Azahomoaminopterin, a New Type of Folic Acid Antimetabolite

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SUMMARY

Several new folic acid and aminopterin analogues were synthesized, in which the aliphatic chain binding the pteridine and aromatic nucleus was extended by 1 nitrogen atom. Condensation of 2-amino-4-hydroxypteridine-6-aldehyde with *p*-hydrazinobenzoylglutamate gave a hydrazone whose carbon-nitrogen double bond was reduced by sodium borohydride to form 11-azahomofolic acid. 11-Azahomopteroic acid was prepared by the condensation of 2-amino-4-hydroxypteridine-6-aldehyde with *p*-hydrazinobenzoic acid and subsequent reduction of the hydrazone with sodium borohydride. 11-Azahomoaminopterin and 4-aminoazahomopteroic acid were prepared in the same manner, using 2,4-diaminopteridine-6-aldehyde as the starting compound. The 5,6,7,8-tetrahydro derivatives of azahomofolic acid and azahomoaminopterin were prepared by catalytic hydrogenation of the parent compounds in glacial acetic acid.

Azahomopteroic acid and related compounds showed no significant inhibitory effect on HeLa cells grown in tissue culture, or any toxicity for mice in doses up to 100 mg/kg. Azahomoaminopterin, its parent hydrazone, and analogous pteroic acid derivatives exhibited a marked cytostatic effect on HeLa cells and were toxic for mice in doses exceeding 100 mg/kg, whereas 4-aminoazahomopteroic acid and its parent hydrazone showed no toxicity when administered in the same doses.

Among the enzymes interconverting folic acid or involving tetrahydrofolate as a coenzyme, thymidylate synthetase was inhibited slightly by azahomoaminopterin and more strongly by its tetrahydro derivative, the inhibition being noncompetitive versus tetrahydrofolate.

Folate reductase from mouse liver was inhibited by all the analogues containing an NH_2 group in position 4 (azahomoaminopterin and related substances). Azahomofolic acid and its derivatives were ineffective. Other tetrahydrofolate-interconverting enzyme systems and purine biosynthesis were not inhibited by any of the compounds studied.

INTRODUCTION

In a previous communication (1) concerning the specificity of thymidylate synthetase we confirmed that tetrahydroaminopterin and some of its derivatives are potent inhibitors of thymidylate synthetase of *Escherichia coli,* as had already been shown by Wahba and Friedkin (2). A more detailed study, using different analogues of tetrahydrofolic acid containing either the OH or NH_2 group in position C^4, led to the conclusion that the glutamic acid moiety and the hydrogenated $C^7=N^8$ double bond of the pyrazine ring are necessary for the binding of tetrahydrofolate and its analogues to thymidylate synthetase. Their elimination or structural change abolishes the coenzymatic activity of

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tetrahydrofolate and the inhibitory activity of its analogues. In contrast, structural change in the 1-carbon-bearing moiety represented by the atoms N^{5}, C^{9}, and N^{10} leads to loss of the coenzymatic activity of tetrahydrofolate but stimulates the inhibitory activity of its analogues. This effect can be demonstrated by substitution of any of these three atoms or by extension of the methylene moiety bridging the pteridine and aromatic nucleus. The latter effect was demonstrated by Friedkin *et al.* (3, 4) in the case of homotetrahydrofolic acid. Furthermore, Baker and Ho (5, 6), using anilinopropylpyrimidine models, showed that the drop in nucleophilicity of the N^{10} atom strongly suppresses the inhibitory effect on folate reductase and thymidylate synthetase. All these findings led us to prepare a group of homofolate and tetrahydrohomofolate analogues in which the C^{10} atom is substituted by a nitrogen atom, i.e., azahomofolic acid (V) and its 4-amino analogue azahomoaminopterin (IX). We considered that these compounds might be suitable models for studying the substrate and inhibition specificity of folate reductase and thymidylate synthetase. The synthesis of these substances and their basic properties are the subject of this paper.

EXPERIMENTAL PROCEDURE

Syntheses

p-Hydrazinobenzoylglutamic acid (III). This compound was prepared from *p*-aminobenzoylglutamic acid by nitrosylation and subsequent reduction (7, 8). *p*-Aminobenzoylglutamic acid (10.5 g) was suspended in 45 ml of water, and 9.5 ml of 37% HCl were added. The solution was chilled in ice, and 2.9 g of sodium nitrite were added with vigorous stirring. A saturated solution of 21.3 g of sodium sulfite was added immediately, whereupon the deep red color turned to orange. After 5 min the solution was acidified with 30 ml of glacial acetic acid and the flask was transferred to a boiling water bath. Powdered zinc was added until the orange color of the solution disappeared. The residual zinc was filtered off, and the pale yellow solution was used directly for preparation of the hydrazones.

