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Inhibition of Serine Metabolism by Tetrahydrohomofolate in L1210 Mouse Leukemia Cells

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SUMMARY

SCANLON, K. J., A. R. CASHMORE, B. A. MOROSON, R. N. DREYER, AND J. R. BERTINO. INHIBITION OF SERINE METABOLISM BY TETRAHYDROHOMOFOLATE IN L1210 MOUSE LEUKEMIA CELLS. *Mol. Pharmacol.* 19:481–490 (1981).

Tetrahydrohomofolate, a reduced folate analogue, inhibited mouse leukemia L1210 grown *in vitro*. However, it was found to be a substrate for the following enzymes: methylene tetrahydrofolate reductase (EC 1.1.1.68), methylene tetrahydrofolate dehydrogenase (EC 1.5.1.5), 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), and thymidylate synthetase (EC 2.1.1.45). Serine transhydroxymethylase (EC 2.1.2.1) was markedly inhibited by the analogue (I_{50} = 62.5 μM). In whole cell studies, incorporation of [3-^{14}C]serine into both nucleic acids and methionine was significantly inhibited by tetrahydrohomofolate. The uptake of [^{3}H]methionine was also inhibited at concentrations of tetrahydrofolate of 50 μM or higher. The inhibition of serine transhydroxymethylase together with the inhibition of methionine uptake may explain, at least in part, the ability of tetrahydrohomofolate to inhibit growth of L1210 cells.

INTRODUCTION

FHH_4,[3] (NSC 89473), a reduced folate analogue, has an additional methylene group between the C-9 and N-10 atoms of the folate molecule (Fig. 1). The more stable reduced derivative, 5-methyl-tetrahydrohomofolate, will be going into clinical trial shortly. The homofolate analogues have been shown to be effective against bacteria and tumor cell growth (1, 2), especially against lines resistant to methotrexate (3).

Although the site of action of these compounds is still poorly understood, FHH_4 inhibited thymidylate synthetase from bacteria (1, 4) and, to a lesser extent, this enzyme from L1210 cell extracts (5). In L1210 cells, FHH_4 did not block [^{3}H]deoxyuridine incorporation into DNA, indicating that thymidylate synthetase inhibition was not the site of action of this compound (6). More recently, FHH_4 was shown to have substrate activity with thymidylate synthetase from mouse erythrocytes (6), Chinese hamster ovary cells (7), HeLa cells (7) and, to a lesser degree, *Lactobacillus casei* (4). An alternate site of action was proposed by Hakala (2) who reported that this compound affected purine biosynthesis at the 5-aminoimidazole-4-carboxamide ribotide transformylase level in Sarcoma 180 cells.

In this communication, the effect of FHH_4 on several folate coenzymes from extracts of L1210 cells is reported. Evidence is presented to indicate that inhibition of serine transhydroxymethylase activity and methionine uptake could explain, at least in part, the effects of this compound on cellular replication.

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[3] The abbreviations used are: FHH_4, (dl)-5,6,7,8-tetrahydrohomofolate [(dl) refers to a mixture of the natural and unnatural disastereoisomers as a result of the asymmetrical center introduced at C-6 during chemical reduction and does not indicate optical rotation]; HF, homofolate; $N^5CH_3FHH_4$, N^5-methyltetrahydrohomofolate; $N^{11}CH_3HF$, N^{11}-methylhomofolate; H[^{14}C]HO, [^{14}C]formaldehyde; HPLC, high-performance liquid chromatography; TMP synthetase, thymidylate synthetase; FH_4, (dl)-5,6,7,8-tetrahydrofolate; CH_2FH_4, 5,10-methylenetetrahydrofolate; 10-CHO-FH_4, 10-formyltetrahydrofolate; 5,10-CH_2FH_4, 5,10-methylenetetrahydrofolate; Hyp, hypoxanthine; DHFR, dihydrofolate reductase; AICAR, 5-amino-imidazole-4-carboxamide ribotide.

MATERIALS AND METHODS

Chemicals

HF (NSC 79249), FHH_4 (NSC 89473), $N^5CH_3FHH_4$ (NSC 139490), and $N^{11}CH_3HF$ (NSC 164953) were obtained from the National Cancer Institute. [5-^{3}H]dUMP (12.7 Ci/mmole), H[^{14}C]HO (15.6 mCi/mmole), [^{3}H-*methyl*]thymidine (41 Ci/mmole), L-[^{3}H-*methyl*]methionine (15 Ci/mmole), [3-^{14}C]serine (56 mCi/mmole), and [^{3}H]leucine (53 Ci/mmole) were purchased from Amersham Corporation, Arlington Heights, Ill. L-[^{14}C-*methyl*]-

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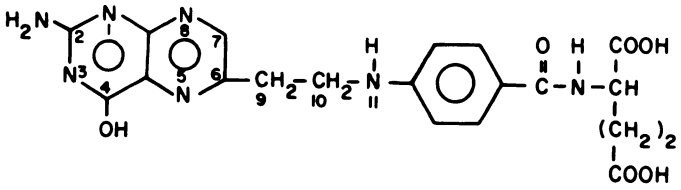

FIG. 1. *Structure of homofolic acid, NSC 79240*

methionine (58 mCi/mmole) was purchased from New England Nuclear Corporation, Boston, Mass.

dUMP, NADP, NADPH, pyridoxal phosphate, FAD, and ATP were purchased from Sigma Chemical Company, St. Louis, Mo. Folic acid was obtained from Nutritional Biological Chemicals, Cleveland, Ohio. Thiodiglycol was purchased from Pierce Chemical Company, Rockford, Ill. All buffers were made in deionized distilled water and filtered through a 0.2-μm GA-8 Metrical membrane (Gelman, catalogue No. 60301). Acetonitrile, distilled in glass, was purchased from Burdick & Jackson, Muskegon, Mich.

Reduction of Folates and Homofolates

Folic acid, homofolate, and $N^{11}CH_3HF$ were reduced by sodium dithionite (8) to the dihydroderivatives, followed by sodium borohydride reduction to the tetrahydrofolate derivatives (9). Purification of the reduced homofolates was accomplished by HPLC. Purity was checked by spectral analysis of the products on a Cary spectrophotometer (Model 15) and HPLC. To minimize oxidation of the reduced compounds during the *in vitro* assays, the compounds were maintained in 50 mM 2-mercaptoethanol, the solution having been purged previously with N_2.

Reduced homofolates used in either tissue culture or for short-term assays with cells were prepared in ascorbic acid-bicarbonate solution (pH 6.0) with a final concentration of ascorbate of 6 μg/ml. This concentration afforded protection for at least 15 min. Higher concentrations inhibited cell growth and macromolecular biosynthesis.[4] The ascorbate solution was prepared fresh daily. Experiments were carried out in dim light to minimize decomposition of FHH_4 and ascorbate.

