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EVIDENCE FOR A LOCALIZED CONVERSION OF ENDOGENOUS TETRAHYDROFOLATE COFACTORS TO DIHYDROFOLATE AS AN IMPORTANT ELEMENT IN ANTIFOLATE ACTION IN MURINE LEUKEMIA CELLS

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Abstract—The inhibition of *de novo* nucleotide, serine, and methionine biosynthesis in mammalian cells treated with antifolates has been attributed generally to a reduction in the levels of tetrahydrofolate cofactors. In L1210 leukemia cells grown in tritiated folic acid (1 μM), most of the endogenous radiolabeled folates were present as formyl-substituted tetrahydrofolates (60–73%, including 10- and 5-formyl and 5,10-methenyl tetrahydrofolate), with lower levels of tetrahydrofolate (including 5,10-methylene tetrahydrofolate), 5-methyl tetrahydrofolate, and non-metabolized folic acid. Trimetrexate (1 μM) caused an elevation of dihydrofolate levels within 5 min following drug addition, from approximately 1 to 20% of the total folates. Whereas total reduced folates were preserved, losses in the levels of individual forms ranged from minor changes in the formyl tetrahydrofolates (approx. 10% decrease), to significant losses in the levels of tetrahydrofolate (approx. 60%) and 5-methyl tetrahydrofolate (95%). Under these conditions, the incorporations of [^{3}H]deoxyuridine into TMP and [^{14}C]glycine into purines or of [^{14}C]formate into biosynthetic products were inhibited (69–95%). The majority (59–100%) of the endogenous radiolabeled folates in L1210 cells grown in various concentrations (0.2 to 3 μM) of [^{3}H]folic acid was bound to soluble intracellular proteins when cell-free extracts were fractionated by rapid gel filtration or charcoal adsorption. Total intracellular folate levels increased in proportion to the changes in medium folic acid concentration; however, cofactor binding was saturable. At low concentrations, below that which supported maximal growth (less than 0.75 μM), all of the intracellular folates were protein-bound; only when maximal growth was achieved, could unbound folates be detected. Incubation with trimetrexate (1 or 10 μM), methotrexate (10 μM), or calcium leucovorin (50 μM) did not alter significantly the levels of total and protein-bound [^{3}H]folates in cells grown in 1 μM [^{3}H]folic acid. Under all conditions, formyl tetrahydrofolates were the major intracellular derivatives; however, these forms were poorly represented in the bound fraction. Conversely, all of the other intracellular folate forms were completely bound. Tetrahydrofolate was the predominant protein-bound derivative in control cells; in antifolate-treated cells, both bound tetrahydrofolate and 5-methyl tetrahydrofolate were largely replaced by protein-bound dihydrofolate. This interconversion in drug-treated cells was independent of (i) sustained levels of [^{3}H]formyl tetrahydrofolates, or (ii) high extracellular concentrations of unlabeled calcium leucovorin (50 μM). Hence, protein-bound tetrahydrofolates must not only be substrates for enzyme-mediated reactions (i.e. TMP synthesis) but also must slowly equilibrate with unbound cofactors. In this fashion, binding of endogenous folates to soluble proteins may function to "segregate" intracellular cofactor pools. The depletion of such a localized fraction of tetrahydrofolate cofactors in antifolate-treated cells could contribute to the inhibition of biosynthetic processes yet would not necessarily be accompanied by significant changes in the levels of other derivatives, nor the total size of the pool of reduced folates.

The antifolates, represented by methotrexate and its lipid-soluble counterpart, trimetrexate, are an important class of antitumor agents which have generally been considered to derive their cytotoxic effects from their tight binding to dihydrofolate reductase. When dihydrofolate reductase is inhibited, the continued conversion of 5,10-methylene tetrahydrofolate to dihydrofolate (Fig. 1) during TMP synthesis should significantly "deplete" the pool of this reduced folate [1]. Moreover, since various other tetrahydrofolate cofactor forms freely equilibrate with 5,10-methylene tetrahydrofolate, the levels of these derivatives should decrease markedly, as well, resulting in a suppression of *de novo* serine, methionine, and nucleotide biosynthetic pathways.

While long an attractive model for the metabolic effects of these drugs, only recently have studies been reported which attempt to correlate antifolate effects on cellular processes with changes in the distribution of endogenous folates [2–4]. In cultured MCF-7 human breast cancer cells, for instance, treatment with growth-inhibitory concentrations of methotrexate or trimetrexate potently suppresses purine nucleotide and TMP biosynthesis; however, total tetrahydrofolate cofactor pools are largely preserved since their net conversion to dihydrofolate is small [2, 5]. Similarly, in murine leukemia cells treated with these agents, inhibitory effects on amino acid and nucleotide biosynthesis are sustained, even when high concentrations of leucovorin [i.e. (6*R*,*S*)5-formyl tetrahydrofolate] are provided [6, 7]. Since