Azahomofolic acid hydrazone (IV). The hydrazone was prepared by condensation of 2-amino-4-hydroxypteridine-6-aldehyde with *p*-hydrazinobenzoylglutamic acid. 2-Amino-4-hydroxypteridine-6-aldehyde (II) (8) (3 g) was suspended in 1200 ml of water, acidified with concentrated HCl to

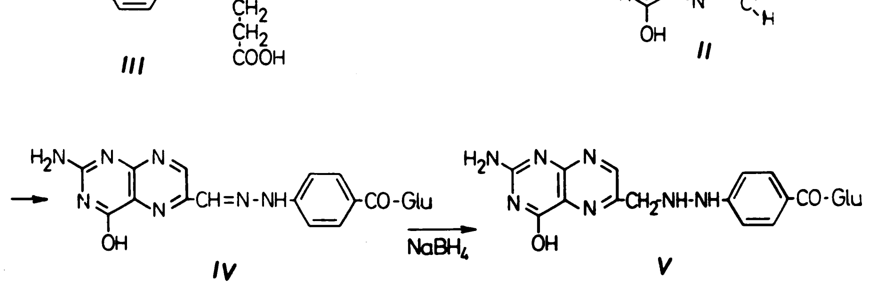

FIG. 1. *The route of synthesis of 11-azahomofolic acid, starting material through V*

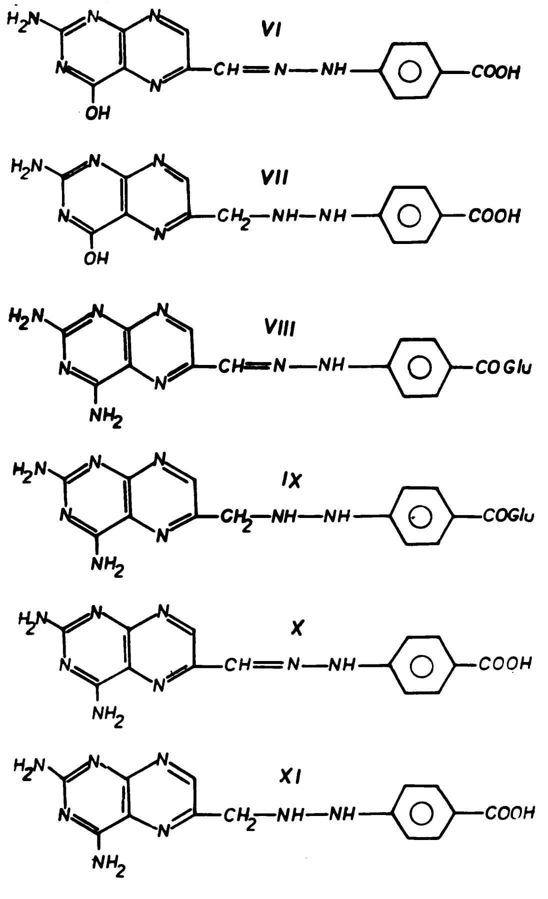

FIG. 2. *The route of synthesis of 11-azahomofolic acid, VI-XI*

pH 2, and heated to 70°, and the insoluble portion was filtered off. The filtrate was brought to pH 3 by adding solid sodium acetate, and a small amount of precipitate was removed by filtration. The solution of *p*-hydrazinobenzoylglutamic acid described above was added to the filtrate, and the resulting solution was allowed to stand for 4 hr. The orange, flocculent precipitate of the hydrazone was removed by suction and dried in a vacuum desiccator (yield, 2.2 g). The mother liquor was evaporated at 40° in a vacuum to about one-third its original volume. On cooling in the refrigerator, the second yield of the hydrazone crystallized. It was filtered off and dried in a vacuum desiccator (yield, 1.3 g).

Both portions of azahomofolic acid hydrazone were combined and dissolved in 120 ml of glacial acetic acid at 80°; 0.8 g of active charcoal was added, and the solution was filtered. The purified azahomofolic acid hydrazone crystallized, on cooling, in the form of red platelets in chromatographically pure form (Fig. 1).

