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Isohomoaminopterin and Isohomofolic Acid

Synthesis and Basic Biochemical and Biological Properties

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SUMMARY

Two new analogues of folic acid, isohomofolic acid and isohomoaminopterin, have been synthesized. Both compounds were prepared by reductive condensation of corresponding pteridine-6-carboxaldehydes with *p*-aminomethylbenzoylglutamic acid diethyl ester. Both compounds proved to be inhibitors of both HeLa and Sarcoma 180 cells in tissue culture, and also inhibited the growth of *Lactobacillus casei* and *Streptococcus faecium*. Isohomoaminopterin penetrated into Sarcoma 180 cells, whereas uptake of isohomofolic acid was not observed. The effects of both compounds on formiminotransferase, tetrahydrofolate formylase, dihydrofolate reductase, thymidylate synthetase, and the biosynthesis of inosinic acid were tested. Of all these enzymes only dihydrofolate reductase was inhibited; the inhibition was competitive with respect to folate and dihydrofolate. The inhibition by isohomoaminopterin (K_i = 12 nM with folate at pH 5.5) was two orders of magnitude greater than that by isohomofolate.

INTRODUCTION

In order to prepare specific inhibitors of folate-interconverting enzymes, especially dihydrofolate reductase and thymidylate synthetase, several compounds have been synthesized in which the bridge linking the pteridine and *p*-aminobenzoate moieties is structurally different from the analogous bridge in the coenzyme, dihydrofolic acid.

The first compounds of this group synthesized were homofolic acid and its tetrahydro derivative (1). In an *Escherichia coli* system homofolic acid was reduced by folate reductase as an artificial substrate, and the resulting tetrahydro derivative appeared to be a strong competitive inhibitor of thymidylate synthetase from the same source (2). In contrast, homofolate inhibited the transport of folate into *Streptococcus faecium* (3) and was an artificial substrate for dihydrofolate reductase.

In previous papers (4, 5) we reported that 11-azahomoaminopterin was a weak inhibitor of folate reductase and that its tetrahydro derivative exerted different inhibitory effects on thymidylate synthetases from various sources.

Similar results showing the importance of the aliphatic bridge for binding of the analogues to folate reductase and thymidylate synthetase were obtained with a series of 2,4-diamino-6-arylalkylpyrimidines prepared by Baker (6). These compounds possess a selective group for alkylation of folate reductase and thymidylate synthetase. Although they showed irreversible alkylating activity toward folate reductase and thymidylate synthetase, in contrast to their effects on enzymes from liver and L1210 leukemia cells, their structures were quite different from folate and tetrahydrofolate. This finding precludes definite statements

concerning the binding sites of these analogues on the enzyme.

In this paper folate analogues have been studied, in which the basic structure of the natural substrate remained unchanged and only the chain linking the pteridine and aromatic nuclei was modified. The synthesis and basic biochemical and biological properties of two antifolates, isohomofolic acid and isohomoaminopterin, are the main subject of this report.

EXPERIMENTAL PROCEDURE

Chemicals

2 - Amino - 4 - hydroxypteridine - 6 - carboxaldehyde. This compound was prepared according to Weygand *et al.* (8). *p*-Aminomethylbenzoic acid was kindly provided by Dr. Jarý, Laboratory of Monosacharides, Institute of Chemical Technology, Prague. Other chemicals were of commercial origin.

N - (o - Nitrophenylsulfenyl) - p - aminomethylbenzoic acid. *p*-Aminomethylbenzoic acid (13 g, 85 mmoles) was suspended in 70 ml of water and dissolved by addition of 2 N NaOH. After the addition of 70 ml of dioxane, a solution of 18 g (95 mmoles) of *o*-nitrophenylsulfenyl chloride in 110 ml of dioxane was added dropwise with vigorous mixing at 20°. The pH of the reaction mixture was maintained at about 8 by the continuous addition of 2 N sodium hydroxide.

A yellow precipitate was gradually formed during nitrophenylsulfenylation. The reaction mixture was diluted with 870 ml of water, and the precipitate was removed by suction and discarded. The pH of the filtrate was lowered to 2 with dilute sulfuric acid, and the yellow precipitate was removed by suction, washed with water, and dried in a desiccator; yield, 20–28 g.

$C_{14}H_{12}N_2O_4S$ (mol wt 304.33)

Calculated:

C 55.25, H 3.97, N 9.21, S 10.59

Found:

C 55.14, H 3.95, N 9.37, S 10.60

p-Aminomethylbenzoylglutamic acid diethyl ester hydrochloride. *N*-(*o*-Nitrophenylsulfenyl)-*p*-aminomethylbenzoic acid (7.5 g, 24.7 mmoles) was suspended by gradual mixing with 750 ml of dry ethyl acetate, and the suspension was heated until the compound was nearly completely dissolved. A small amount of unsolved residue was filtered off.