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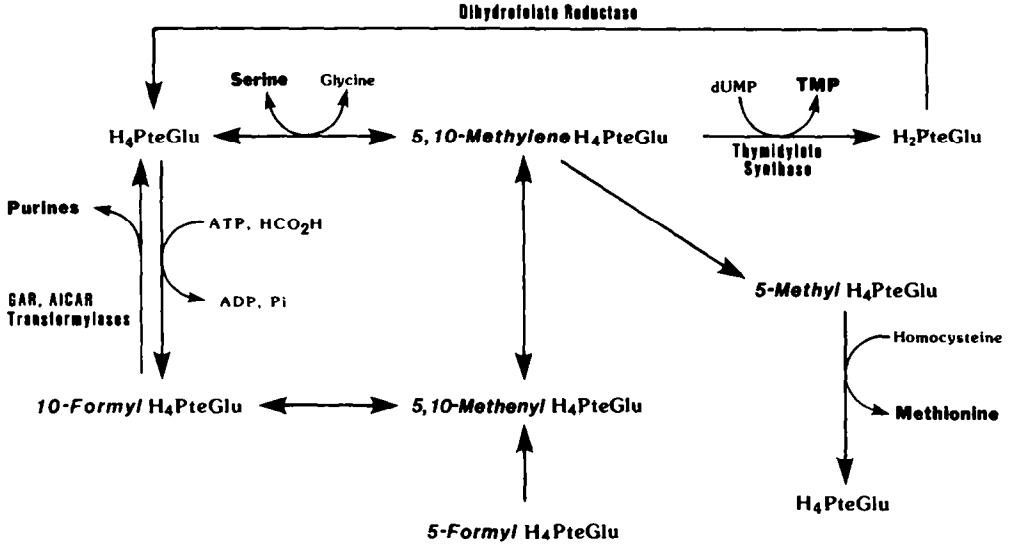

Fig. 1. Interconversion and biosynthetic ultilization of the natural folates. Abbreviations: AICAR, aminoimidazolecarboxamide ribonucleotide; GAR, glycinamide ribonucleotide; H_2PteGlu, dihydrofolate; and H_4PteGlu, tetrahydrofolate.

(6*S*)5-formyl tetrahydrofolate is readily transported into tumor cells and metabolized to other tetrahydrofolate cofactor forms which accumulate intracellularly [6, 8], it appears that suppression of biosynthetic processes under these conditions does not simply arise from decreased levels of reduced folates required for one-carbon transfer reactions.

It is not established to what extent the minor changes in the total tetrahydrofolate cofactor pools in cells treated with diaminoantifolates accurately reflect the biochemical consequences of drug action since cell populations may be quite heterogeneous [3] and/or intracellular folates may be compartmentalized through their binding to folate-dependent enzymes [9–11] or other proteins [12–20]. A number of "folate binding proteins" have been described in mammalian systems in soluble [13, 15–17, 20] or membrane-bound forms [12–14, 17, 18]; however, in most instances, their functions are unclear. In any case, if an appreciable fraction of intracellular folates is protein-bound, this may render measurements of the changes in *total* cofactor pools largely irrelevant to the metabolic events which actually contribute to the suppression of biosynthetic pathways by antifolates.

This report focuses on the biochemical bases for the inhibitory effects of diaminoantifolates on *de novo* pathways leading to purine nucleotides, TMP, serine, and methionine in the murine L1210 leukemia cell model. Data are presented which establish that a large component of the endogenous folates in tumor cells grown in [^{3}H]folic acid are bound to soluble folate-binding proteins. The specific association of particular endogenous folate derivatives with intracellular proteins may serve a previously unrecognized role in cofactor metabolism, and/or may constitute an important element in the pharmacologic activity for this class of drugs.

MATERIALS AND METHODS

Chemicals. [3′,5′,7,9-^{3}H]Folic acid and [6-^{3}H]2′-deoxyuridine were purchased from Moravek Biochemicals (Brea, CA). [2-^{14}C]Glycine and [^{14}C]formate were obtained from ICN Radiochemicals (Irvine, CA). Methotrexate and trimetrexate (glucuronate salt) were acquired from the Drug Development Branch, National Cancer Institute, Bethesda, MD. Assorted biochemicals, including leucovorin, folic acid, (6*R*,*S*)5-methyl tetrahydrofolate, (6*R*,*S*)tetrahydrofolate, and 2′-deoxyuridine were purchased from the Sigma Chemical Co. (St. Louis, MO). Other unlabeled folate derivatives including 10-formyl tetrahydrofolate [21], 5,10-methenyl tetrahydrofolate [21], and dihydrofolate [22] were prepared as previously described. Unlabeled and [^{3}H]folic acid were purified prior to use by HPLC [6]. Methotrexate was purified by DEAE-cellulose chromatography prior to use [23]. Other reagents were obtained in the highest purity available and used without further purification.

Cell culture. Murine L1210 leukemia cells were maintained in a humidified atmosphere at 37° in RPMI 1640 medium supplemented with 10% heat-inactivated undialyzed sera, 2 mM l-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL), all purchased from Gibco Laboratories (Grand Island, NY). 2-Mercaptoethanol (20 μM) was also required for growth. For all studies, cells were previously depleted of endogenous folates by growing the cells for several weeks in folate-free RPMI 1640 containing 10% dialyzed fetal bovine serum in the presence of adenosine (100 μM) and thymidine (10 μM; [6, 7]). Inocula were made at 1×10^5 cells/mL, and cell transfers were made every other day.

Cell numbers were determined with a Coulter Counter or by direct counting with a hemocytometer.

Incubation techniques and quantitation of protein-bound folates. Folate pools were replenished in folate-depleted L1210 cells by growing cells in the presence of known levels of unlabeled or radiolabeled folic acid, using folate-free RPMI 1640 and dialyzed sera. To assess the effects of various treatments on endogenous folate pools, cells (1×10^6/mL) were incubated in complete folate-free medium at 37° in specially-designed flasks stirred with Teflon paddles under an atmosphere of 95% O_2/5% CO_2. Incubations were routinely 2 hr. Metabolic fluxes were terminated by dilution of cells into 5–10 vol. of ice-cold Dulbecco's phosphate-buffered saline (DPBS) followed by centrifugation (500 *g*, 5 min at 4°). The cell pellets were washed twice with 20 vol. of DPBS (0°). For quantitation of total intracellular radioactivity, the cell pellets were digested in 0.3 N NaOH (1 mL). Direct measurements of ^3H and protein were performed on samples of the alkaline extract.