High-Performance Liquid Chromatography (HPLC)

The modular HPLC instrumentation used was that described by Dreyer (10). A weak anion exchanger, Partisil-10-PAC (Whatman, Inc., Clifton, N. J.), 4.6 × 250 mm, was used for separating the homofolate derivatives. The elution conditions were as follows: linear gradient 0–10% acetonitrile in 0.02 M sodium phosphate buffer, pH 6.4 (v/v) over 30 min; flow rate 0.8 ml/min; and column temperature, 40°. A composite elution profile is shown in Fig. 2. Homofolates were injected singly (1–5 μg/injection). Each migrated as a single peak beyond the initial peak of 2-mercaptoethanol with the exception of $5CH_3FHH_4$, which had an unidentified early eluting peak. The dihydro- and tetrahydrohomofolates could be separated by approximately 1-min difference in retention time only when the two compounds were injected simultaneously. These two compounds could not be resolved when injected with the other derivatives.

The separation of dTMP from [^{14}C]formaldehyde utilized a column 4.5 × 100 mm which had been slurry-packed with BA-X4, a strong anion exchanger of quaternary ammonium ions bonded to polystyrene, cross-linkage of approximately 4%, 7- to 10-μm particles (James B. Benson, Reno, Nev.). The elution mode was linear from 0.1 to 0.4 M ammonium acetate, pH 7.0, over 30 min; column temperature, 50°; and a flow rate 0.8 ml/min. One-minute fractions (800 μl) were collected in an Isco fraction collector (Instrument Specialities, Model 328). To each fraction, 1 ml of water and 10 ml of Aquasol (New England Nuclear Corporation) were added. The samples were counted in a Beckman LSC 7000 with a constant counting efficiency of 92% for ^{14}C throughout the gradient. Recovery of radioactivity from the column was >95%.

Labeled amino acids were separated on a 4.6 × 250 mm stainless steel column which had been slurry-packed with nominally 7.5% cross-linked polystyrene cation exchange resin (HPAN 90, Hamilton, Reno, Nev.). The elution conditions were as follows: freshly filtered mobile phase composed of 0.3 M NH_4ClO_4/0.1 M citric acid/2.0% thiodiglycol, titrated to pH 2.2 with 2 N NaOH (the final pH after filtering was 2.37); flow rate, 1.0 ml/min; column temperature, 55°. The elution profile was monitored at 280 and 230 nm. Retention times for standard amino acids under these conditions were 7.0, 43.0, 67.0, and 123.0 min for serine, methionine, tryptophan, and histi-

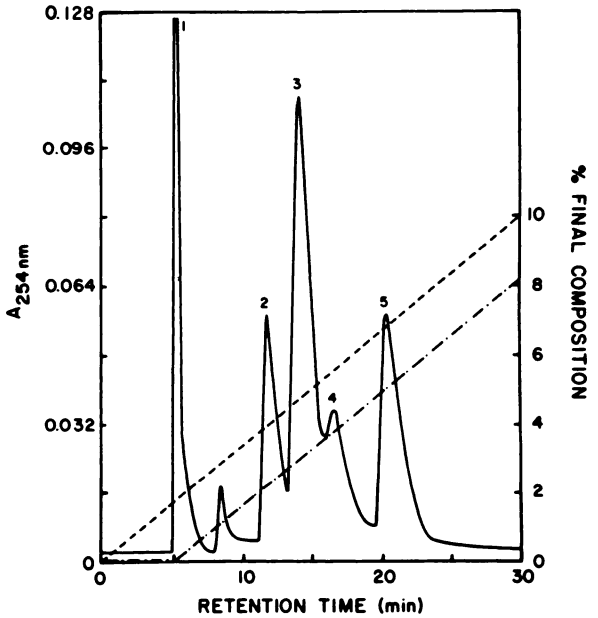

FIG. 2. *The composite elution profile for the homofolate derivatives from a Partisil-10-PAC column*

Each derivative, 1–5 μg, was run individually for determination of homogenity. Elution conditions are given under Materials and Methods. - - -, The gradient profile on the LKB template; -·-·-, the actual gradient at the head of the column. The sample components were as follows: *1*, 2-mercaptoethanol; *2*, homofolate; *3*, di- and tetrahydrohomofolate; *4*, 5-methyltetrahydrohomofolate; and *5*, 11-methylhomofolate. The unmarked peak between *1* and *2* was an unidentified impurity associated with the 5-methyltetrahydrohomofolate.

[4] K. J. Scanlon, A. R. Cashmore, B. A. Moroson, R. N. Dreyer, and J. R. Bertino, unpublished data.

dine, respectively. Fractions (1 ml) were collected and counted for ^{14}C in 10 ml of Aquasol in a Beckman LSC 7000 with 90% counting efficiency. Recovery of radioactivity from the column was >90%.

Sources of TMP Synthetase

Purified TMP synthetase from a dichloromethotrexate-resistant line of *L. casei* was the generous gift of Dr. Henry Blair, New England Enzyme Center, Tufts University Medical School, Boston, Mass. The specific activity was 0.5 μmole/min/mg of protein. L1210 TMP synthetase, specific activity of 0.015 μmole/min/mg of protein, was purified by affinity chromatography as described by Rode *et al.* (11).

Crude Enzyme Extracts

Crude enzyme extracts were prepared by thawing the cultured L1210 cell packs in 50 mM Tris-HCl buffer, pH 7.5, at a density of 25–30 × 10^6 cells/ml. The cells were disrupted by sonic oscillation (Biosonik, Bronwill Scientific). The sonic extract was then centrifuged at 37,000 × *g* for 20 min at 4° in a Sorvall RC-2 centrifuge to remove cell debris. The supernatant was used to assay folate enzyme activities.

TMP Synthetase Assays

Tritium release assay. TMP synthetase was assayed using the procedure of Roberts (12). The standard reaction mixture contained, in a final volume of 40 μl, 1.8 nmoles [5-^{3}H]dUMP (about 3 × 10^7 cpm/μmole); 5.2 nmoles of 5,10-CH_2FH_4; 0.1 μmole of formaldehyde; 6 μmoles of 2-mercaptoethanol; 2 μmoles of NaF; 2 μmoles of phosphate buffer, pH 7.5; and enzyme. The assay for *L. casei* contained 22 mM $MgCl_2$ for optimal enzyme activity. The mixture was incubated for 30 min at 37° and was linear for at least 90 min. The addition of 200 μl of a charcoal suspension in 2 N trichloroacetic acid (Norit, 100 mg/ml) terminated the reaction. The mixture was centrifuged at 1000 × *g* for 5 min and a 100-μl aliquot was added to 3.5 ml of ACS (Amersham) counting fluid and counted in a Beckman LS230. All assays were performed in duplicate. One unit of enzyme activity is defined as the amount of enzyme required to form 1 μmole of TMP/min at 37° under the assay conditions.

[^{14}C]Formaldehyde assay. The L1210 TMP synthetase was assayed using H[^{14}C]HO as the labeled substrate. The standard reaction mixture was similar to that described in the tritium release assay, except that the final volume was 50 μl. The cofactors FH_4 or FHH_4, at 150 μM final concentration, unlabeled dUMP, and labeled formaldehyde (25 nCi/1.6 nmoles) were added to the reaction mixture. Appropriate blanks minus dUMP and minus enzyme were also run. The assay mixture was incubated for 90 min at 37° and was terminated by placing the reaction tubes on ice. The products of the reaction were examined utilizing the BA-X4 column as previously described.

Spectrophotometric assay. TMP synthetase was assayed according to the method of Wahba and Friedkin (13) except that the final concentration of FHH_4 or FH_4 was 0.2 μM and the concentration of formaldehyde was 150 μM.