α,γ-Diethyl glutamate (6.25 g, 30.8 mmoles) dissolved in ethyl acetate was added to the filtrate. Then 5.2 g (23 mmoles) of solid dicyclohexylcarbodiimide were added, and the solution was chilled at 4° in a moisture-free atmosphere for 2 days. The resulting crystals of dicyclohexylurea were filtered off and washed with ethyl acetate. The filtrate was washed in a separatory funnel successively with 100 ml of water, three 100-ml portions of 0.5 N H_2SO_4, 100 ml of water, three 100-ml portions of saturated aqueous sodium bicarbonate, and finally three times with 100 ml of water. The ethyl acetate solution was dried with Na_2SO_4, and the solvent was removed in a flash evaporator. The remaining yellow, viscous oil was mixed with 80 ml of ethanol saturated with HCl. The insoluble part was removed by filtration and extracted with pure hot ethanol. The filtrate and extract were each poured into 12 volumes of ether, and the white precipitate of the desired product was filtered by suction and washed with ether. The product was purified by dissolution in ethanol and precipitation with ether. Yield, 2.0–2.5 g.

Chromatographic analysis was performed on Whatman No. 3 paper with butanol–ethanol–water (4:2:1, v/v), and the products were detected with ninhydrin. The product consisted from a mixture of *p*-aminomethylbenzoylglutamate diethyl ester, the corresponding α- and γ-monoesters, and free *p*-aminomethylbenzoic acid in varying ratios. This crude product was used for further synthesis.

Isohomofolic acid diethyl ester. Sodium bicarbonate (2 g) and 4 g of sodium acetate were dissolved in 200 ml of water, and 500 mg of 2-amino-4-hydroxypteridine-6-carboxaldehyde were suspended in this solution. A solution of 700 mg of *p*-aminomethylbenzoylglutamic acid diethyl ester hydrochloride in 200 ml of ethanol and 400 mg of Adams catalyst (PtO_2) was added. After approximately 180 ml of hydrogen had been consumed by reductive condensation, plati-

num was removed by filtration in a carbon dioxide-filled chamber. I_2, 6.3 g/500 ml of 1.25% KI, was gradually added to the filtrate, until iodine reduction was no longer apparent (starch reaction). Part of the crude product precipitated as a dark brown material. The entire suspension was concentrated under vacuum to approximately 10 ml and immediately hydrolyzed.

Isohomofolic acid. A solution of sodium hydroxide was added to the suspension of crude isohomofolic acid diethyl ester, yielding a final concentration of 0.1 N. After heating for 3 min at 100°, the hydrolysate was removed by filtration and the filtrate was acidified to pH 3–4 with concentrated hydrochloric acid. A yellow-brown precipitate, representing the first yield of crude isohomofolic acid, was separated by filtration and washed with water, ethanol, and ether.

As the insoluble residue remaining after alkaline hydrolysis contained a large amount of nonhydrolyzed isohomofolic acid diethyl ester, it was dissolved in ethanol–water (10:1); 2 N NaOH was added to a final concentration of 0.1 N, and the hydrolysis was repeated. The filtrate was acidified to pH 3–4 and evaporated to 10 ml. This second isohomofolic acid product was separated by filtration and dried with ethanol and ether as described above.

The mother liquors from both purification steps still contained considerable amounts of the product. Therefore activated charcoal was added to the combined mother liquors until the yellow fluorescence disappeared. The charcoal was removed by centrifugation, washed with water, and extracted with two 50-ml portions of ethanol and 2.5% aqueous ammonia (1:1 by volume). The yellow-green fluorescent extract was freed of charcoal by centrifugation and evaporated to dryness. This third product was combined with the first two for final purification.

Isohomofolic acid was purified twice by preparative chromatography on sheets of Whatman No. 3 paper, first with 0.05 N aqueous ammonia and then with 0.1 N acetic acid–2-propanol (3:7). After each run the strips containing the desired product were eluted with water and freeze-dried.

The lyophilized product was dissolved in a minimal amount of 0.05 N NH_3, filtered, mixed with 10 volumes of concentrated acetic acid, and filtered again. Isohomofolic acid was precipitated by addition of 12–13 volumes of ether. The precipitate was separated by centrifugation, washed with ether, and dried.

$C_{20}H_{21}N_7O_6 \cdot \frac{1}{2}H_2O$ mol wt 464.44

Calculated:

C 51.72, H 4.77, N 21.11

Found:

C 51.30, H 4.59, N 23.08

The pathway of synthesis of isohomofolic acid is depicted in Fig. 1.

Isohomoaminopterin. Isohomoaminopterin was prepared in the same way as isohomofolic acid, starting with 2,4-diaminopteridine-6-carboxaldehyde and *p*-aminomethylbenzoyldiethylglutamate hydrochloride. The procedure and purification steps were essentially the same as for isohomofolic acid. However, since isohomoaminopterin is more soluble in water than isohomofolic acid, the latter could be crystallized from hot water without considerable loss. When isohomoaminopterin was crystalized, a considerable fraction of the product remained in the mother liquors.