For the quantitation of the protein-bound folates, the cell pellets were suspended in 10 mM Tris–HCl, pH 7.4, containing 1% 2-merceptoethanol. Cells were broken by sonic oscillation (3 × 15 sec) or nitrogen cavitation (500 psi, 20 min). The cell-free extracts were centrifuged (20,000 *g* or 200,000 *g* respectively). Samples of the supernatant (typically 800 μL) were fractionated by rapid centrifugation on columns of BioGel P6 (200–400 mesh; BioRad Laboratories, Richmond, CA), prepared in 5-mL disposable syringes and equilibrated with 10 mM Tris–HCl and 1% 2-mercaptoethanol. This procedure has been described previously in detail [24] and has been used to quantitatively separate protein-bound from unbound folate derivatives [25–27]. Cell homogenates and column effluents were sampled for radioactivity and proteins. Alternatively, cell free homogenates (800 μL) were treated with 200 μL of a charcoal slurry (5% Norit A, treated with 1% high molecular weight dextran and suspended in Tris–mercaptoethanol). After mixing and centrifuging in a microcentrifuge to sediment the charcoal, aliquots were removed for determination of radioactivity and proteins. Proteins were determined using a modification of the Lowry protein assay [28]. With both fractionation methods, the levels of the total and protein-bound radioactivity were expressed in units of picomoles per milligram protein and were essentially identical.

Extraction and HPLC analysis of cellular folates. For the analysis of the intracellular radiolabeled folates following growth in [^3H]folic acid, a modification of the methods of Matherly *et al.* [6] was employed. Briefly, a portion of the washed cell pellet was suspended in 0.8 mL of nitrogen-saturated 0.1 M sodium maleate, pH 6, containing 1% 2-mercaptoethanol and 20 nmol of assorted unlabeled folate standards (i.e. 10-formyl, 5-methyl, and unsubstituted tetrahydrofolate, and dihydrofolate). The centrifuge tube was sealed with a serum stopper, and the contents were evacuated thoroughly with a vacuum pump and a syringe needle. The cellular suspension was boiled (90 sec) and cooled (0°), then incubated at 37° for 3 hr (under nitrogen) with an aliquot of conjugase (10 mg), purified approximately 12-fold from fresh hog kidneys [29]. Following the enzymic conversion of polyglutamyl to "monoglutamyl" folate forms, the extracts were again boiled (90 sec) and centrifuged. The clarified samples were analyzed immediately by HPLC as described below. For quantitation of the protein-bound folate derivatives, cell free homogenates (in 10 mM Tris–HCl, pH 7.4, 1% 2-mercaptoethanol) were fractionated, as described above; the protein-bound fraction was added to 0.1 vol. of 1 M sodium maleate, pH 6. 2-Mercaptoethanol was added to a final concentration of 1% along with the unlabeled carrier folates. Extraction and enzymic deconjugation steps for these derivatives were identical to those described above.

HPLC analysis of radioactive folates was routinely performed using a reversed phase method [6] and an ISCO gradient liquid chromatograph equipped with a 5 μm octadecylsilyl column (4.5 × 250 mm; ISCO, Inc.). The flow rate was 2 mL/min; fifty 0.8-mL fractions were collected and determined for radioactivity as described below. The elution positions of the unlabeled carrier folate standards were monitored at 254 nm and could be correlated with those of the radioactive folates. Good resolution of all derivatives with the exception of folic acid and dihydrofolate was obtained. In experiments where quantitation of these unresolved forms was necessary, an ion-pairing HPLC method was employed [2].

Under the conditions used to extract the radioactive folates, some nonenzymic conversion of 10-formyl to 5-formyl tetrahydrofolate and 5,10-methenyl tetrahydrofolate occurred (approx. 50% of the 10-formyl tetrahydrofolate). Because of the large extent of this nonenzymic interconversion at pH 6, these separate forms were routinely treated throughout this study as a single pool. From experiments using standard known quantities of 10-formyl tetrahydrofolate to establish the extent of these interconversions, it appeared that essentially all of the 5-formyl tetrahydrofolate in our cell extracts arose from 10-formyl tetrahydrofolate. The 5,10-methylene tetrahydrofolate pool was quantitatively converted to tetrahydrofolate during the extraction procedure. With these exceptions, net recoveries of radiolabeled folates were typically 75–80%.

Measurements of folate-dependent biosynthetic reactions. In situ measurement of incorporations of radioactivity originating with [^{14}C]formate or [^{14}C]glycine into nucleotides and amino acids were performed as described Matherly *et al.* [7]. Intracellular thymidylate synthase activity was assessed by quantitating the incorporation of [6-^3H]-2′-deoxyuridine into trichloroacetic acid-precipitable nucleic acids as previously detailed by Goldman [30].

Radioactivity measurements. Radioactivity was measured with an LKB 1209 liquid scintillation counter using Ready Value scintillation mixture (Beckman, Irving, CA). Corrections for counting efficiencies were made by internal standardization with ^3H or ^{14}C toluene.

