mercaptoethanol. dUMP methotrexate, chlorasquin, and triazinate were also dissolved in the Tris-HCl-β-mercaptoethanol buffer, pH 7.5.

After addition of compound to enzyme, each fraction was placed in a 37° water bath. In each experiment, 1 fraction received only the Tris-HCl-β-mercaptoethanol buffer, pH 7.5. After 90 sec to reach 37°, aliquots were removed over an interval of 20 min and cooled. When all aliquots were collected, they were assayed for thymidylate synthetase or thymidine kinase activity. Control activity was determined from enzyme which received an appropriate volume of the Tris-HCl-β-mercaptoethanol buffer, pH 7.5, but was not subjected to heat denaturation. Results were expressed as percentage of control activity.

RESULTS

RPMI 4265 growth and dihydrofolate reductase activity are shown in Chart 1. Following an initial lag of 24 hr, the cells divided exponentially for 72 hr, after which they entered a stationary phase which was monitored for 48 hr. Cells could remain in the stationary phase for an additional 48 hr without loss of viability. Dihydrofolate reductase activity rose to a 3- to 5-fold peak at the mid-log stage of growth (72 hr) and declined as the cells entered the later growth stages. In the experiment shown in Chart 1, dihydrofolate reductase activity increased 4.5-fold by the mid-log stage.

The concentration of antifolate necessary to produce a 50% inhibition of growth by 96 hr was 5.9 × 10⁻⁸, 1.7 × 10⁻⁸, and 9.0 × 10⁻⁸ M for methotrexate, chlorasquin, and triazinate, respectively. Use of each antifolate at a level of 10⁻⁸ M did not affect growth, but it had an effect on dihydrofolate reductase activity, as shown in Chart 2.

Dihydrofolate reductase activity in extracts of control cells, when assayed at pH 7.0 or pH 8.5, rose to the expected peak at the mid-log stage of growth and then declined. A slight activation was evident when the pH of the assay was 8.5. Reductase activity in extracts of drug-treated cells, when assayed at pH 7.0, showed no pronounced increase during the growth cycle and was about one-third lower than the activity in extracts of control cells. This activity was measured at pH 7.5.

Chart 1. Dihydrofolate reductase activity in RPMI 4265 cells. RPMI 4265 cells were set up in fresh medium, 15% with respect to fetal calf serum. At 24-hr intervals, cells were counted and harvested. Dihydrofolate reductase was extracted and assayed at pH 7.0. Each point is the mean of 2 samples differing not more than 10%. o, cell growth; □, dihydrofolate reductase activity. This experiment has been repeated at least 24 times with similar results.
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that of the control cells at mid-log. The activity in these same extracts, when assayed at pH 8.5, was quite different depending upon the antifolate. In cells grown with 10^{-8} M methotrexate, enzyme activity rose to a level twice that of the controls by the mid-log stage of growth and remained elevated throughout the remainder of the growth cycle. In cells grown with 10^{-6} M chlorasquin, assay of dihydrofolate reductase at pH 8.5 revealed an increase in activity in cells harvested in the late-log and stationary phases of growth. This increase in activity was not as great as that found in cells grown with methotrexate. No increase in enzyme activity was found at pH 8.5 in extracts of cells grown with 10^{-8} M triazinate. At 10^{-8} M, the antifolates had no significant effect on the activities of the other enzymes under consideration (Charts 4 and 5).

Higher levels of each antifolate were used to produce an immediate and complete inhibition of growth. At 10^{-7} M or higher, methotrexate or chlorasquin completely inhibited growth. At 10^{-7} M triazinate produced a 25% inhibition; at 10^{-6} M or higher, immediate and complete inhibition occurred. Experiments involving these higher concentrations were terminated at 48 hr. Cells grown without antifolate and with 10^{-8} M antifolate were always included as controls.

Dihydrofolate reductase in cells grown for 48 hr with 10^{-8} M antifolate is shown in Chart 3. Assay of reductase at pH 8.5 again revealed the presence of elevated levels in both methotrexate- and chlorasquin-treated cells. In confirmation of the results shown in Chart 2, enzyme activity in cells grown with 10^{-8} M methotrexate was lower than in the controls at pH 7.0 and higher at pH 8.5. More activity was detected in cells grown with 10^{-7} M methotrexate when assayed at pH 8.5 than at pH 7.0. After growth with 10^{-6} M, activity could only be detected in the 36- and 48-hr samples assayed at pH 8.5. No activity was detected at either pH in cells grown with 10^{-5} M methotrexate. Some increase in reductase activity was again detectable at pH 8.5 in cells grown with 10^{-6} and 10^{-7} chlorasquin. In cells grown with 10^{-6} M chlorasquin, only minimal activity was detected in the 24- to 48-hr samples, and activity was not affected by the pH of the assay. No activity was detected in cells grown with 10^{-5} M chlorasquin. Dihydrofolate reductase activity was present in cells grown with 10^{-6} M triazinate and was measurable in cells grown with 10^{-7} to 10^{-5} M. Reassay at pH 8.5 did not reveal additional activity.