$C_{20}H_{21}N_8O_5 \cdot 3H_2O$ (mol wt 508.49, trihydrate)

Calculated:

C 47.24, H 5.55, N 22.05

Found

C 47.80, H 5.61, N 20.73

Enzyme Inhibition Studies

The effects of all substances on the activity of tetrahydrofolate formylase and formiminotransferase were studied by spectrophotometric methods described earlier (9, 10). Purine biosynthesis was determined as [^{14}C]formate incorporation into inosinic acid (9). A 5% aqueous extract of acetone powder prepared from pigeon liver was employed for checking the activities of all these systems.

Reduction of folate was assayed by determination of diazotizable amine (11) and

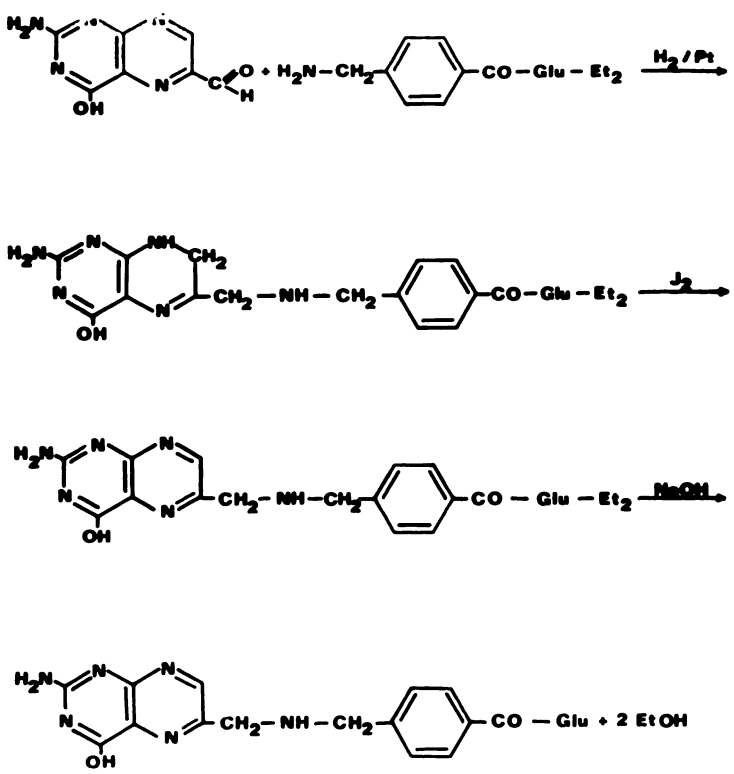

FIG. 1. *Synthesis of isohomofolic acid*

dihydrofolate reduction, by the decrease in absorbance at 340 nm. A 30–65% saturated ammonium sulfate fraction of mouse liver homogenate was employed as the enzyme preparation.

Thymidylate synthetase was investigated by a modification of the spectrophotometric method of Wahba and Friedkin (12) described previously (13). As the enzyme source, an ammonium sulfate fraction (50–60% saturation) of calf thymus extract was employed. Before ammonium sulfate fractionation the crude thymus extract was freed of nucleic acids by precipitation with 0.25 volume of 5% streptomycin sulfate.

Tissue culture procedures were the same as described previously (5).

Penetration Studies

The penetration of isohomoaminopterin and isohomofolic acid was determined using Sarcoma 180 ascitic cells. The tumors were transplanted every 7 days using 0.2 ml of tumor cell suspension diluted with an equal volume of 5.3% glucose solution, into 6–10-week-old male SPF-H mice. The ascitic fluid was withdrawn into 10.7 mM sodium phosphate buffer, pH 7.4, containing 95 mM NaCl, 6.1 mM KCl, and 10 mM EDTA. The suspension was centrifuged, and the concentration of cells was adjusted to 15% with the same buffer. Each tube contained 1.8 ml of the cell suspension and 0.2 ml of the isohomofolic acid or isohomoaminopterin solution, both were added after 10 min of incubation at 37°. At intervals the cells were cooled and washed three times with ice-cold buffer. The pellet was extracted three times for 5 min each, using 1 ml of 0.1 M citrate buffer, pH 5.5, in a boiling water bath. The uptake of isohomofolic acid and isohomoaminopterin was determined from the fluorescence in the extract, using a Shimadzu spectrophotometer with a fluorescence accessory. The concentration of the compound in the medium was 0.134 mM, and uptake was determined after 40 min of incubation.

RESULTS

Synthesis

Both isohomoaminopterin and isohomofolic acid are yellow, microcrystalline powders, slightly soluble in water and easily soluble in alkali, forming yellow-green fluorescent salts.

The absorption spectrum of isohomofolic acid showed peaks at 255 and 385 nm in 0.1 N NaOH and at 229, 278, 333, and 391 nm in 0.1 N HCl (Fig. 2A). Isohomoaminopterin displayed absorption maxima at 249, 261, and 410 nm in 0.1 N NaOH and at 255 and 400 nm in 0.1 N HCl (Fig. 2B).