Other Folate Enzymes

CH_2FH_4 reductase. CH_2FH_4 reductase was assayed as described by Kutzbach *et al.* (14). The complete reaction mixture contained, in a final volume of 0.6 ml, 0.1 ml of enzyme (0.1–0.85 mg of protein); 40 nmoles of potassium phosphate, pH 7.2; 2.0 nmoles of FAD; 160 nM NADPH; 20 nM 2-mercaptoethanol; 1 mM 5,10-CH_2-FH_4 or 5,10-CH_2-FHH_4, generated *in situ* by the addition of 10 mM HCHO and 1 mM reduced folate. After a preincubation period of 5 min the reaction was initiated by the addition of NADPH and incubated at 37°. Enzyme activity was expressed as nanomoles per milligram of protein.

Serine transhydroxymethylase. Serine transhydroxymethylase was assayed as described by Taylor and Weissbach (15). Complete assay systems contained 0.25 mM [3-^{14}C]-L-serine, 0.1 μmole of pyridoxal phosphate, 0.8 μmole of potassium phosphate, and enzyme (0.1 ml, 0.1–0.5 mg of protein) in a total volume of 0.4 ml, pH 7.4. All components except serine were first incubated for 5 min at 37°. Reactions were then initiated by addition of the substrate and were terminated 15 min later with 0.3 ml of 1.0 M sodium acetate, pH 4.5; 0.2 ml of 100 mM formaldehyde and 0.3 ml of 400 mM dimedon [Pfaltz and Bauer, Flushing, N. Y. (in 50% ethanol)] were added in succession. The conical centrifuge tubes were heated for 5 min in a boiling water bath to accelerate formation of the HCHO dimedon derivative. The tubes were then cooled for 5 min in an ice bath before the dimedon compound was extracted by vigorous shaking with 5.0 ml of toluene at room temperature. Centrifugation for 2 min separated the phases, and 3.0 ml of the upper phase were removed for counting. Enzymatic activity was linear for 30 min over a protein range of 0.05–0.4 mg. Radioactivity was counted in Aquasol.

10-CHO-FH_4 synthetase. 10-CHO-FH_4 synthetase was determined as described by Bertino *et al.* (16). The assay mixture contained 50 μmoles of Tris-HCl buffer (pH 7.5), 10 μmoles of mercaptoethanol, 20 μmoles of $MgCl_2$, and enzyme (0.1–0.16 mg of protein) in a total volume of 1.0 ml. After incubation at 37° for 15 min, 0.5 ml of 10% trichloroacetic acid was added and the denatured protein was removed by centrifugation. Acidification with 2 N trichloroacetic acid converted the product, N^{10}-formyltetrahydrofolate, to N^5,N^{10}-methenyltetrahydrofolate, which was estimated by light absorption at 355 nm (ϵ = 22 × 10^6 cm^2/mole). The blank contained all components except formate. Enzymatic activity was linear for 30 min over a protein range of 0.1–0.4 mg. Enzyme activity was expressed as nanomoles of 5,10-methenyl FH_4 per milligram of formed protein.

5,10-CH_2FH_4 dehydrogenase. Mixtures containing 1.0 μmole of *dl*-tetrahydrofolate, 5.0 μmoles of HCHO, 100 μmoles of phosphate buffer (pH 7.5), and 10 μmoles of mercaptoethanol in a volume of 0.5 ml were allowed to stand at room temperature for 5 min to permit the chemical synthesis of N^5, N^{10}-methylenetetrahydrofolate. TPN (0.6 μmole) and the enzyme (0.1 ml, 0.1–0.85 mg of protein) were added to a final volume of 1.0 ml, and the reaction mixture was incubated at 37° for 15 min. Trichloroacetic acid (0.5 ml) was added, and the denatured protein was removed by centrifugation. The amount of N^5,N^{10}-methylenetetrahydrofolate formed

was determined by light absorption at 355 nm (16). Enzyme activity was expressed as nanomoles of 5,10-methenyltetrahydrofolate formed per milligram of protein.

Incorporation of Labeled Precursors into Macromolecules

Logarithmically growing L1210 cells (3×10^5 cells/ml), in Fischer's medium supplemented with 10% horse serum, were incubated with FHH_4 (50 μM) in ascorbate (1.0 μg/ml). After the addition of either [^{3}H]methionine, 4.1 μCi/μmol, or [^{3}H]uridine, 5.0 μCi/μmole; thymidine, 1 μCi/μmole; [^{3}H]leucine, 5.3 μCi/μmole; [^{3}H]methionine, 0.15 μCi/μmole; or [^{14}C]serine, 1.5 μCi/μmoles, to 1 ml of cell suspension, the cells were incubated for 30 min at 37°. The reaction was terminated by chilling the cells (4°). The cells were then centrifuged, the supernatant was removed, and the cell pellet was frozen. To the cell pellet, 100 μl of bovine serum albumin (20 mg/ml) were added with 2.0 ml of 0.3 N $HClO_4$. Following centrifugation, the pellet was washed three times with 0.3 N $HClO_4$ and the supernatant was discarded.

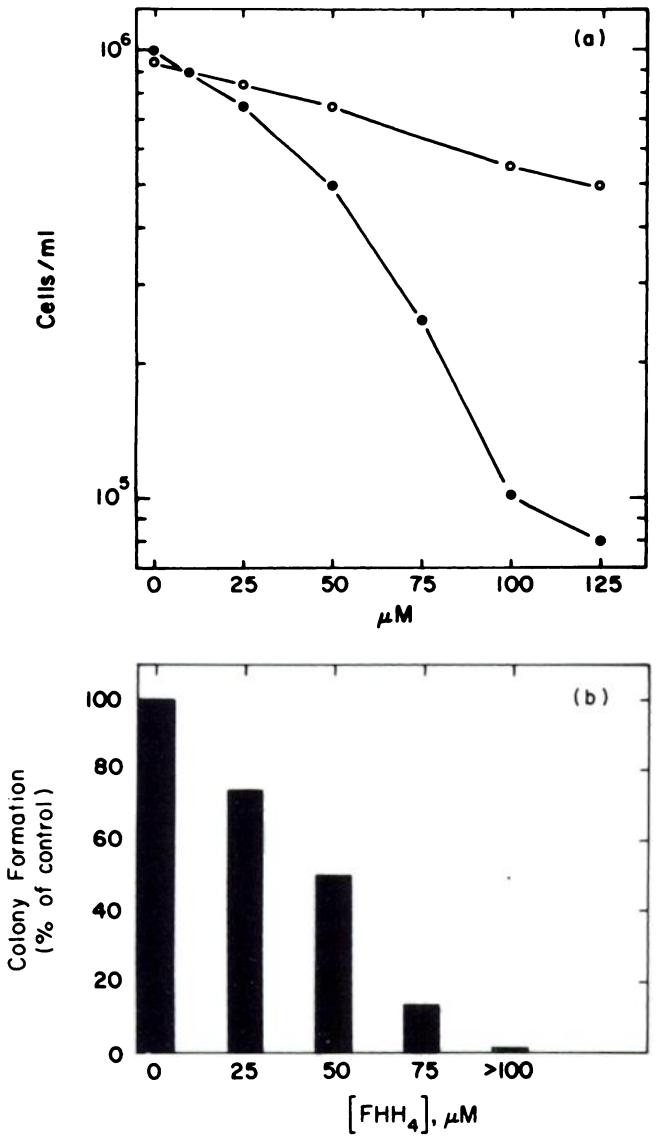

FIG. 3. *ED_{50} determinations and cell viability*

a. Exponentially growing L1210 cells were treated for 48 hr with the indicated concentrations of FHH_4, ●——●; or HF, ○——○. The final concentration of ascorbic acid in the growth media for all experiments was 6.0 μg/ml. Each point represents duplicate determination.

b. Cell viability. L1210 cells were exposed to the indicated concentrations of FHH_4 for 12 hr. Cells were removed from the drug by washing twice in drug-free medium and cloned in soft agar. Colony formation measured on day 10 was expressed as percentage of control.