The effect of 10^{-8} to 10^{-5} M methotrexate, chlorasquin, and triazinate on thymidylate synthetase activity in RPMI 4265 cells is shown in Chart 4. Thymidylate synthetase activity in control cells increased approximately 3-fold during the 48-hr experimental period and was not affected when cells were grown with 10^{-6} M. However, when cell growth was inhibited by methotrexate (10^{-7} to 10^{-5} M), chlorasquin (10^{-7} to 10^{-5} M), or triazinate (10^{-6} to 10^{-5} M), there was an additional 2-fold increase in synthetase activity at 36 hr after exposure to the drug. This additional activity actually represented a 6-fold increase as referenced to initial

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**Chart 2.** Dihydrofolate reductase activity in cells grown 10^{-8} M methotrexate, chlorasquin, or triazinate. RPMI 4265 cells were set up in fresh medium, 15% with respect to fetal calf serum, containing no drug (○), 10^{-6} M methotrexate (●), 10^{-6} M chlorasquin (△), or 10^{-6} M triazinate (▲). Duplicate cell packs were harvested every 24 hr for 144 hr. Dihydrofolate reductase was extracted and assayed at pH 7.0 (-----) and pH 8.5 (---). Each point represents the mean enzymatic activity determined from the duplicate cell packs. This experiment has been repeated 3 times with similar results.

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**Chart 3.** Dihydrofolate reductase activity in cells grown with 10^{-6} to 10^{-1} M methotrexate, chlorasquin, or triazinate. RPMI 4265 cells were set up in medium, 15% with respect to fetal calf serum, containing no drug (○), 10^{-6} M (●), 10^{-5} M (△), 10^{-4} M (▲), or 10^{-3} M (□) methotrexate, chlorasquin, or triazinate. Duplicate cell packs were harvested every 12 hr for 48 hr. Dihydrofolate reductase was extracted and assayed at pH 7.0 (-----) and pH 8.5 (---). Each point represents the mean enzymatic activity determined from the duplicate cell packs. A separate experiment was performed for each drug and was repeated at least once with similar results. A, dihydrofolate reductase activity in cells grown with methotrexate; B, dihydrofolate reductase activity in cells grown with chlorasquin; C, dihydrofolate reductase activity in cells grown with triazinate.
that observed in the completely inhibited cultures.

folate synthetase, 5,10-methylenetetrahydrofolate dehydro-

duce an intermediate elevation of thymidine kinase activity

enzymatic activity and the concentration of drug used to

were not done. At 10^{-7} M, triazinate produced only a slight

inhibition of growth (25%) and a slight elevation of

thymidylate synthetase. The increase was also maximum at

points which produced an elevation of thymidylate syn-

these higher levels was only one-half that produced by 10^{-5}

M dUMP. At 10^{-4} M dUMP, the enzyme lost less than 20% of its activity (Chart 6A). Comparable levels of dihydrofolate (Chart 6, A and B) and 5,10-methylenetetrahydrofolate (Chart 6, A and C) gave no protection. At 10^{-4} and 10^{-3} M, 5,10-methylenetetra-

hydrofolate produced a graded protection (Chart 6C), whereas

no protection was observed with dihydrofolate until 10^{-3} M

(Chart 6B). However, the maximum stability produced by

these higher levels was only one-half that produced by 10^{-5}

M dUMP. At 10^{-4} M dUMP (not shown), the concentration

normally used for assay, no significant heat inactivation was

observed.

The ability of the antifolates to stabilize thymidylate syn-

thetase in vitro is shown in Chart 6, D, E, and F. With 10^{-7}

and 10^{-6} M methotrexate (Chart 6D), 10^{-7} M chlorasquin

(Chart 6E), and 10^{-6} and 10^{-5} M triazinate (Chart 6F), con-

centrations that produced an elevation of thymidylate syn-

thetase in vivo, no protection from heat denaturation was

observed in vitro. At 10^{-5} M methotrexate (Chart 6D) and

10^{-6} M chlorasquin (Chart 6E), some protection occurred.