RESULTS

Effects of inhibition of dihydrofolate reductase on

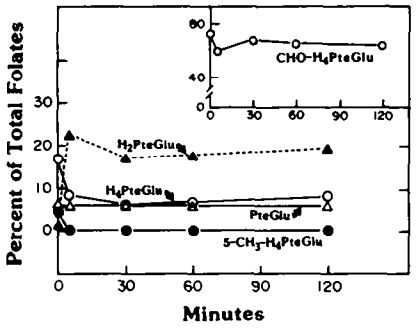

Fig. 2. Time course of trimetrexate effects on the distribution of endogenous folate cofactors in L1210 cells. The endogenous folates were radiolabeled by growing folate-depleted L1210 cells for 48 hr in [^{3}H]folic acid (1 μM). Cells were washed and suspended in fresh folate-free RPMI 1640 containing dialyzed sera. At various times following the addition of trimetrexate (1 μM), portions of the cell suspension were removed, and the total cellular radioactive folates were extracted and quantitated as described in Materials and Methods. The data shown represent the mean results of three separate experiments. Abbreviations: CHO-H_4PteGlu, formyl tetrahydrofolate; H_2PteGlu, dihydrofolate; H_4PteGlu, tetrahydrofolate; PteGlu, folic acid; and 5-CH_3-H_4PteGlu, 5-methyl tetrahydrofolate. Total folate levels in trimetrexate-treated cells were 29.11 pmol/mg protein.

total cell folates in L1210 cells. Like methotrexate, trimetrexate is a potent inhibitor of dihydrofolate reductase in L1210 cells [31] and inhibits cell growth (IC_{50} = 2.5 nM; [32]). However, trimetrexate is neither metabolized in tumor cells nor appears to bind to folate-dependent enzymes other than dihydrofolate reductase [5, 31]. Moreover, uptake of trimetrexate into cells is not limiting to binding to dihydrofolate reductase [33]. This allows the analysis of the time-dependent changes in distributions of endogenous folate pools accompanying enzyme suppression without considerations of the rates of drug transport. L1210 cells were grown in [^{3}H]folic acid (1 μM) and incubated over 2 hr with a cytotoxic concentration of trimetrexate (1 μM; Fig. 2). Under these experimental conditions, the total intracellular level of radiolabeled folates was essentially unchanged (31.02 vs 29.11 pmol/mg protein for untreated and trimetrexate-treated cells respectively). Prior to trimetrexate treatment, most of the folate derivatives were present as formyl-substituted tetrahydrofolates (approx. 73%, including 10- and 5-formyl, and 5,10-methenyl tetrahydrofolate; inset), with lower levels of unsubstituted tetrahydrofolate (17%, including 5,10-methylene tetrahydrofolate), and 5-methyl tetrahydrofolate (4%). Small amounts of unmetabolized folic acid were also detected (6%). Within 5 min following drug addition, dihydrofolate pools were increased maximally, from 1% to nearly 20% of the total, consistent with the inhibition of the major portion of dihydrofolate reductase. This was accompanied by a corresponding decrease in the levels of the reduced folates. The losses among the different forms were variable and disproportionate since the formyl tetrahydrofolate pool was decreased minimally (11.47 ± 4.14%) relative to its large size, whereas marked losses were observed in the already low levels of 5-methyl tetrahydrofolate (95% decrease) and tetrahydrofolate (60% decrease).

Correlations between drug-mediated changes in folate distributions and cofactor-dependent de novo *biosynthesis.* Whereas only small amounts of the *total* reduced folate pool were converted to dihydrofolate during an exposure to 1 μM trimetrexate, cofactor-dependent *de novo* thymidylate and purine nucleotide biosynthesis was nonetheless inhibited (95 and 91%, respectively; data not shown), when these pathways were assayed after 2 hr by following the incorporation over 30 min of [^{3}H]2'-deoxyuridine and [2-^{14}C]glycine into these products. Similarly, trimetrexate treatment markedly suppressed the appearance of [^{14}C]formate in newly synthesized purine nucleotides and TMP (84 and 91% respectively) and, likewise, the radioactivity in perchloric acid-insoluble proteins (69%), representing *de novo* synthesized methionine and serine [7]. Virtually identical effects on *de novo* biosynthetic pathways were observed in L1210 cells incubated over 2 hr with 10 μM methotrexate (not shown).

Protein binding of endogenous folates. The lack of correlation between *de novo* biosynthetic activity and the levels of reduced folates in antifolate-treated L1210 cells could conceivably arise if folate derivatives were, in some fashion, compartmentalized. In this instance, drug treatment could cause a "localized" depletion of particular derivatives as dihydrofolate accumulates, resulting in inhibition of biosynthetic activity, yet only minor changes in the levels of total reduced cofactors. One mechanism by which folates could be compartmentalized is through their binding to cellular folate-binding proteins, a possibility for which there is ample precedent [9–20].

To evaluate this possibility in L1210 cells, endogenous folate pools were radiolabeled by growing folate-depleted cells in [^{3}H]folic acid, as described above; cell free homogenates were prepared, and the supernatants, following centrifugation, were chromatographed by gel filtration using a rapid centrifugation technique [24], or fractionated by adsorption onto activated charcoal [27]. Both of these procedures facilitate the rapid separation of protein-bound from unbound folates [25–27]. Identical results were obtained by both methods. Data are illustrated in Table 1 for L1210 cells grown in 1 μM folic acid and analyzed by gel filtration. As summarized in Table 1, 77% of the intracellular [^{3}H]folates were associated with a high molecular weight fraction during BioGel P6 gel filtration (exclusion limit 6000 daltons). The amount of the radiolabeled folate–binder complex could be reduced significantly by boiling (88%), or by treatment with 4 M guanidine-HCl (91%). In addition, radioactive folates could be dissociated by digestion with proteinase K (300 μg/mL) for 30 min (80%), indicating that the folate binder is a protein.