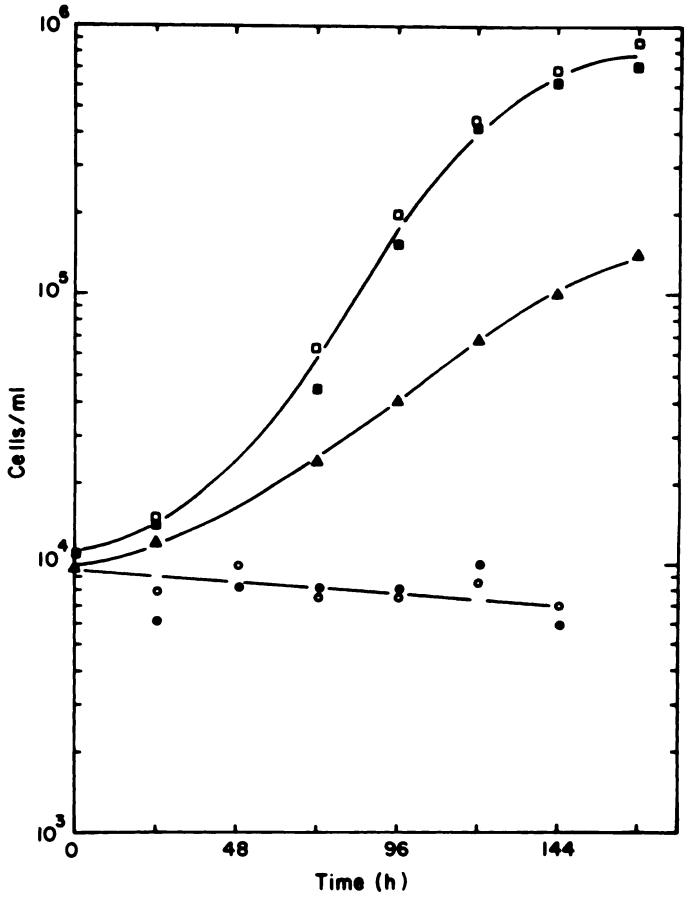

FIG. 4. *Growth requirements for L1210 cells*

□——□, Control; ■——■, cells grown in the absence of folic acid for 3 weeks, resupplemented with folic acid, 10 mg/liter; ▲——▲, cells grown in the absence of folic acid supplemented with 10 μM dThd and 50 μM Hyp; ○——○, cells grown in the absence of folic acid, as above, supplemented with homofolate, 10 mg/liter; ●——●, cells grown in the absence of folic acid, as above, supplemented with 10 μM dThd, 50 μM Hyp, and homofolate, 10 mg/liter.

Protein synthesis was measured as either leucine or methionine incorporation into the perchloric acid-insoluble material. Thymidine incorporation into DNA was measured in the acid/heat-soluble fraction, following treatment of the pellet for 30 min in 1.0 ml of 2 N $HClO_4$ at 80°. Uridine incorporation into RNA was measured into the alkaline/heat-soluble fraction (1 N KOH, 37°, 20 hr).

Serine incorporation into nucleic acids and protein was measured as follows: the 0.3 N $HClO_4$ pellet was alkaline/heat-extracted as above; the resulting pellet was then acid/heat-extracted for DNA. The final pellet was either counted for total labeled protein solubilized in NCS (Amersham) or it was hydrolyzed (in 6 N HCl for 20 hr at 110°) for analysis on HPLC using the cation exchange

system to determine the distribution of the carbon label into amino acids.

Uptake of Methionine or Serine into L1210 Cells

Uptake of radiolabeled methionine or serine in the presence or absence of reduced folate analogues was studied in L1210 cells as previously described by Wohlheuter *et al.* (17). After the radioactivity was added to the cell suspension [methionine (0.5 μCi/μmole) or serine (1.5 μCi/μmole)], 200-μl samples containing 1×10^6 cells were removed at various time intervals, layered at the top of 400-μl plastic Eppendorf microfuge tubes containing 50 μl of 0.6 N $HClO_4$, overlaid by 100 μl of an oil mixture (84 parts of silicone fluid to 16 parts of light mineral oil, by weight, final density 1.034 g/ml). The cells were then centrifuged at 12,000 × *g* for 5 sec. The tubes were frozen and the perchloric acid fraction, sliced off with a razor blade, was solubilized in 0.5 ml of NSC solution (5 parts of NSC, 1 part of water, v/v) overnight at 37°. Samples were counted in 10 ml of toluene-2,5-diphenyloxazole scintillation fluid using a Beckman LSC7000 counter.

Protein Estimation

Samples were estimated for protein using the protein dye assay of Bradford (18).

Growth Requirements for L1210 Cells

L1210 cells maintained in Fischer's medium supplemented with 10% horse serum, had a generation time of approximately 12 hr. These cells did not grow in the absence of methionine. Cells grown in the absence of folic acid (L1210-FA) could grow in medium supplemented with 10 μM dThd and 50 μM Hyp with a generation time of 36 hr. Under these conditions dThd gave optimal growth at concentrations between 0.5 and 10 μM, while higher concentrations of Hyp above 50 μM were inhibitory. The growth of the L1210-FA cells upon supplementation with folic acid returned to the normal rate.

ED_{50} Determinations

Exponentially growing L1210 cells were treated with the indicated compounds for 48 hr and cells were counted with a Coulter counter, Model B. ED_{50} values were determined by plotting cell growth versus inhibitor concentration, and extrapolating to 50% inhibition (19). Several drug concentrations and a control, in duplicate, were used for each determination.

Agar Cloning

Cloning was performed according to the method of Chu and Fischer (20). L1210 cells were treated with FHH_4 for 12 hr, washed free of drug, and then allowed to grow in a drug-free, soft agar medium. Colony formation was measured on day 10 and expressed as percentage of control.

RESULTS

Effect of HF and FHH_4 on cell growth. Both HF and FHH_4 inhibited growth of L1210 cells propagated in Fischer's medium containing 10% horse serum. The ED_{50} for inhibition of growth was 125 μM for HF and 50 μM for FHH_4, as determined by measuring cell growth after 48 hr (Fig. 3a) and by agar cloning (Fig. 3b).