The fluorescence spectrum of isohomofolic acid showed a maximum at 488 nm at pH 12 and at 474 nm at pH 2, using excitation at 254 nm (Fig. 3A). Isohomoaminopterin exhibited maximal fluorescence at 494 nm in 0.1 N NaOH and at 474 nm in 0.1 N HCl at the same wavelength of excitation (Fig. 3B).

The structures of both compounds were confirmed by both elemental and chromatographic analysis of the products obtained by hydrolysis with 0.5 N HCl and 0.5 N NaOH at 100° for 4 hr. In both acidic and alkaline media with isohomofolic acid, the glutamate residue was predominantly split off. Therefore isohomopteroic acid appeared as the main product of hydrolysis. Isohomoaminopterin gave isohomofolic acid as the product of hydrolysis; 4-aminoisohomopteroic acid was also detectable. Alkaline hydrolysis proceeded more rapidly than acidic but gave principally the same products. In addition, an unidentified pteridine was formed, which was detected using 0.05 N aqueous ammonia as the solvent.

However, in a butanol–acetic acid–water mixture (4:1:4 by volume), several compounds reacting with ninhydrin were detected, i.e., glutamate, *p*-aminomethylbenzoate, and *p*-aminomethylbenzoylgluta-

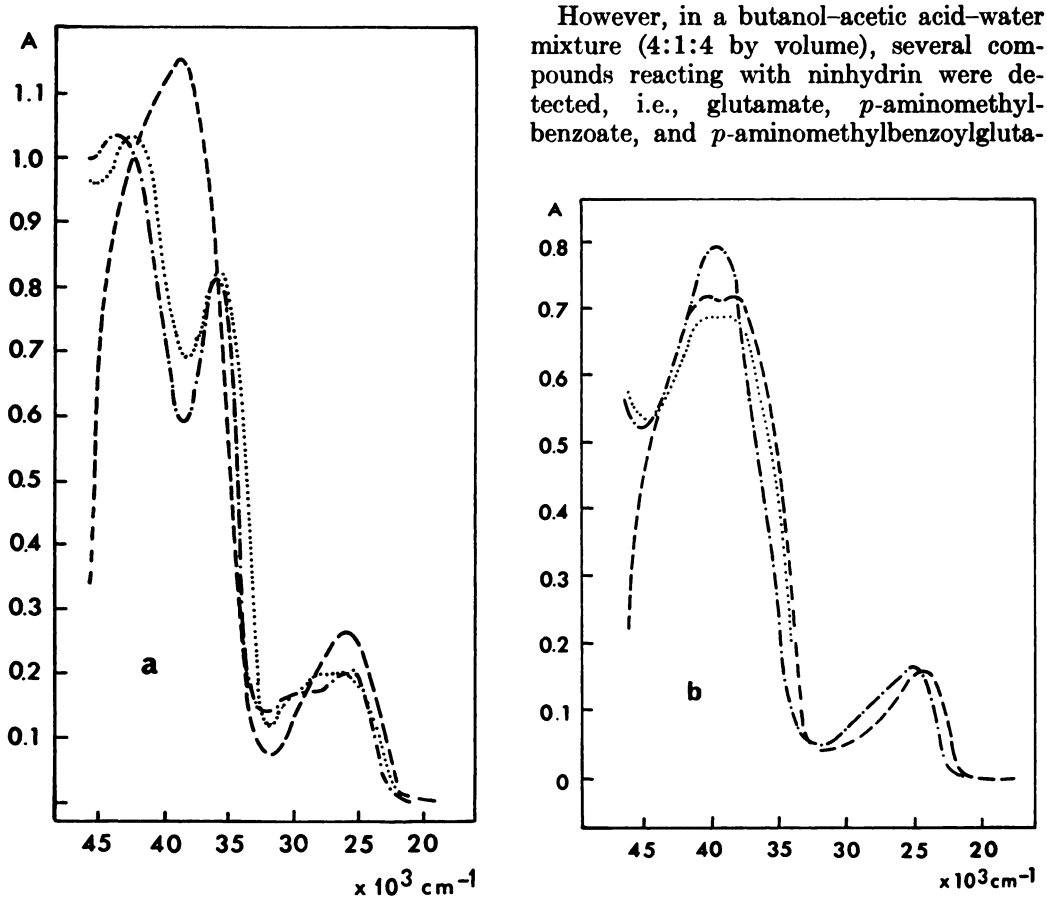

FIG. 2. *Absorption spectra of isohomofolic acid (A) and isohomoaminopterin (B) at different pH values*

– – –, pH 12; –·–, pH 2; ···· pH 7.

mate, if hydrolysis was carried out in 0.5 N sodium hydroxide. The ninhydrin-reactive products of acid hydrolysis were glutamate and a small amount of *p*-aminomethylbenzoylglutamate.

Cleavage using powdered zinc in 1 N HCl yielded only one or two unidentified fluorescent compounds. Chromatography with butanol–acetic acid–water yielded a larger ninhydrin spot of *p*-aminomethylbenzoylglutamate and a smaller one of *p*-aminomethylbenzoate.