In further studies, experiments were performed to establish the intracellular disposition of the folate-binding protein(s). Cells, previously grown in [^{3}H]folic acid (1 μM), were broken using a Parr nitrogen cell disruption bomb, and the cell-free homogenate was separated into soluble and crude

Table 1. Effects of various treatments on the protein-binding of endogenous folate cofactors

Treatment	Bound [^{3}H]folates (pmol/mg)	% of Total intracellular [^{3}H]folates bound
None	23.83 ± 1.99	76.75
Boiled	2.97 ± 0.40	9.57
Proteinase K	4.66 ± 1.12	15.01
Guanidine-HCl	2.06 ± 0.52	6.63

L1210 cells were grown in 1 μM [^{3}H]folic acid. Cell-free homogenates were prepared and subjected to various treatments including boiling (2.5 min), digestion with proteinase K (300 μg/mL; 30 min at 37°), or treatment with 4 M guanidine-HCl (30 min at 23°). All treatments were accompanied by untreated controls and were performed under a nitrogen atmosphere. Data are presented as the means ± SE of bound tritiated folates from 4 to 13 experiments or as the percent bound of the total intracellular tritiated folates. The total level of tritiated folates in cells grown in 1 μM [^{3}H]folic acid was 31.05 ± 2.05 pmol/mg (N = 13).

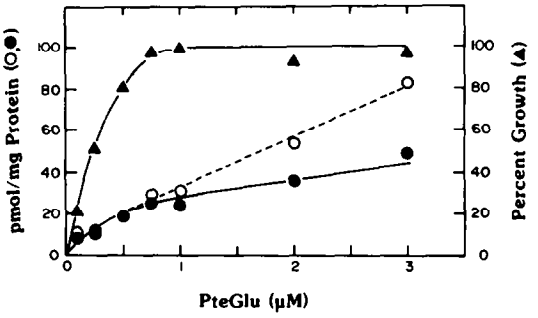

Fig. 3. Relationships among folic acid-dependent growth, total folate accumulation, and protein-binding of folates. Folate-depleted L1210 cells (approx. 50,000 cells/mL, initial density) were grown in various concentrations of [^{3}H]folic acid. After 48 hr, cell growth (▲——▲), as well as total intracellular (O- - -O) and protein-bound (●——●) folates were quantitated as described in Materials and Methods. The data shown are the mean results of three separate experiments. PteGlu = folic acid.

membrane fractions by ultracentrifugation (200,000 *g*, 1 hr). Approximately 80% of the radioactivity in L1210 cells grown in 1 μM folic acid was recovered in the soluble fraction; of this soluble folate pool, 53% was protein-bound. The residual insoluble fraction, which contained not only membrane-associated but also "trapped" radioactivity from the soluble supernatant fraction, consisted of approximately one-half protein-bound folates.

Relationships among folate-dependent growth requirements for L1210 cells, intracellular accumulation, and binding to soluble proteins. The relationships among folate cofactor requirements for growth, net intracellular accumulation, and binding to soluble protein(s) were determined by growing L1210 cells, previously depleted of endogenous folates, in the presence of various concentrations of [^{3}H]folic acid, following which total and protein-bound folates were measured (Fig. 3). Only a slight correlation was observed between the level of total intracellular [^{3}H]folates accumulated, and the extent of growth achieved after 48 hr at various medium folic acid concentrations, since growth was maximal at 0.75 μM, at which point intracellular tritium was only 21 pmol/mg protein. Above this concentration, total cellular folate levels continued to increase with increasing medium concentrations; at 3 μM folic acid, a level of 82.5 pmol [^{3}H]folates/mg protein was attained. In contrast to these findings, the protein-bound fraction was saturable as medium folic acid concentration increased. At all concentrations, protein-bound folates comprised the major portion of the total derivatives, ranging from essentially 100% of the cofactors at growth-limiting concentrations to 59% of the total at 3 μM folic acid as the binding approached a maximum of 61.25 pmol/mg protein (calculated from reciprocal plot analysis of the data presented in Fig. 3; not shown). Only when maximal growth was achieved could unbound intracellular folates be demonstrated.

Effects of antifolate treatment on the distributions of total and protein-bound folates. The apparent relationship between the growth requirements for folic acid and the extent of binding of endogenous cofactors to soluble proteins suggests a possible metabolic role for these associations. Binding may also have a role in influencing the net extent of conversion of intracellular reduced folates to dihydrofolate in cells incubated with antifolates such as trimetrexate.

To consider this possibility, L1210 cells were grown in 1 μM [^{3}H]folic acid and, following different treatments, the compositions of the total and protein-bound fractions were analyzed by HPLC. In Fig. 4 is illustrated a representative HPLC chromatogram of the endogenous folate components for untreated L1210 cells. Whereas all the major derivatives could be identified in the analysis of the total cell fraction, a marked specificity was observed in the bound fraction since only tetrahydrofolate, folic acid, and 5-methyl tetrahydrofolate were present to appreciable extents relative to their total intracellular levels.