The ability of HF to serve as a folate source for growth was tested. Cells were depleted of folates by growing them in media supplemented with dThd and Hyp for 3 weeks in the absence of folic acid. The cells were then

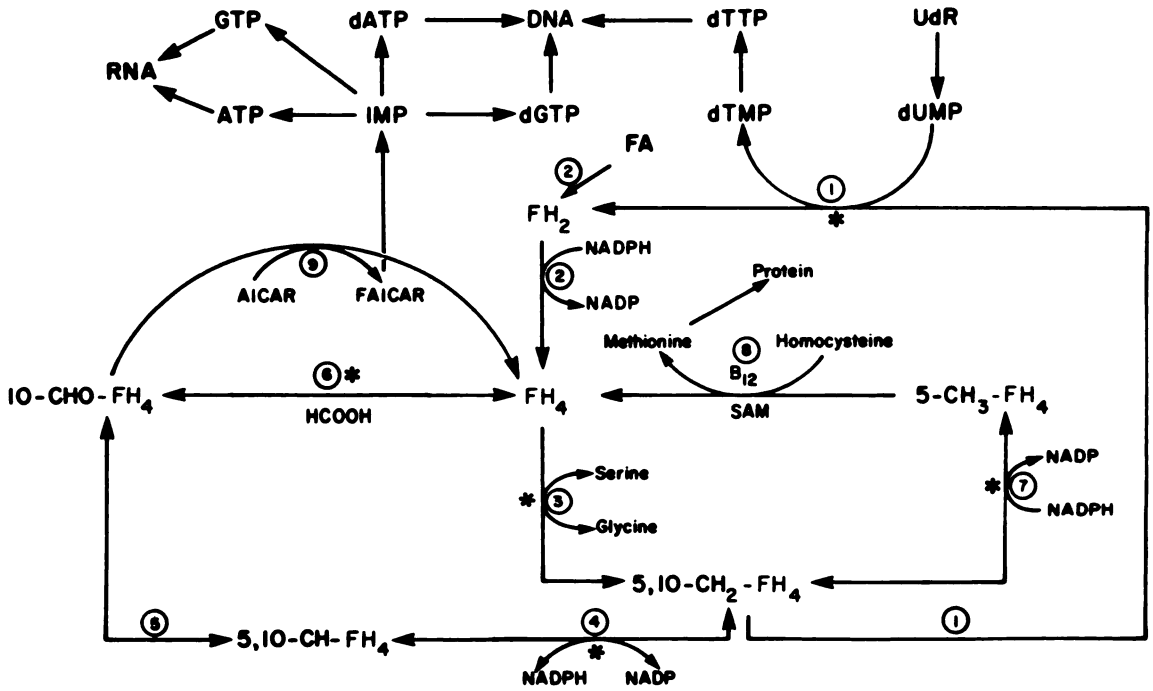

FIG. 5. *Metabolic pathways of folate metabolism*

1, Thymidylate synthetase; *2*, dihydrofolate reductase; *3*, serine transhydroxymethylase; *4*, 5,10-methylenetetrahydrofolate dehydrogenase; *5*, 5,10-methylenetetrahydrofolate cyclohydrolase; *6*, 10-formyltetrahydrofolate synthetase; *7*, 5,10-methylenetetrahydrofolate reductase; *8*, methionine synthetase; and *9*, AICAR transformylase. The *asterisks* indicate the enzymes studied.

transferred to complete Fischer's medium or to Fischer's medium less folic acid supplemented with HF (10 mg/liter). Under these circumstances, complete Fischer's medium restored cell growth to normal, while there was no cell growth observed when homofolate replaced folic acid in the growth media (Fig. 4). Cells propagated in dThd and Hyp without folic acid grew slowly with a generation time of 36 hr. When homofolate was added (10 mg/liter) to cells grown under these conditions (i.e., folate-free media), marked inhibition of cell growth was observed.

FHH_4 as a coenzyme for certain folate-requiring enzymes. The pathways of folate metabolism are shown in Fig. 5. The *asterisks* indicate the enzymes studied. Two of the folate enzymes tested which used CH_2FH_4 as a substrate utilized CH_2FHH_4 equally as well, CH_2FH_4 reductase (Fig. 6a) and CH_2FH_4 dehydrogenase (Fig. 6b). 10-CHO-FH_4 synthetase utilized FH_4 and FHH_4 at approximately the same rate, 2.4 versus 2.6 nmoles/min/mg of protein (Fig. 6c). In contrast, FHH_4 did not have activity as a substrate for serine transhydroxymethylase

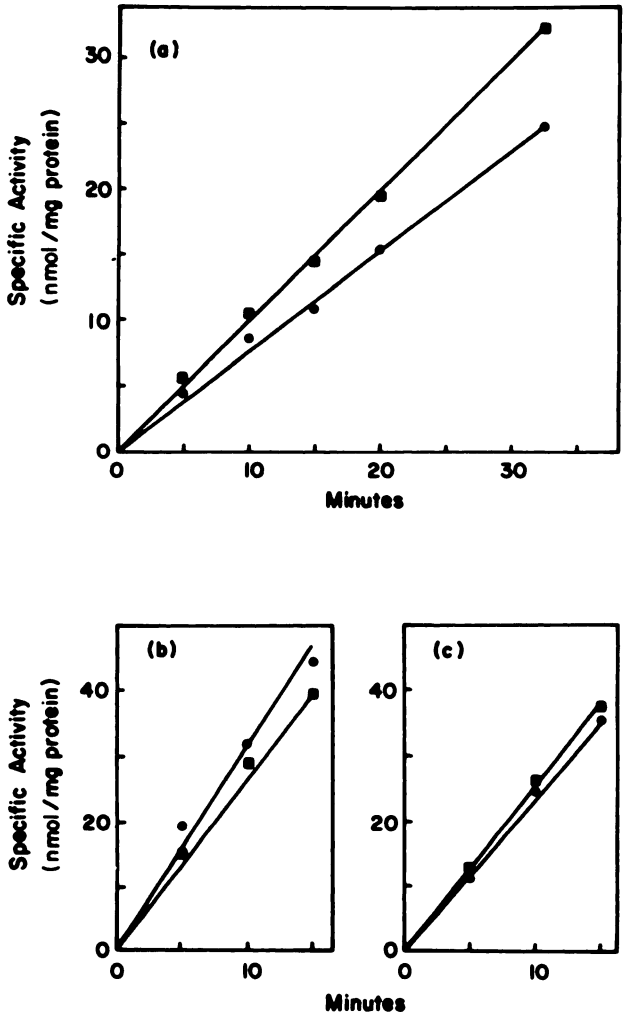

FIG. 6. *Determination of enzyme activity with time for extracts of L1210 cells*

FHH_4 (150 μM) as substrate (■——■) or FH_4 (150 μM) as substrate (●——●). Assay conditions are described under Materials and Methods: a, methylene tetrahydrofolate reductase; b, methylenetetrahydrofolate dehydrogenase; c, 10-formyltetrahydrofolate synthetase.