Oxidative cleavage with potassium permanganate at pH 3–4 yielded no components of use in confirming the structure.

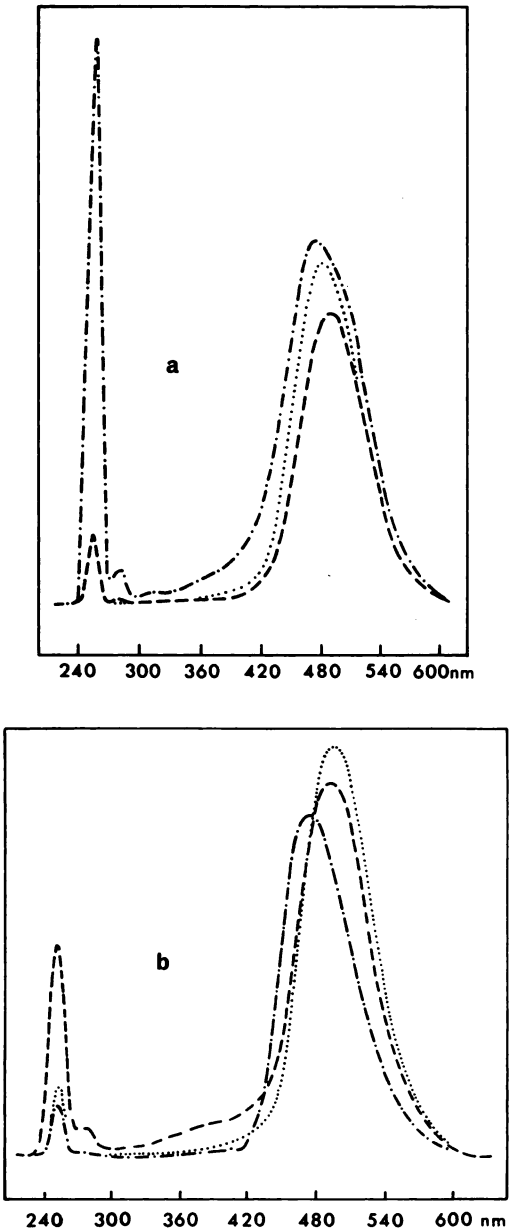

FIG. 3. *Flourescent spectra of isohomofolic acid (A) and isohomoaminopterin (B) at different pH values*

– – –, pH 12; ·–·–, pH 2; ····, pH 7.

Basic Biological Properties

Isohomoaminopterin inhibited the growth of *Lactobacillus casei* and *Streptococcus faecium* by about 50% at concentrations of 1 pM and 1 nM, respectively. Isohomofolic acid was more than 100 times weaker than isohomoaminopterin in inhibiting *L. casei* growth, and more than 10-fold weaker for *S. faecium* (Table 1).

In HeLa cells cultured in Eagle's medium, isohomofolic acid produced growth inhibition at 1 μM, which reached 50% at 0.1 mM.

Isohomoaminopterin inhibited HeLa cells under the same conditions, the inhibitory effect being observed at 0.1 μM; 50% inhibition was observed at 10 μM.

The growth of Sarcoma 180 cells was inhibited 50% by 0.1 mM isohomofolic acid and by 50 μM isohomoaminopterin. Morphological changes were similar to those due to the action of aminopterin or methotrexate: appearence of pycnotic nuclei, pro-

TABLE 1

Inhibition of growth of strains of L. casei and S. faecium

Microorganisms were cultivated on Difco medium. The level of folic acid was 0.2 μg/ml in the medium for *L. casei* and 3 μg/ml in that for *S. faecium*.

Antimetabolite	Half-maximal inhibition	
	L. casei ATCC 7830	*S. faecium* ATCC 8043
	M	*M*
Aminopterin	6.0×10^{-15}	9.0×10^{-11}
Isohomoaminopterin	1.4×10^{-12}	1.6×10^{-9}
Isohomofolic acid	4×10^{-9}	1.0×10^{-7}

nounced vacuolization, hyperplasia, and finally growth inhibition.

Reversal of the inhibitory effect of isohomoaminopterin by either N^5-formyltetrahydrofolate or folate was studied using the latter agents at 100 and 10 μM each. Isohomoaminopterin was used at a concentration which exerts about 50% growth inhibition. The inhibitory effect of isohomoaminopterin was reversed by N^5-formyltetrahydrofolate. Folic acid reversed the inhibition to a lesser extent under the same conditions (Fig. 4).

Permeation Studies

Permeation by both compounds was followed spectrophotometrically. Permeation by isohomofolic acid could not be observed at concentrations varying from 5 to 50 μM. Isohomoaminopterin entered the cells, as shown in Fig. 5, and its influx was enhanced by increasing the temperature (Fig. 6). Thus the temperature quotient, Q_{10}, between 25° and 35°, was determined to be 10.5 kcal/mole.