In Fig. 5, the HPLC data from replicate experiments are normalized to total cell protein to allow a

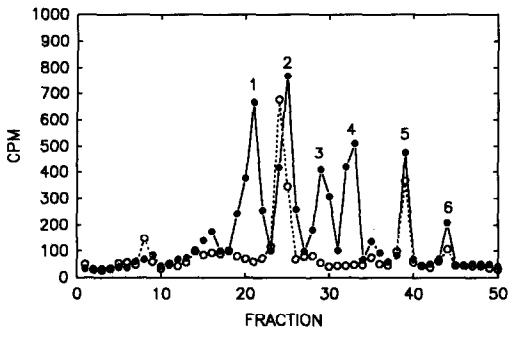

Fig. 4. HPLC analysis of total and protein-bound folates in L1210 cells. Cells were grown in [^{3}H]folic acid (1 μM). Total (●——●) and protein-bound (○——○) folate derivatives were fractionated by charcoal adsorption, followed by extraction and HPLC analysis of "monoglutamyl" folates, as described in Materials and Methods. The peaks are as follows: (1) 10-formyl tetrahydrofolate; (2) tetrahydrofolate; (3) 5-formyl tetrahydrofolate; (4) 5,10-methenyl tetrahydrofolate; (5) folic acid and dihydrofolate; and (6) 5-methyl tetrahydrofolate.

Table 2. Effects of folate and antifolate additions on total intracellular and protein-bound folate cofactors

Addition	N	[^{3}H]Folates (pmol/mg) Total	Protein-bound
None	13	31.05 ± 2.05	23.83 ± 2.60
1 μM TMQ	4	29.11 ± 0.99	19.01 ± 1.81
10 μM TMQ	3	33.23 ± 6.63	22.51 ± 3.63
10 μM TX	3	37.55 ± 7.30	24.83 ± 0.12
50 μM LCV	3	37.21 ± 5.65	23.48 ± 3.61

L1210 cells were grown in 1 μM [^{3}H]folic acid as described in Materials and Methods. Cells were washed and suspended in fresh folate-free RPMI 1640 containing dialyzed serum and the various additions shown. Incubations were for 2 hr at 37° at which time cell-free homogenates were prepared and assayed for total and protein-bound [^{3}H]folates as described in Materials and Methods. Data shown are the means ± SE from 3 to 13 experiments (N). Abbreviatons: LCV, leucovorin; MTX, methotrexate; and TMQ, trimetrexate.

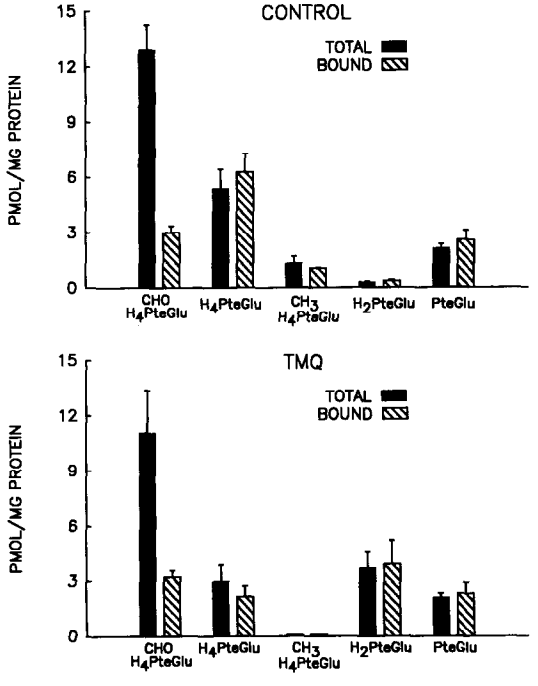

Fig. 5. Effects of trimetrexate on the distribution of total and protein-bound folate cofactors. Folate-depleted L1210 cells were grown for 48 hr in the presence of 1 μM [^{3}H]folic acid. Cells were washed with DPBS, and resuspended in complete folate-free RPMI 1640. A portion of the cells was incubated with 1 μM trimetrexate for 2 hr at which time the total (solid bars) and protein-bound (striped bars) folate derivatives were quantitated as described in Materials and Methods. The results shown are the mean (±SE) of data from three experiments. Abbreviations are defined in the legend for Fig. 2.

direct comparison of the *absolute* levels of total and protein-bound folates. By this analysis, formyl-substituted tetrahydrofolates (includes 5-formyl, 10-formyl, and 5,10-methenyl tetrahydrofolate peaks from the HPLC) represented the bulk of the total (approx. 60% in Fig. 5), yet constituted a much smaller component (approx. 23%) of the protein-bound derivatives. Rather, as evident in the elution profile depicted in Fig. 4, the predominant bound folate was tetrahydrofolate (approx. 47%, including 5,10-methylene tetrahydrofolate), even though this form contributed less than 25% to the total. In contrast to the formyl tetrahydrofolates, virtually all of the tetrahydrofolate pool was protein-bound (Fig. 5). Quantitative binding was also observed for other less abundant derivatives, including 5-methyl tetrahydrofolate, folic acid, and dihydrofolate.

In the bottom panel of Fig. 5 are illustrated the effects of 1 μM trimetrexate on the distributions of total and protein-bound folates. Treatment with trimetrexate (1 or 10 μM) had no significant effect on the levels of total and bound folates (Table 2). Under these conditions, however, bound tetrahydrofolate and 5-methyl tetrahydrofolate levels significantly decreased (65 and 95%, respectively, Fig. 5) and were replaced by an essentially equivalent amount of bound dihydrofolate. As described above (i.e. Fig. 2), the total formyl tetrahydrofolate pool was maintained at 90% of the untreated levels in the presence of trimetrexate, and no loss was observed in the already low levels of these derivatives in the protein-bound fraction. Because of the small amounts of these bound forms compared to dihydrofolate and folic acid, the net result of dihydrofolate reductase inhibition was a greater relative accumulation of oxidized cofactors in the protein-bound fraction than was measured when all of the intracellular folates were considered. In these studies, 54% of the protein-bound folates were identified as folic acid or dihydrofolate (34% dihydrofolate); in contrast, only 29% of the total folates were so identified (19% dihydrofolate).