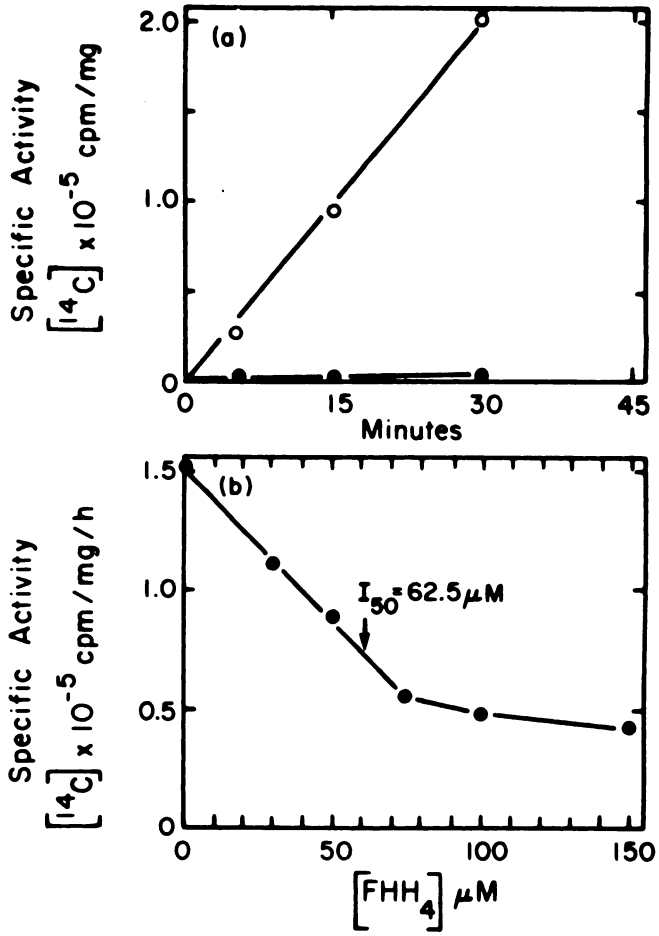

FIG. 7. *Serine transhydroxymethylase activity*

a, FHH_4 (●——●) or FH_4 (○——○) as substrate; b, inhibition of serine transhydroxymethylase by FHH_4 at indicated concentrations. The assay contained 25 μM FH_4 as substrate for the L1210 enzyme.

(Fig. 7a) even when incubated for 60 min with 1 mg of protein. However, the analogue was an inhibitor of this enzyme with an I_{50} of 62.5 μM (Fig. 7b).

The kinetic data for TMP synthetase from L1210, shown in Fig. 8a, yielded a $K_{m(app)}$ of 66 μM for (dl)FH_4 and 45 μM for (dl)FHH_4 with approximately a 2.5-fold higher V_{max} for the latter. Linear plots were obtained for initial velocity studies with 5,10-CH_2FH_4 as the variable substrate with changing fixed concentrations of FHH_4 as inhibitor (Fig. 8b). All lines intersected to the *left* of the *1/V* axis and above the *1/S* axis, indicating that FHH_4 was a noncompetitive inhibitor of the enzyme. Replots of the slopes and intercepts versus inhibitor concentration revealed that the inhibition was *S*-hyperbolic-*I*-hyperbolic. Nonlinearity was confirmed by the plots of *1/V* versus inhibitor concentration at constant substrate concentrations. K_i slope and K_i intercept of 42 and 26 μM, respectively, were determined from 1/Δ slope and 1/Δ intercept versus 1/inhibitor concentration replots (21).

The bacterial TMP synthetase had only 6% of the activity at saturating concentrations of FHH_4 as compared with that of FH_4. The oxidized homofolate compounds or the reduced 5-methyl and 11-methyl homofolates had little or no activity (<1%) as substrates for the TMP synthetase enzymes studied (Table 1). These re-

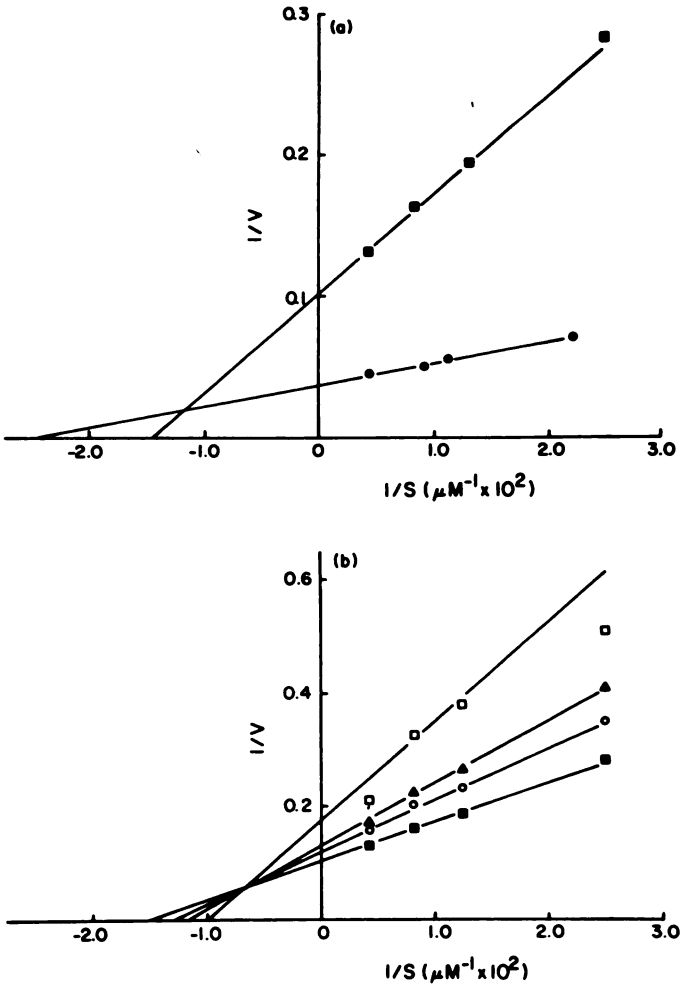

FIG. 8. *Initial velocity studies*

a, For the conversion of dUMP to dTMP by L1210 TMP synthetase as determined by the tritium release assay, using FH_4 as substrate (■——■) or FHH_4 as substrate (●——●). Each point represents the average of at least two determinations. b, With 5,10-CH_2FH_4 as the variable substrate (40–240 μM) at varying fixed concentrations of FHH_4. The concentrations of FHH_4 were as follows: ■——■, control, 0 μM, ○——○, 5 μM; ▲——▲, 10 μM; and □——□, 50 μM. The enzyme concentration was 0.08 nmole/min/mg of protein. Velocity is expressed ass counts per minute × 10^{-3}/15 min. Each point represents the average of at least two determinations.

sults were confirmed using the spectrophotometric assay of Wahba and Freidkin (13).

To demonstrate conclusively that FHH_4 could be used as a substrate, H[^{14}C]HO was used in the assay in the presence of L1210 TMP synthetase. The product of the assay was analyzed on HPLC using the BA-X4 column as described under Materials and Methods. Chromatography of the assay mixture minus enzyme (Fig. 9a) indicated that the labeled formaldehyde, as represented by the *hatched area*, eluted early, while the elution position of dTMP is represented by peak *2*. In the absence of dUMP (Fig. 9b), about 5% labeled thymidylate was formed. This higher blank may be due to the residual amount of dUMP present when the enzyme was purified by affinity chromatography (11). In the presence of the natural substrate, FH_4, about 60% of the total H[^{14}C]HO was converted to [^{14}C]thymidylate monophosphate (Fig. 9c); when FHH_4 was the substrate, about 25% of the total was converted to thymidylate (Fig. 9d).