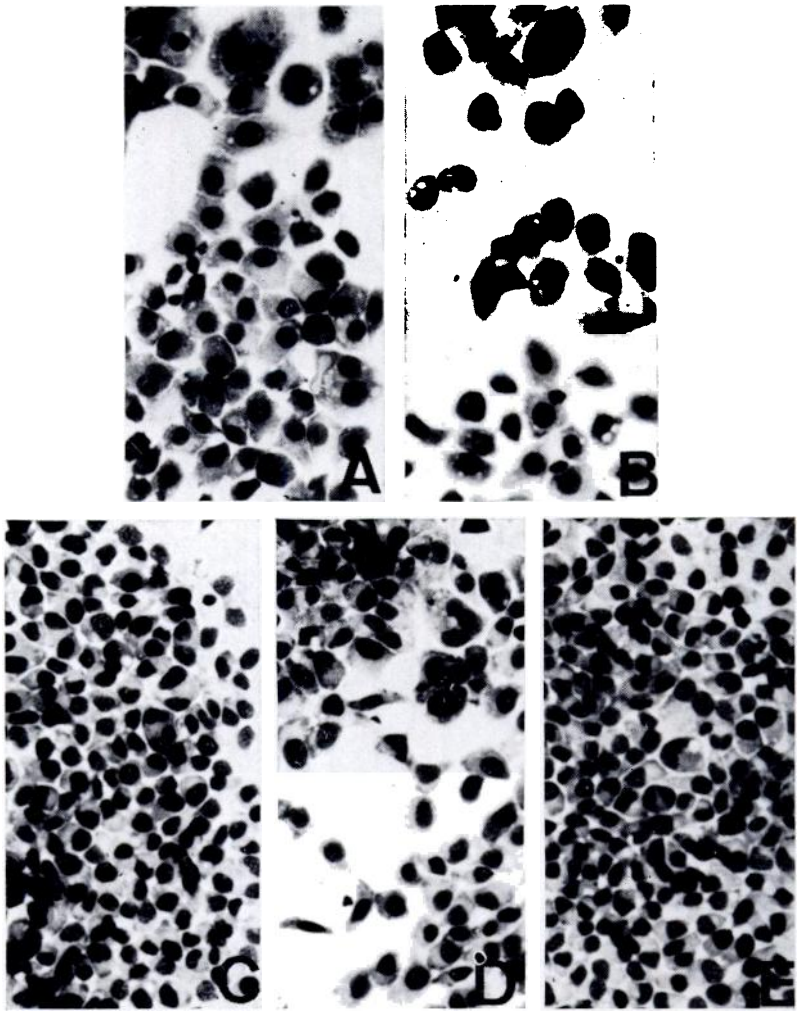

FIG. 4. *Cytostatic effects of various agents*

A. Isohomoaminopterin (10 μM). B. Isohomofolic acid (10 μM). C. Isohomoaminopterin (10 μM) plus N^5-formyltetrahydrofolate (100 μM). D. Isohomoaminopterin (10 μM) plus folic acid (100 μM). E. Culture of HeLa cells without any inhibitor. Giemsa stain (magnification 150 ×).

Enzyme Inhibition

Both isohomofolic acid and isohomoaminopterin were tested as potential inhibitors of enzymes involved in folate and tetrahydrofolate interconversion. Using folate reductase partially purified from mouse liver, both compounds were competitive inhibitors. The K_i values are summarized in Table 2. The reduction of folate in citrate buffer, pH 5.5, was inhibited by isohomofolic acid competitively with respect to folate (Fig. 7a), the K_i being 4 μM. The inhibition of dihydrofolate reductase at pH 7.5 was lower and was also competitive with respect to dihydrofolate (Fig. 7b). Isohomoaminopterin appeared to be a considerably stronger inhibitor of folate reductase (K_i = 12 nM). It competitively inhibited the reduction of folate at pH 5.5 with respect to folate as well as the reduction of dihydrofolate at pH 7.5 with respect to dihydrofolate (Fig. 8a and b). Neither isohomofolate nor isohomoaminopterin served as a substrate of folate reductase.

The reduction of dihydrofolate was inhibited by both isohomofolate and isohomoaminopterin between pH 4.5 and 7.5. Figure 9a shows that the inhibition was not strongly pH-dependent. In contrast, the reduction of folate was strongly pH-dependent, with a maximum at pH 6 for isohomoaminopterin and a maximum at about pH 5.5 for isohomofolate. Above pH 6 the inhibition dropped rapidly to nearly zero for both inhibitors (Fig. 9b).