$C_{19}N_8O_6H_{18}$ (mol wt 454.317)

Calculated: C 50.23%, H 3.989%, N 24.66%
Found: C 49.78%, H 3.93%, N 24.17%

Azahomofolic acid (*V*). This compound was prepared by reduction of azahomofolic acid hydrazone with sodium borohydride. One gram of hydrazone (IV) was dissolved in 200 ml of 0.1 N NaOH, 3 g of sodium borohydride were added, and the mixture was heated in a boiling water bath for 2 hr. A small amount of the precipitate that formed was filtered off, the pH of the filtrate was brought to 7, and another small portion of precipitate was filtered off. The warm solution was brought to pH 4 with hydrochloric acid, and the orange, crystalline precipitate was removed by filtration after cooling in the refrigerator. The crude product was purified by dissolving it in 0.1 N HCl at 40°; crystallization was induced by the addition of a saturated solution of ammonium acetate to pH 3 and cooling. The crystals were separated by centrifugation and washed three times with water, and the water suspension was dried in a vacuum from the frozen state (yield, 0.7 g) (Fig. 1).

$C_{19}N_8O_6H_{20}$ (mol wt 456.433)

Calculated: C 50.00%, H 4.417%, N 24.55%
Found: C 49.76%, H 4.48%, N 25.29%

Azahomoaminopterin hydrazone (*VIII*). The method of preparation was analogous to that of the azahomofolic acid hydrazone; 2,4-diaminopteridine-6-aldehyde was the starting compound. 2,4-Diaminopteridine-6-aldehyde (3 g) was dissolved in 400 ml of water with the addition of HCl to pH 2. The pH was brought to 3 with ammonium acetate, and the solution was filtered. A solution of hydrazinobenzoylglutamic acid prepared from 10 g of *p*-aminobenzoylglutamic acid as described above was added, and the pH was again brought to 3 with ammonium acetate. The hydrazone precipitated immediately as an orange-brown solid. After standing in the refrigerator overnight, the precipitate was filtered by suction. Another portion of hydrazone was obtained by evaporation of the mother liquor in a vacuum to one-fourth its original volume (total yield, 3.5 g). The crude product was recrystallized from hot glacial acetic acid and dried in a vacuum (Fig. 2).

$C_{19}N_9O_7H_{23}$ (dihydrate) (mol wt 489.450)
Calculated: C 46.63%, H 4.73%, N 25.76%
Found: C 46.59%, H 5.19%, N 25.63%

Azahomoaminopterin (*IX*). To 1 g of azahomoaminopterin hydrazone (VIII), dissolved in 200 ml of 0.1 N NaOH, were added 3 g of solid sodium borohydride, and the solution was heated in a boiling water bath for 2 hr. The small precipitate was filtered off, the pH was brought to 7 with hydrochloric acid, the mixture was filtered again, and the product was precipitated from the hot (60°) filtrate by adding solid ammonium acetate to pH 4. The product was left to crystallize overnight in the refrigerator, filtered by suction, and dried with ethanol and ether. The yield of crude product was 0.6 g.

The crude product was purified by dissolving it in 200 ml of water at 60° by addition of 2 N HCl to pH 3. Some dark impurities were filtered off, and the hot filtrate was brought to pH 5 with a saturated ammonium acetate solution. After crystallization overnight, the yellow product was washed with water and dried with ethanol and ether (Fig. 2).

$C_{19}N_9O_7H_{25}$ (dihydrate) (mol wt 491.450)
Calculated: C 46.43%, H 5.127%, N 25.65%
Found: C 46.46%, H 5.77%, N 26.30%

Azahomopteroic acid hydrazone (*VI*). Four grams of hydrazinobenzoic acid hydrochloride, prepared according to Fischer (7), were dissolved in 300 ml of water, and an aqueous solution of 3 g of 2-amino-4-hydroxypteridine-6-aldehyde in 300 ml, prepared as previously described, was added. The precipitated hydrazone was purified in the same manner as azahomofolic acid hydrazone (IV) (total yield, 3 g) (Fig. 2).

$C_{14}N_7O_3H_{12}$ (mol wt 324.310)
Calculated: C 51.69%, H 3.408%, N 30.15%
Found: C 50.94%, H 3.77%, N 28.68%

Azahomopteroic acid (*VII*). One gram of azahomopteroic acid hydrazone was reduced in 200 ml of 0.1 N NaOH with 3 g of $NaBH_4$ in the same manner as described for the synthesis of azahomofolic acid. After removal of impurities by filtration, the solution was diluted to 800 ml to prevent precipitation of the sodium salt, and then azahomopteroic acid was precipitated by acidification to pH 3 at 60°. The crystals obtained on cooling were filtered, washed with water, and dried with ethanol and ether. The crude product was recrystallized by dissolving it in water at 60°, adding ammonium hydroxide, and precipitating it with hydrochloric acid at pH 3 (total yield, 600 mg) (Fig. 2).