Effect of FHH_4 on macromolecular synthesis. Preincubation of FHH_4 with L1210 cells for 30 min had very little effect on subsequent DNA or RNA synthesis, but had a greater effect on the protein synthesis (Table 2). Thymidine incorporation into DNA was stimulated slightly by the presence of low levels (less than 60 μM) of FHH_4. Inhibition of RNA synthesis by FHH_4, measured by the incorporation of [^{3}H]uridine into alkaline heat-soluble material, was essentially nil. Protein synthesis as measured by [^{3}H]leucine incorporation into acid-insoluble material was inhibited by 35% at 100 μM FHH_4. The methionine incorporation was markedly affected at lower levels (58% at 20 μM FHH_4).

Examination of the effect of FHH_4 on serine and methionine incorporation into protein revealed that 50 μM FHH_4 had little effect on the uptake of serine into L1210 cells (Fig. 10a). Under these conditions, serine was taken up rapidly within 5 sec and during the next 10 sec there was a slow decrease in the intracellular level of the amino acid. Methionine, on the other hand, was taken up at a much slower rate, and reached equilibrium at 90 sec (Fig. 10b). The uptake of methionine by FHH_4-treated cells was markedly decreased (by 75%) at 90 sec and had not reached equilibrium at this time point.

[β-^{14}C]serine incorporation into macromolecules was studied in the absence and presence of varying concentrations of FHH_4 (Table 3). In untreated cells, after 30 min, 90% of the serine incorporated was into the acid-insoluble material (protein), and 10% was in the nucleic acid fraction (heat-soluble material). Increasing concentrations of FHH_4 from 10 to 100 μM progressively decreased the percentage of serine incorporation into protein, and into nucleic acids by 70% (Table 3). Hydrolysis of the protein and analysis by HPLC revealed approximately 25% of the [^{14}C]serine label was incorporated into methionine and 66% remained unmetabolized in the control cells (Table 4). For FHH_4-treated cells (50 μM), incorporation of the radiolabel into methionine was markedly inhibited.

DISCUSSION

Sarcoma 180 cells were killed equally well with either the oxidized or the reduced form of HF (ED_{50} = 10–15 μM) (2). In contrast, L1210 cells were less sensitive to HF (ED_{50} = 125 μM), but showed a greater sensitivity to

TABLE 1

Comparison of homofolate derivatives as substrates for TMP synthetase

TMP synthetase from *Lactobacillus casei* (5.1 nmoles/min/mg of protein) and from L1210 (0.08 nmole/min/mg of protein) was assayed for activity using the tritium release assay as described under Materials and Methods. The final concentration of the homofolate substrates was 150 μM. Each assay was run in duplicate. Activity is expressed as percentage of control, FH_4 as substrate.

Source of TMP synthetase	Substrate			
	HF	FHH_4	N^5-CH_3FHH_4	N^{11}-CH_3FHH_4
L. casei	<1	6	<1	<1
L1210	<1	51	<1	<1

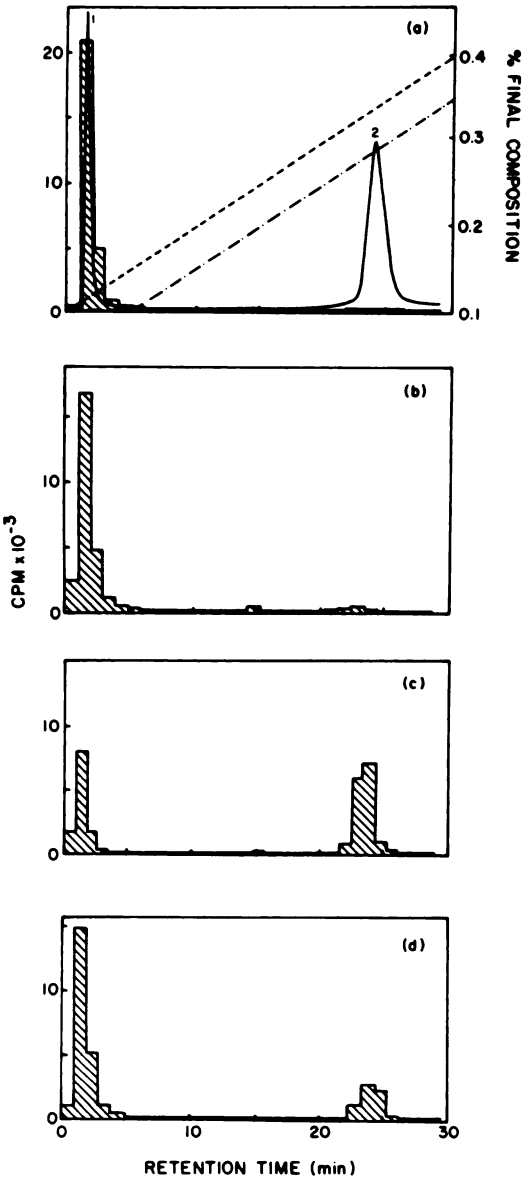

FIG. 9. *Product analysis of L1210 TMP synthetase assays on a BA-X4 column*

The gradient conditions are given under Materials and Methods. – – –, The gradient profile on the LKB template; -·-·-·, the gradient at the head of the column. The sample components were: *1*, 2-mercaptoethanol, coeluting with [^{14}C]formaldehyde; *2*, dTMP. The complete assay mixture contained 1.8 nmoles of dUMP, 5.2 nmoles of folate source, 0.1 μmole of formaldehyde, 6 μmoles of 2-mercaptoethanol, 2 μmoles of NaF, 2 μmoles of sodium phosphate buffer (pH 7.5), and enzyme. a, minus enzyme; b, minus exogenous dUMP; c, complete system with FH_4 as substrate; and d, complete system with FHH_4 as substrate.

FHH_4 (ED_{50} = 50 μM). The low concentrations of ascorbate used (6 μg/ml) may have been insufficient for complete stability of FHH_4 so that these ED_{50} data may underestimate its potency. The differences between the oxidized and reduced homofolate may be due to differences in uptake or the limited ability of the cells to reduce homofolate with dihydrofolate reductase (22–24).

The mechanism of action of HF in bacterial systems proposed by Goodman *et al.* (1) was initial reduction to FHH_4 with subsequent inhibition of TMP synthetase. The inhibitory effects of HF could be reversed by supplementing the growth medium with dThd (11). This reversal phenomenon was not observed by Hakala (2) using Sarcoma 180 cells, and a different mechanism of action involving purine biosynthesis was suggested. The lack of inhibition of dUrd incorporation into DNA (6) is another argument against TMP synthetase as the site of action of this inhibitor in mammalian cells.

The present studies show that CH_2FHH_4 was utilized as a substrate by the L1210 TMP synthetase ($K_{m(app)}$ = 45 μM) and was an *S*-hyperbolic-*I*-hyperbolic noncompetitive inhibitor with respect to 5,10-CH_2FH_4. This type of inhibition has been previously demonstrated by Dolnick (25) for human TMP synthetase using 5-$CHOFH_4$ as inhibitor. The hyperbolic functions are indicative of partial inhibition of two binding sites on the enzyme. The K_i data suggest that FHH_4 binds more tightly to free enzyme than to the enzyme-substrate complex (21).