This, however, was less than that seen with 10^{-3} M 5,10-

methylenetetrahydrofolate, which was included as a refer-

cence. Chlorasquin was not studied at higher concentrations

because of its inhibitory effect on thymidylate synthetase

(see below).

values. Of note is that synthetase activity in the drug-treated
cells declined toward control levels after 36 hr.

The 2-fold elevation of thymidylate synthetase activity was
reproducible, but there was no apparent correlation be-
tween the amount of synthetase elevation and the concentra-
tion of antifolate used to produce the complete inhibition of
growth, i.e., once an adequate concentration of inhibitor
was present to cause complete inhibition, 10- to 100-fold
higher levels produced no further elevation of activity.

When equimolar concentrations (10^{-5} M) of each antifolate
were compared, there were no significant differences in the
use of methotrexate; B, thymidine synthetase activity in cells grown with chlorasquin; C,
thymidine synthetase activity in cells grown with triazinate.

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seen. Chlorasquin was not studied at higher concentrations

The effect of the 3 antifolates on thymidine kinase activity
is shown in Chart 5. Thymidine kinase activity in control
cells increased 3- to 4-fold in 48 hr and was not affected by
growth with 10^{-8} M antifolate. Antifolate concentrations that
completely inhibited growth elevated the thymidine kinase
activity an additional 3-fold by 48 hr. Again, as with the
use of thymidylate synthetase, repeat experiments
failed to demonstrate a correlation between the elevation of
enzymatic activity and the concentration of drug used to
achieve complete inhibition of growth. With the exception of
the studies with 10^{-7} M triazinate, experiments to deter-
mine whether an intermediate growth inhibition would pro-
duce an intermediate elevation of thymidine kinase activity
were not done. At 10^{-7} M, triazinate produced only a slight
inhibition of growth (25%) and a slight elevation of thymi-
dine kinase activity. The pattern of elevation was similar to
that observed in the completely inhibited cultures.

Growth inhibition by either of the antifolates did not ele-
vote the activities of 4 other enzymes: 10-formyltetrahydro-
folate synthetase, 5,10-methylenetetrahydrofolate dehydro-
genase, serine trans-hydroxymethylase, and thymidylate ki-

nase. In control cells, the trans-hydroxymethylase and the
synthetase increased approximately 2- and 3-fold in 48 hr,
whilst the activities of the other 2 enzymes remained
constant. At 10^{-6} M, the antifolates had no significant effect,
whilst at higher levels there was a slight depression in the
activity of each enzyme.

Since the elevation of thymidylate synthetase activity was
possibly the result of intracellular stabilization from antifolate or
from compounds accumulating as a result of dihydrofolate
reductase inhibition, heat stability studies were initiated to
determine which compounds could prevent denaturation in
vivo. The results are shown in Chart 6. The enzyme lost 5 to
15% of its activity while being heated from 0 to 37 \degree C
and an additional 60 to 70% during the 20-min interval when it was
kept at 37 \degree C. dUMP was a very effective protectant. At 10^{-5}
M dUMP, the enzyme lost less than 20% of its activity (Chart 6A). Comparable levels of dihydrofolate (Chart 6, A and B) and
5,10-methylenetetrahydrofolate (Chart 6, A and C) gave no protection. At 10^{-4} and 10^{-3} M, 5,10-methylenetetra-
hydrofolate produced a graded protection (Chart 6C), whereas
no protection was observed with dihydrofolate until 10^{-3} M
(Chart 6B). However, the maximum stability produced by
these higher levels was only one-half that produced by 10^{-5}
M dUMP. At 10^{-4} M dUMP (not shown), the concentration
normally used for assay, no significant heat inactivation was
observed.