Table 3. Effect of unlabeled leucovorin on total and protein-bound dihydrofolate accumulation in trimetrexate-treated L1210 cells

Addition	Dihydrofolate (pmol/mg)	
	Total	Bound
None	0.42	0.22
10 μM TMQ	6.64	5.02
50 μM LCV	0.33	0.26
50 μM LCV + 10 μM TMQ	6.75	5.51

L1210 cells were grown in 1 μM [^{3}H]folic acid. Cells were washed and placed in fresh folate-free RPMI 1640 containing dialyzed sera and incubated at 37° for 2 hr in the presence and absence of 50 μM unlabeled calcium leucovorin. At this time, trimetrexate was added to a portion of the cells, and after an additional 30 min at 37°, cells were washed and cell-free homogenates prepared. Total and protein-bound folates were quantitated as described in Materials and Methods. Abbreviations are as defined for Table 2. Values are means of two experiments.

Methotrexate treatment (10 μM) had no appreciable effect on the levels of total or protein-bound [^{3}H]folates in L1210 cells during a 2-hr incubation with drug (Table 2). The effects of methotrexate, moreover, on the distributions of the individual derivatives were virtually identical to those observed in the presence of 1 μM trimetrexate (data not shown).

Effects of leucovorin on intracellular binding of endogenous folates and net conversion of tetrahydrofolate cofactors to dihydrofolate. Addition of 50 μM calcium leucovorin had no significant effect on the level of protein-bound folates over 2 hr (Table 2), even though the (6*S*)5-formyl tetrahydrofolate is rapidly transported into cells and metabolized [6, 8], resulting in a 5-fold increase in the intracellular folate pool. Preincubation of cells previously grown in 1 μM [^{3}H]folic acid with 50 μM unlabeled leucovorin, moreover, had no effect on the net level of accumulation of [^{3}H]dihydrofolate from endogenous bound [^{3}H]tetrahydrofolate forms upon treatment with trimetrexate (10 μM; Table 3). In both the presence and absence of added leucovorin, the majority of the radiolabeled dihydrofolate which accumulated was protein-bound.

DISCUSSION

Early models for the metabolic effects of dihydrofolate reductase inhibition by diaminoantifolates have presumed that an essentially complete conversion of endogenous tetrahydrofolates to dihydrofolate occurs when cells are incubated with these drugs [1]. However, it is now established that *de novo* folate-dependent biosynthetic reactions can be suppressed even though total reduced cofactor pools are preserved.

These findings have been interpreted as evidence for direct inhibitory effects on enzymes other than dihydrofolate reductase in drug-treated cells [2, 5–7], mediated directly by the antifolyl (i.e. methotrexate; [34, 35]) and/or by dihydrofolyl polyglutamates [35, 36]. As demonstrated in the present and earlier reports [2, 3], dihydrofolate can accumulate to considerable intracellular levels when dihydrofolate reductase is blocked, even though the net extent of conversion of total reduced folates to this form is small.* Whereas direct inhibitory effects at the level of thymidylate synthase [34], or the purine biosynthetic enzymes [2, 5, 6, 35] could conceivably account for the extent of biosynthetic inhibition observed when tumor cells are treated with antifolates, their pharmacologic importance to drug action has been difficult to assess.

It has been suggested that the extent of tetrahydrofolate cofactor conversion to dihydrofolate in antifolate-treated cells may reflect, in part, the cell cycle heterogeneity of the cell population, relating to the varying levels of thymidylate synthase activity among cells at different stages of the cell cycle [3]. In this report, data were presented which suggest that an additional, previously unrecognized, element may be involved in the preservation of tetrahydrofolate cofactor forms in antifolate-treated cells, involving a compartmentation of intracellular folates through their binding to soluble proteins. In L1210 cells grown in [^{3}H]folic acid (0.1 to 3 μM), the majority (59–100%) of the radioactive folate derivatives was protein-bound. Binding was saturable with increasing medium folate and showed a correlation with folate-dependent growth since its level approached a maximum as peak growth was achieved. As described elsewhere [37], a similar relationship between growth and protein binding of folate metabolites was observed in L1210 cells grown in increasing concentrations of (6*S*)5-formyl[^{3}H]tetrahydrofolate. Further, the maximum level of binding measured in these studies was virtually identical to that observed in L1210 cells grown in [^{3}H]folic acid.

Folate-binding proteins or protein-bound folates have been described previously in both soluble and

* From the data presented in Fig. 5 for intracellular dihydrofolate levels in the presence of 1 μM trimetrexate, and the values for intracellular water (0.054 mL/10^8 cells; [6]) and protein content of L1210 cells (1.38 mg/10^7 cells), a theoretical dihydrofolate concentration of 1 μM can be calculated. This, of course, assumes that all of the dihydrofolate is osmotically active (i.e. unbound) within the intracellular space.

particulate forms from a number of mammalian sources [9–20]. Whereas a few reports have documented the existence of protein-bound folate cofactors in cellular systems [11, 12, 17], in general, the identification of folate-binding proteins has rested on the ability of these purified or partially-purified species to bind radioactive folates *in vitro*. Consequently, little direct evidence for a biochemical function, if any, for these associations has been provided. Suggestions have been made for a role for folate-binding proteins in membrane transport of folate derivatives [15, 17], in stabilizing labile cofactor forms [9, 11], or as a storage depot for cellular folates [17]. Moreover, these proteins may also regulate the biosynthetic availability of folate derivates for one-carbon transfer reactions [17, 19], or may themselves represent folate-dependent enzymes [9, 10, 11]. This suggested diversity of function may account for the apparent molecular weight heterogeneity of these proteins (40,000–210,000 daltons) among various mammalian systems. By comparison, initial studies indicate that the major binding protein from L1210 cells has an approximate molecular weight of 200,000 daltons [37].