Examination of several other folate enzymes demonstrated that FHH_4 had substrate activity for CH_2FH_4 reductase, CH_2FH_4 dehydrogenase, and 10-CHO-FH_4 synthetase. 5-CH_3FHH_4 has been shown to have substrate activity for methionine synthetase in Chinese hamster ovary cells (7). In contrast, FHH_4 was not a substrate, but rather an inhibitor, of serine transhydroxymethylase (I_{50} = 62.5 μM) from L1210 cells.

Serine catabolism has been shown to be an essential pathway for methionine biosynthesis in bacterial (26), rat liver (27), and human systems (28). The primary route for methionine biosynthesis is through serine transhydroxymethylase, a major source of one-carbon units for the cells (16, 28). By transfer of the β-carbon from serine to FH_4 via serine transhydroxymethylase, 5,10-CH_2FH_4 may be converted to 5-CH_3FH_4, to coenzyme for methionine synthetase (Fig. 5). In L1210 cells, 90% of 5-

TABLE 2

Inhibition of macromolecular synthesis by FHH_4 in L1210 cells

L1210 cells (3 × 10^5/ml) were incubated without and with FHH_4 for 30 min; the cells were then incubated with the radioactive precursors for an additional 30 min at 37°. After chilling the cells to terminate the reaction, the mixture was centrifuged, the supernatant was removed, and the cell pellets were frozen. Albumin (2 mg) and 1.0 ml of 0.3 N $HClO_4$ were added to this pellet. Following centrifugation, the perchloric acid precipitate was processed as described under Materials and Methods. Ascorbate was added (1 μg/ml) to stabilize FHH_4 (any higher concentration inhibited incorporation of the label). The assays were run in duplicate. The values in parentheses indicate the counts per minute incorporated per 10^6 untreated cells.

Precursor	[FHH_4], μM					
	0	10	20	40	60	100
% of control						
Acid-insoluble (protein)						
[^{3}H]Leucine	100 (77,902 cpm)	96	—[a]	80	77	65
[^{3}H]Methionine	100 (9,636 cpm)	108	42	—	33	25
Acid/heat-soluble,						
[^{3}H]thymidine	100 (140,904 cpm)	102	108	123	105	99
Alkaline/heat-soluble,						
[^{3}H]Uridine	100 (36,762 cpm)	111	100	93	—	85

[a] —, Not determined.

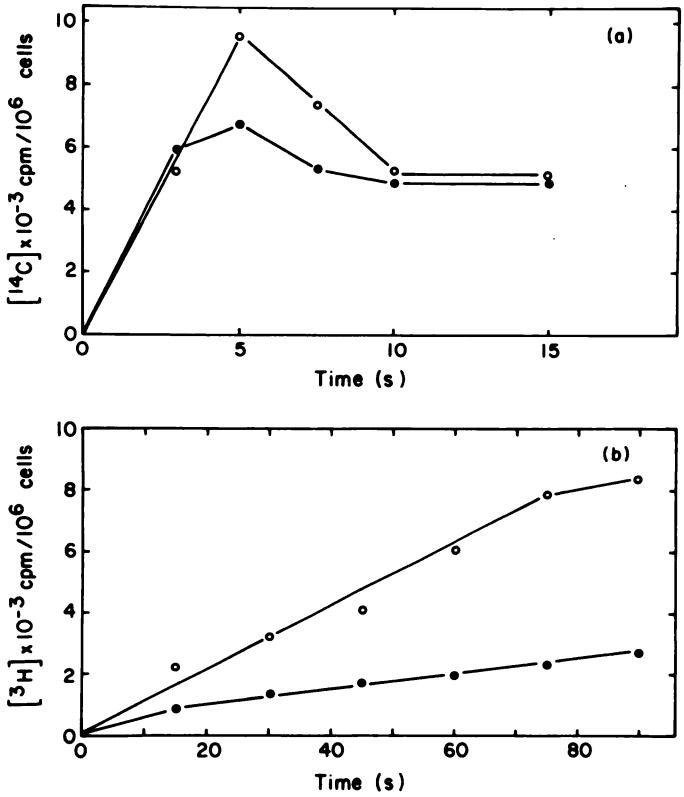

FIG. 10. *Uptake of labeled amino acids into L1210 cells*

Cells (10^6) were incubated with [^{14}C]serine, 5.6 μCi/μmole (a), or [^{3}H-*methyl*]methionine, 0.5 μCi/μmole (b), in Fischer's medium. Aliquots were removed at the indicated time points and processed through a discontinuous gradient. After centrifugation, the perchloric acid fraction, sliced off with a razor blade, was solubilized in NCS solution overnight at 37°. Samples were counted in toluene-2,5-diphenyloxazole scintillation fluid: ○——○, control; ●——●, 50 μM FHH_4.

[^{14}C]methyl of 5-CH_3FH_4 was converted to methionine during the first 10 min of incubation (29). Inhibition of serine transhydroxymethylase by FHH_4 would limit the catabolism of serine, ultimately causing a reduction in the amount of methionine available for any methionine-rich essential proteins.

L1210 cells have been shown to be methionine auxotrophs (30). Even in the presence of high concentrations of exogenous methionine (0.7 mM), the cytotoxic effect of FHH_4 may be explained in part by (a) the inhibition of serine transhydroxymethylase, (b) the inhibition of uptake of methionine into L1210 cells; and (c) the inhibition of the incorporation of [^{14}C]serine into methionine and nucleic acids.

TABLE 3

Inhibition by FHH_4 of [β-^{14}C]serine incorporation into acid-precipitable material in L1210 cells

L1210 cells (2×10^5 cells/ml) were incubated without or with FHH_4 for 30 min; the cells were then incubated for an additional 30 min at 37° with [β-^{14}C]serine. The cells were chilled, washed, and processed through the perchloric acid step as described in Table 2. This acid pellet was then heat-extracted for nucleic acids as described under Materials and Methods. The resulting pellet was counted for total protein. Inhibition is expressed as percentage of control (without FHH_4 in the assay). The values in parentheses indicate the cpm per 10^6 cells incorporated by untreated cells.

Fraction	[FHH_4] μM					
	0	10	20	40	60	100
	% of control					
Acid-insoluble (protein)	100 (16,183 cpm)	71	66	56	—[a]	28
Heal-soluble (nucleic acids)	100 (1,868 cpm)	90	70	60	40	30

[a] —, Not determined.

TABLE 4

HPLC analysis of radiolabeled amino acids from [β-^{14}C]serine after hydrolysis of protein in L1210 cells

L1210 cells were preincubated for 30 min without or with FHH_4 (50 μM) followed by addition of [β-^{14}C]serine and further incubation for 30 min. Cells were chilled, washed, and processed through the perchloric acid step as described in Table 2. The heat/acid-insoluble material was hydrolyzed for 20 hr at 110° in 6 N HCl. The solution was lyophilized and taken up in mobile phase. The amino acids were separated on HPLC using the cation exchange system described under Materials and Methods. The values in parentheses indicate the counts per minute per 10^6 cells incorporated in each sample.

Amino acid	Control (10,350 cpm)	50 μM FHH_4 (10,609 cpm)
	%	%
Unknown (void volume)	5.3	4.5
Serine	66.0	92.0
Glycine and/or glutamate	2.9	2.3
Methionine	25.5	0.1
Histidine	0.2	0.9
Tryptophan	0.1	0.3

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