$C_{14}N_7O_3H_{12}$ (mol wt 326.326)
Calculated: C 51.37%, H 4.003%, N 29.96%
Found: C 49.28%, H 4.34%, N 29.00%

4-Aminoazahomopteroic acid (*XI*) *and its hydrazone* (*X*). These compounds were prepared in the same manner as described for the 4-hydroxy derivatives, using 2,4-diaminopteridine-6-aldehyde instead of 2-amino-4-hydroxypteridine-6-aldehyde (Fig. 2). Analysis of the 4-aminoazahomopteroic acid gave the following results:

$C_{14}N_8O_4H_{18}$ (dihydrate) (mol wt 325.328)
Calculated: C 46.40%, H 5.00%, N 30.93%
Found: C 46.66%, H 5.37%, N 28.66%

Tetrahydro derivatives. The tetrahydro derivatives of all the compounds described above were prepared in the following way: 100 mg of the compound were dissolved in 60 ml of glacial acetic acid at 80°. The solution was cooled quickly to room temperature; in several cases the product separated as an orange, microcrystalline precipitate. PtO_2 (70 mg) was added, and the hydrogenation was carried out under atmospheric pressure in an ordinary hydrogenation apparatus under vigorous stirring with a Teflon-coated magnetic stirrer. After the hydrogen consumption had ceased, 2 moles of hydrogen being absorbed per mole of the hydrogenated compound (the consumption of H_2 for the reduction of PtO_2 has been subtracted), the catalyst was filtered off. The catalyst was filtered in a large chromatographic chamber filled with carbon dioxide, and the filtrate was allowed to drop directly into a flask containing 1000 ml of anhydrous ether, with occasional stirring. The hydrogenated compounds formed white or lemon-yellow precipitates in ether. Ether was decanted, and the precipitate was filtered quickly by suction, washed thoroughly with ether, and dried in a vacuum. The preparation was sealed in evacuated vials and kept at −10° in a dark chamber.

Under all the conditions described (using a large excess of the catalyst and a sufficient period of reduction), the tetrahydro derivatives formed were almost 90% pure. The content of dihydro derivatives was about 5% as shown by chromatography on DEAE-cellulose using a 2 M ammonium bicarbonate–water linear gradient for the elution (Fig. 3).

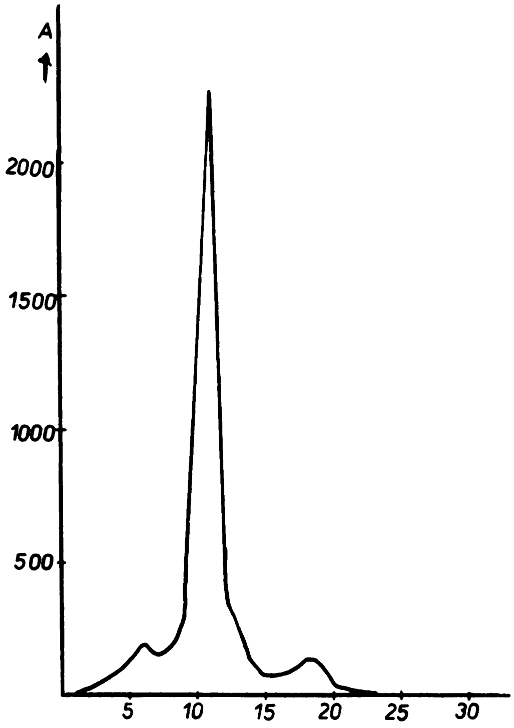

FIG. 3. *Elution profile of tetrahydroazahomoaminopterin (10 mg) on a DEAE-cellulose column with a linear water–2 M ammonium bicarbonate gradient*

The ordinate shows optical density at 290 nm; the abscissa, number of tubes.

Methods of Chromatography

All nonhydrogenated compounds were tested for purity by paper chromatography on Whatman No. 3 paper with three different solvents: (a) 5% aqueous ammonia–ethanol–dimethylformamide, 5:5:1 and 1:1:2 (by volume), and (b) 5% aqueous ammonia.

11-Azahomofolic acid (V), azahomoaminopterin (IX), and the analogous pteroic acid derivatives showed a lemon-yellow fluorescence in neutral or alkaline solution, whereas the parent hydrazones did not and were visible on paper chromatograms only as dark-absorbing spots.