Hence, the relationships between the soluble folate-binding protein(s) from L1210 cells and those previously described from other mammalian systems are uncertain. It is of interest, however, that the apparent specificities for endogenous radiolabeled folate binding to the L1210 protein(s) were similar to those reported for the folate-binding proteins isolated from human leukemia [16, 20] or cultured KB cells [17] with "monoglutamyl" folate derivatives. Only low levels of formyl-substituted tetrahydrofolates (i.e. 5- and 10-formyl tetrahydrofolate, and 5,10-methenyl tetrahydrofolate) were protein-bound in L1210 cells grown in 1 μM [^{3}H]folic acid, even though these derivatives were the most abundant intracellular forms. Essentially all the remaining derivatives were protein-bound, including tetrahydrofolate, 5-methyl tetrahydrofolate, dihydrofolate, and unmetabolized folic acid. Only at higher folic acid concentrations could appreciable unbound levels of these derivatives be detected (Matherly LH, unpublished observations).

Trimetrexate treatment had no significant effect on the level of protein-bound folates in L1210 cells. In the presence of this agent, moreover, the apparent folate binding specificities were preserved. Indeed, because of this specificity, the net effect of drug treatment was an accumulation of a majority of the bound derivatives (folic acid and dihydrofolate) which are incapable of associating with, and transferring, one-carbon units in enzyme reactions. When all of the intracellular folates were considered in this fashion, the relative accumulation of these biosynthetically "inert" forms was diminished markedly.

From these data, a mechanism for antifolate action can be envisaged which includes a key role for a metabolically important fraction of protein-bound folates. Since virtually all of the endogenous cofactors were bound under growth-limiting conditions, protein-bound folates must be available for enzyme reactions required for cell replication. For the 5-methyl and tetrahydrofolate pools this relationship appears to be specific, since in cells grown in 1 μM folic acid, these forms (i) were bound to a greater extent than expected on the basis of their intracellular levels and, upon treatment with trimetrexate, (ii) were disproportionately "depleted" as dihydrofolate accumulated. Conversely, only minor losses occurred in the total intracellular formyl tetrahydrofolates, and, no losses, at all, were detected in the already low levels of these derivatives bound to proteins.

The interconversion of bound folate cofactors appears to be essentially "direct", without significant equilibration with unbound derivatives since neither the level of endogenous [^{3}H]folate binding nor the extent of bound [^{3}H]dihydrofolate accumulation during TMP synthesis was diminished appreciably in the presence of 5-fold elevated pools of unlabeled metabolites of 5-formyl tetrahydrofolate. This lack of equilibration of protein-bound and unbound folates in this experiment may, of course, in part reflect the slow rate of conversion of unlabeled folate metabolites to their long chain polyglutamyl congeners likely required for optimal binding.

Hence, binding of endogenous folates to soluble proteins may "segregate" particular cofactor derivatives and, thereby, contribute to a localized depletion of certain tetrahydrofolate forms when dihydrofolate reductase is inhibited. If protein-bound folates are preferentially used for biosynthetic reactions, this localized effect could have profound metabolic consequences on one-carbon transfer reactions even though the majority of the reduced cofactors are preserved. Since the majority of the protein-bound folates are forms required for the biosynthesis of TMP, serine, and methionine, these processes would be most greatly affected. The conversion to dihydrofolate may still be influenced, of course, by the number of cells in S-phase and, hence, actively synthesizing TMP [3]. Indeed, such cell kinetic considerations may be relevant in explaining the lack of complete conversion of *protein-bound* tetrahydrofolates to dihydrofolate in drug-treated cells.

The finding that formyl tetrahydrofolates required for *de novo* purine biosynthesis were preserved essentially intact in drug-treated cells, despite the extensive depletion of 5-methyl and tetrahydrofolate pools, implies that the equilibration between formyl-substituted and nonformylated folates is slow. Nonetheless, purine nucleotide biosynthesis was inhibited. Clearly, the metabolic basis for the antipurine consequences of antifolate treatment remains an enigma which cannot be assessed simply through measurements of the total levels of the individual intracellular folates.

In conclusion, these studies demonstrate that a large fraction of the intracellular folate cofactors in L1210 cells grown in [^{3}H]folic acid are bound to protein(s) localized primarily in the soluble cellular mileau. While studies are underway to further establish both the identities and detailed metabolic functions of these proteins, these results indicate that the minor redistributions in the levels of total cellular folates which accompany dihydrofolate reductase inhibition by antifolates do not likely reflect the actual causative factors which contribute to the suppression of *de novo* pathways. These findings may also have ramifications beyond those specifically

addressed in this report, relating to antifolate resistance, leucovorin "rescue", and the potentiation of fluoropyrimidine antitumor activity by reduced folates. The latter issue has been considered recently in a preliminary form [37].

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