Chart 6A. Comparable levels of dihydrofolate (Chart 6, A and B) and
5,10-methylenetetrahydrofolate (Chart 6, A and C) gave no protection. At 10^{-4} and 10^{-3} M, 5,10-methylenetetra-
hydrofolate produced a graded protection (Chart 6C), whereas
no protection was observed with dihydrofolate until 10^{-3} M
(Chart 6B). However, the maximum stability produced by
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The ability of the antifolates to stabilize thymidylate syn-
thetase in vitro is shown in Chart 6, D, E, and F. With 10^{-7}
and 10^{-6} M methotrexate (Chart 6D), 10^{-7} M chlorasquin
(Chart 6E), and 10^{-6} and 10^{-5} M triazinate (Chart 6F), con-
centrations that produced an elevation of thymidylate syn-
thetase in vivo, no protection from heat denaturation was
observed in vitro. At 10^{-5} M methotrexate (Chart 6D) and
10^{-6} M chlorasquin (Chart 6E), some protection occurred.
This, however, was less than that seen with 10^{-3} M 5,10-
methylenetetrahydrofolate, which was included as a refer-
cence. Chlorasquin was not studied at higher concentrations
because of its inhibitory effect on thymidylate synthetase
(see below).
### Enzyme Elevation by Antifolates


After extraction from cells, thymidylate synthetase was placed on ice and split into fractions. After addition of the compounds under investigation (see "Materials and Methods" for preparative techniques), the enzyme fractions were placed in a 37°C water bath. After 90 sec (hatched portion) was allowed for the enzyme fraction to reach 37°C (0 time), aliquots were removed for 20 min, placed on ice, and assayed for thymidylate synthetase activity. Non-heat-treated enzyme received only Tris-HCl, B-mercaptoethanol buffer, pH 7.5, and was kept on ice until thymidylate synthetase activity was measured. Each section represents a separate experiment, repeated at least once. Each point is the mean activity of 2 assays on the same aliquot, expressed as percentage of activity of non-heat-treated control activity. A, no additions except buffer (O—O), 10⁻⁴ M dihydrofolate ([—[—]), 10⁻³ M 5,10-methylenetetrahydrofolate (O—O), 10⁻² M dUMP (x—x—x). B, no additions except buffer (O—O), 10⁻⁴ M dihydrofolate (O—O), 10⁻³ M dihydrofolate (x—x—x). C, no additions except buffer (O—O), 10⁻³ M 5,10-methylenetetrahydrofolate (O—O), 10⁻² M 5,10-methylenetetrahydrofolate (O—O). D, no additions except buffer (O—O), 10⁻³ M methotrexate (x—x). E, no additions except buffer (O—O), 10⁻³ M methotrexate (x—x—x). F, no additions except buffer (O—O), 10⁻³ M triazinate (x—x).  

Heat stability studies with thymidine kinase were difficult because the enzyme was extracted with thymidine (0.16 mM). Without thymidine, the enzyme lost 70 to 90% of its activity during extraction. In the presence of thymidine, the enzyme was completely protected from heat inactivation at 37°C. Heat stability studies with the residual activity remaining after extraction in the absence of thymidine indicated that, at a concentration 10⁻⁵ M, neither methotrexate, chlorasquin, or triazinate provided protection. Also, antifolate concentrations of 10⁻⁵ M did not protect the enzyme from denaturation during extraction.

Inhibitor-enzyme interaction was also investigated by the inhibition of thymidylate synthetase and thymidine kinase. Kinetic studies, graphed by the method of Lineweaver and Burk (30), showed that both methotrexate and chlorasquin, in relation to 5,10-methylenetetrahydrofolate (Kᵢ = 3 to 4 × 10⁻⁵ M using Sigma dL-tetrahydrofolate and assuming one-half to be the active L-form), were noncompetitive inhibitors of RPM14265 thymidylate synthetase, with approximate Kᵢ values of 4.5 × 10⁻⁵ and 4.9 × 10⁻⁶ M, respectively. Triazinate produced no significant inhibition of RPM14265 thymidylate synthetase at 10⁻³ M. Any enzyme inhibition obscuring a heat stabilization at the methotrexate (10⁻⁷ and 10⁻⁸ M) and the chlorasquin (10⁻⁷ M) concentrations that produced no stabilization would have been inconsequential during both the actual stability studies and in the subsequent as-
say, where the drugs were further diluted by 50%. At 10^-5 M methotrexate (final assay concentration, 5 x 10^-6 M) and 10^-6 M chlorasquin (final assay concentration, 5 x 10^-7 M), some slight inhibition may have occurred. Only negligible inhibition of thymidine kinase was observed at antifolate concentrations of 10^-3 M.

**DISCUSSION**

Exposure to low concentrations of antifolates (10^-6 M) affected only dihydrofolate reductase and indicated that there was more reductase per cell than was necessary for replication, since growth and other enzyme patterns were normal when activity was inhibited to one-third that of the controls. This excess reductase may be a mechanism to ensure the maintenance of the reduced folate pools.