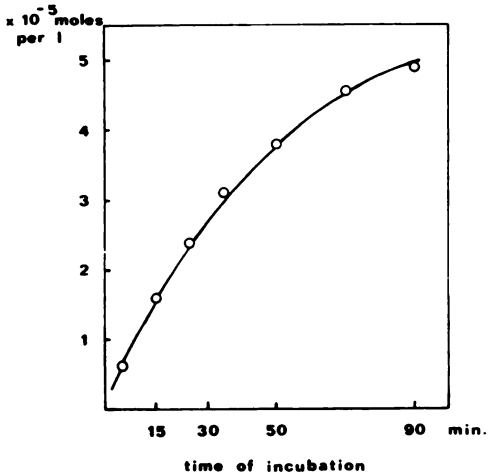

FIG. 5. *Time course of isohomoaminopterin uptake into Sarcoma 180 cells at 37°*

The extracellular concentration of inhibitor was 0.134 mM. The intracellular concentration of the drug was calculated per liter of packed cells.

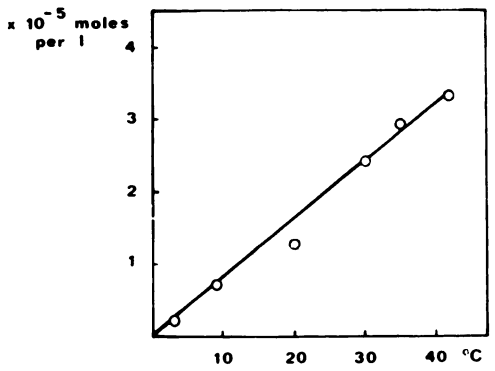

FIG. 6. *Effect of temperature on uptake of isohomoaminopterin into Sarcoma 180 cells*

Cells were incubated in the presence of isohomoaminopterin (0.134 mM) for 40 min. The intracellular concentration of the drug was calculated per liter of packed cells.

Other enzymes converting tetrahydrofolate to its coenzyme forms, i.e., serine hydroxymethylase, formiminotransferase, and tetrahydrofolate formylase, were not inhibited at a 0.1 mM concentration of either compound if an acetone powder extract prepared from pigeon liver was employed as the enzyme source. Inhibition of purine synthesis, followed by incorporation of [^{14}C]-formate, was also not observed with the same enzyme preparation.

Isohomoaminopterin and isohomofolate did not exert any inhibitory effect on thymidylate synthetase prepared from *Escherichia coli*, calf thymus, or Sarcoma 180 cells, but their tetrahydro derivatives in a crude form displayed inhibition of the calf thymus enzyme, as reported earlier (5).

DISCUSSION

Isohomofolic acid and isohomoaminopterin are cytostatics but are less potent than aminopterin against *L. casei* and *S. faecium*. Both isohomofolic acid and isohomoaminopterin also inhibit HeLa and Sarcoma 180 cells in tissue culture to a lesser extent, but the morphological changes are principally the same as those observed with methotrexate. Penetration into Sarcoma 180 cells could be observed only with isohomo-

TABLE 2

Inhibition of dihydrofolate reductase from mouse liver with various substrates

The complete system for folate reduction at pH 5.5 contained 0.1 μmole of folate, 0.125 μmole of NADPH, 10 μmoles of $MnCl_2$, 100 μmoles of sodium citrate, and 0.1 ml of enzyme preparation in a total volume of 0.8 ml. Incubation was carried out at 37° for 1 hr. The complete system for dihydrofolate reduction at pH 7.5 contained 0.3 μmole of dihydrofolate, 0.375 μmole of NADPH, 300 μmoles of sodium citrate, and 0.2 ml of enzyme preparation in a total volume of 3 ml. The decrease in absorbance at 340 nm was measured after 10 min of incubation at room temperature.

Antimetabolite	Folate at pH 5.5		Dihydrofolate at pH 7.5	
	I_{50}	K_i	I_{50}	K_i
	M	*M*	*M*	*M*
Isohomoaminopterin	2.4×10^{-7}	1.2×10^{-8}	5.7×10^{-7}	1.1×10^{-7}
	Competitive		Competitive	
Isohomofolic acid	2.6×10^{-5}	4.0×10^{-6}	7.5×10^{-5}	5.6×10^{-6}
	Competitive		Competitive	
Methotrexate	1.7×10^{-8}		1.8×10^{-8}	4.0×10^{-9}
	Stoichiometric (uncompetitive)		Competitive	

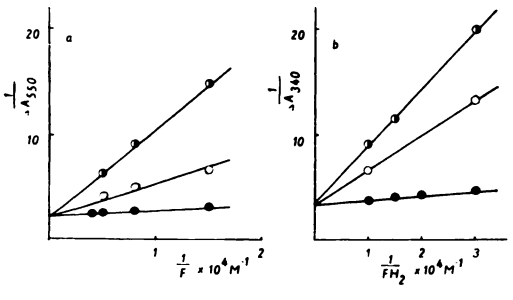

FIG. 7. *Inhibition of folate and dihydrofolate reduction by isohomofolic acid, plotted according to Lineweaver and Burk*

a. Folic acid (F) was used as the substrate, at pH 5.5. Concentration of inhibitor used: ●, none; ○, 25 μM; ◑, 50 μM.

b. Dihydrofolic acid (FH_2) was used as the substrate, at pH 7.5. Concentration of inhibitor used: ●, none; ○, 50 μM, ◑, 0.1 mM.