Hilcoat et al. (24) showed that there were 2 patterns of reductase activity in cells grown with methotrexate and proposed the following explanation. Although enzyme was inactivated by methotrexate, the bound inhibitor simultaneously protected the protein from intracellular degradation, resulting in an accumulation of inactive reductase. At pH 7.0, methotrexate was irreversibly bound (1), so that only noninhibitor-bound enzyme was active in the *in vitro* assay. At pH 8.5, binding was competitive with respect to dihydrofolate (1), and the accumulated enzyme was revealed. Similar studies with chlorasquin and triazinate revealed little additional activity at pH 8.5 as compared to pH 7.0, although we believe inactive enzyme was also stabilized by these antifolates. Since chlorasquin binds tighter to reductase at alkaline pH than does methotrexate (18, 26), whereas triazinate has a uniform pH binding profile (A. R. Cashmore, unpublished observations), very little of the inactive enzyme would be expected to be unmasked at pH 8.5.

Use of higher concentrations of antifolate inhibited growth, presumably by reducing the dihydrofolate reductase activity below the level necessary to maintain reduced folate pools. Within these cells, there was an elevation of thymidylate synthetase and thymidine kinase. Elevation of thymidylate synthetase after methotrexate has been reported in cultured mammalian cells (13, 38, 39), in regenerating liver (8, 29, 31), and in rat hepatomas (31). Both Roberts and Loehr (39) and Bonney and Maley (8) believed that the elevation of thymidylate synthetase was due to stabilization by methotrexate, accumulated enzyme substrates or products, or a combination of these. Conrad and Ruddle (13) proposed that the elevation of thymidylate synthetase was due to synthesis of new enzyme by a regulatory gene sensitive to intracellular levels of TTP. Eker (7) believed that changes in pools of phosphorylated thymidine nucleotides caused a derepression of thymidine kinase synthesis.

Direct enzyme stabilization by either inhibitor is probably not responsible for the elevation of thymidylate synthetase or thymidine kinase activity in RPMI 4265 cells. Methotrexate and chlorasquin inhibit thymidylate synthetase, but triazinate does not. This suggests that the triazine derivative does not bind to the enzyme, yet it also produced the elevation *in vivo*. Although *in vitro* heat denaturation is only an approximation of *in vivo* degradative processes, heat stability studies showed that high levels of methotrexate and chlorasquin partially protected the enzyme, but triazinate did not. Finally, the similarity of synthetase elevation produced by 10^-7 to 10^-5 M methotrexate, considered with the lack of *in vitro* heat stability at less than 10^-5 M, necessitates an antifolate concentative mechanism (40) which may be beyond the capacity of the RPMI 4265 cell. Thymidine kinase was neither inhibited, nor stabilized from heat inactivation, nor stabilized during extraction by any of the antifolates.

Stabilization of thymidylate synthetase by accumulated substrates or products may explain the increase in this enzyme activity. Inhibition of dihydrofolate reductase may result in accumulation of dihydrofolate (monoglutamate or polyglutamate) (32, 34). Heat stability studies showed that neither this folate form nor 5,10-methylene tetrahydrofolate were effective protectants. However, these studies were done with monoglutamates, and recent work has implicated polyglutamate forms as both natural substrates and
inhibitors of folate enzymes (12, 14, 15, 20, 28). Fridland (19) reported that treatment of CCRF-CEM cells with a level of methotrexate known to elevate thymidylate synthetase (38) also increased deoxycytidylate deaminase activity and the pool of dUMP. This nucleotide proved to be an excellent protectant of thymidylate synthetase. Bonney and Maley (8) reported similar findings and suggested that a combination of the nucleotide and a folate form were the natural protectants. Whether increased deoxycytidylate deaminase activity also occurs in RPMI 4265 cells or in other cell types should be investigated.

The role of thymidine, which reversed the methotrexate-induced elevation of thymidylate synthetase in DON Chinese hamster cells (13) and the methotrexate-induced elevation of thymidine kinase in Chang human liver cells (17), but which further elevated the methotrexate-induced elevation of thymidylate synthetase in CCRF-CEM cells (39), has yet to be investigated in RPMI 4265 cells.

REFERENCES

Elevation of Dihydrofolate Reductase, Thymidylate Synthetase, and Thymidine Kinase in Cultured Mammalian Cells after Exposure to Folate Antagonists

Paul L. Chello, Charlene A. McQueen, Lisa M. DeAngelis, et al.


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