The assay system was identical with that described in Table 2.

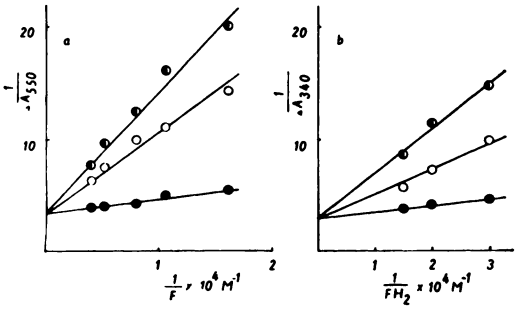

FIG. 8. *Inhibition of folate and dihydrofolate reduction by isohomoaminopterin, plotted according to Lineweaver and Burk*

a. Folic acid was used as the substrate, at pH 5.5. Concentration of inhibitor used: ●, none; ○, 62.5 nM; ◑, 78 nM.

b. Dihydrofolic acid was used as the substrate, at pH 7.5. Concentration of inhibitor used: ●, none; ○, 0.33 μM; and ◑, 0.66 μM.

The assay system was identical with that described in Table 2.

aminopterin. This supports the concept that modification of the bridge in isohomofolate prevents its penetration through the cell wall at the concentrations investigated, in contrast to folate and methotrexate. However, if the 4-hydroxyl group is substituted by a 4-amino group, this phenomenon cannot be observed; i.e., isohomoaminopterin penetrates. The temperature dependence of isohomoaminopterin intake, which permits calculation of the energy of transport, strongly supports the presumption that this substance is transported actively through the cell wall, perhaps with the aid of a specific transport protein which usually is employed in the transport of folic acid and its conventional analogues. The cytostatic effect of isohomofolate in tissue culture could be explained by its inhibition of folate permeation through the cell wall. However, there is no direct experimental evidence for this idea.

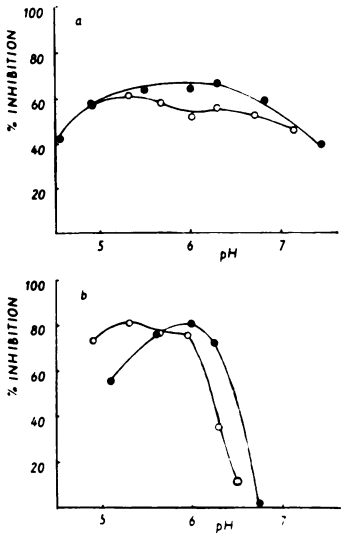

FIG. 9. *Effect of pH on inhibition of dihydrofolate (a) and folate reduction (b) caused by isohomofolic acid* (○) *and isohomoaminopterin* (●)

a. Isohomofolate was used in concentrations of 6.2×10^{-6} M for folate reduction and 1×10^{-4} for dihydrofolate reduction.

b. Isohomoaminopterin was used in concentrations of 2.5×10^{-7} M for folate reduction and 7.5×10^{-7} M for dihydrofolate reduction.

The substrate concentrations for the assay system were the same as shown in Table 2.

The single target enzyme of these new folate analogues seems to be dihydrofolate reductase. Comparison of the effects of isohomofolate and isohomoaminopterin with those of aminopterin and methotrexate leads to the following conclusions.

1. The modification of the aliphatic bridge of folate eliminates its substrate activity for mouse liver folate reductase and leads to inhibition. Substitution of the 4-hydroxyl by a 4-amino group on the pteridine nucleus enhances the inhibitory effect, as observed with isohomoaminopterin.

2. In contrast to methotrexate, the inhibition exerted by isohomoaminopterin is competitive with respect to folate at pH 5.5. This provides evidence that modification of the aliphatic bridge eliminates the "stoichiometric" character of inhibition well known in classical antifolates. Therefore substantial parts of the molecule participating in binding to the enzyme, i.e., the 2,4-diaminopyrimidine ring, aromatic nucleus, and glutamate carboxyls, may be bound reversibly to the binding site for folate or dihydrofolate, respectively. This change in the type of inhibition could be due to elongation of the bridge but, in contrast to homofolic acid, which is an artificial substrate for dihydrofolate reductase, isohomofolic acid loses its substrate properties (at least for the mouse liver enzyme).

3. The small pH dependence of the inhibition of dihydrofolate reduction contrasts with the sharp drop in inhibition of folate reduction with increasing pH. Because Bertino *et al.* (14) suggested that both substrates are bound on the same active site of the enzyme, a different mode of binding for folate and dihydrofolate to the enzyme seems very probable. Both inhibitors tested compete with both folate and dihydrofolate for the active site. Both these facts could explain the differential dependence of inhibition on pH with folate and dihydrofolate as substrates. A more detailed explanation of these observations requires additional experimental support.

The availability of only a small quantity of each compounds has so far prevented their use as chemotherapeutic agents in experimental leukemias and the determination of their toxicity. Nevertheless it would be of interest to test these compounds against a methotrexate-resistant strain of mouse leukemia.

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