Tissue Culture Procedures

HeLa cells were cultured in Eagle's medium containing 20% human serum, 100 international units of penicillin, and 200 μg of streptomycin per milliliter (9). For inoculation, 10^5 cells were suspended in 1 ml of the culture medium in each test tube (16 × 160 mm). After 24 hr of cultivation at 37°, the medium was removed; the cultures were washed with Hanks' solution and overlayered with fresh medium containing azahomofolic acid derivatives in concentrations from 10^{-3} to 10^{-8} M.

The medium containing the cytostatic agent was replaced with the same medium after 24 hr. At the end of a 48-hr cultivation in a rotating drum, the cell proliferation was evaluated according to Sanford *et al.* (10). The growth of HeLa cells cultured in the same way but without the addition of the cytostatic agent served as a basis for the calculations (in percentage of growth inhibition).

Determination of Toxicity

Mice of the H strain, weighing 18–20 g, were employed for the determination of toxicity. The substances tested were administered intraperitoneally as their sodium salts in aqueous solutions; usually 0.2 ml was injected. In the case of pteroic acid analogues, aqueous suspensions were administered because of the poor solubility of the sodium salts. Treated mice were observed for 10 days after application.

Enzyme Inhibition

The effects of all substances on the activity of serine hydroxymethylase, methylenetetrahydrofolate reductase, tetrahydrofolate formylase, and formiminotransferase were studied by spectrophotometric methods described in detail in an earlier communicaton (11). Purine biosynthesis was determined as ^{14}C-formate incorporation into inosinic acid (12). A 5% aqueous extract of acetone powder prepared from pigeon liver was employed for checking the activities of all these systems.

Folate reductase activity was assayed by determination of diazotizable amine (13), and dihydrofolate reductase, by the decrease of absorbance at 340 mμ. As the enzyme source, an ammonium sulfate fraction (30–65% saturation) of mouse liver homogenate was employed.[3]

The effect on thymidylate synthetase was investigated by a modification of the spectrophotometric method of Wahba and Friedkin (14) described previously (1). As the enzyme source, an ammonium sulfate fraction (50–60% saturation) of calf thymus extract was employed. Before ammonium sulfate fractionation, the crude extract of thymus was freed of nucleic acids by precipitation with one-fourth its volume of a 5% streptomycin sulfate solution.

RESULTS

Chemical structure. The most convenient method for extension of the aliphatic chain bridging the pteridine and *p*-aminobenzoate rings by 1 atom of nitrogen appeared to be the condensation of the pteridine-6-aldehyde with *p*-hydrazinobenzoic or *p*-hydrazinobenzoylglutamic acid, leading to the formation of stable hydrazones whose double bond could be reduced by sodium borohydride under appropriate conditions.

The presence of the nitrogen atom in the bridge linking the pteridine and aromatic nucleus introduced a new chromophore group in all the compounds studied. This group caused the appearance of a new high absorption peak in the visible region of the spectrum, as well as the orange or red color of the compounds (see Fig. 4). Because this strong absorption band is present both in azahomofolate and its hydrazone but absent in the tetrahydro derivatives prepared by catalytic hydrogenation, it is probable that sodium borohydride reduces the double bond of the hydrazone without attacking the pyrazine ring of the pteridine nucleus. Additional evidence for this supposition is the consumption of 2 moles of hydrogen in the course of the catalytic reduction of azahomofolate and 3 moles if azahomofolate hydrazone was directly hydrogenated. However, sodium borohydride is able to reduce the pyrazine ring of the pteridine nucleus under certain conditions, as shown by Scrimgeour and Vitols (15). These findings support the proposed structures of azahomofolate, azahomoaminopterin, and related substances.

Biological properties. All the compounds synthesized were tested for toxicity in mice of the H strain. Azahomofolic acid, azahomopteroic acid, and its 4-amino analogues did not show any toxicity in mice if administered intraperitoneally in a single dose up to 100 mg/kg. Nor did the administration of the parent hydrazones of any of the compounds mentioned show remarkable toxic effects. Azahomoaminopterin was toxic if administered in doses of 50–100 mg/kg, and its parent hydrazone was toxic in approximately the same dosage range. Higher doses of the analogues were not used in the preliminary experiments reported in this paper in order to avoid artifacts due to the low solubility of the compounds.

If some pteroic acid derivatives (4-aminoazahomopteroic acid) were admin-

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[3] J. Souček and K. Slavík, unpublished experiments.

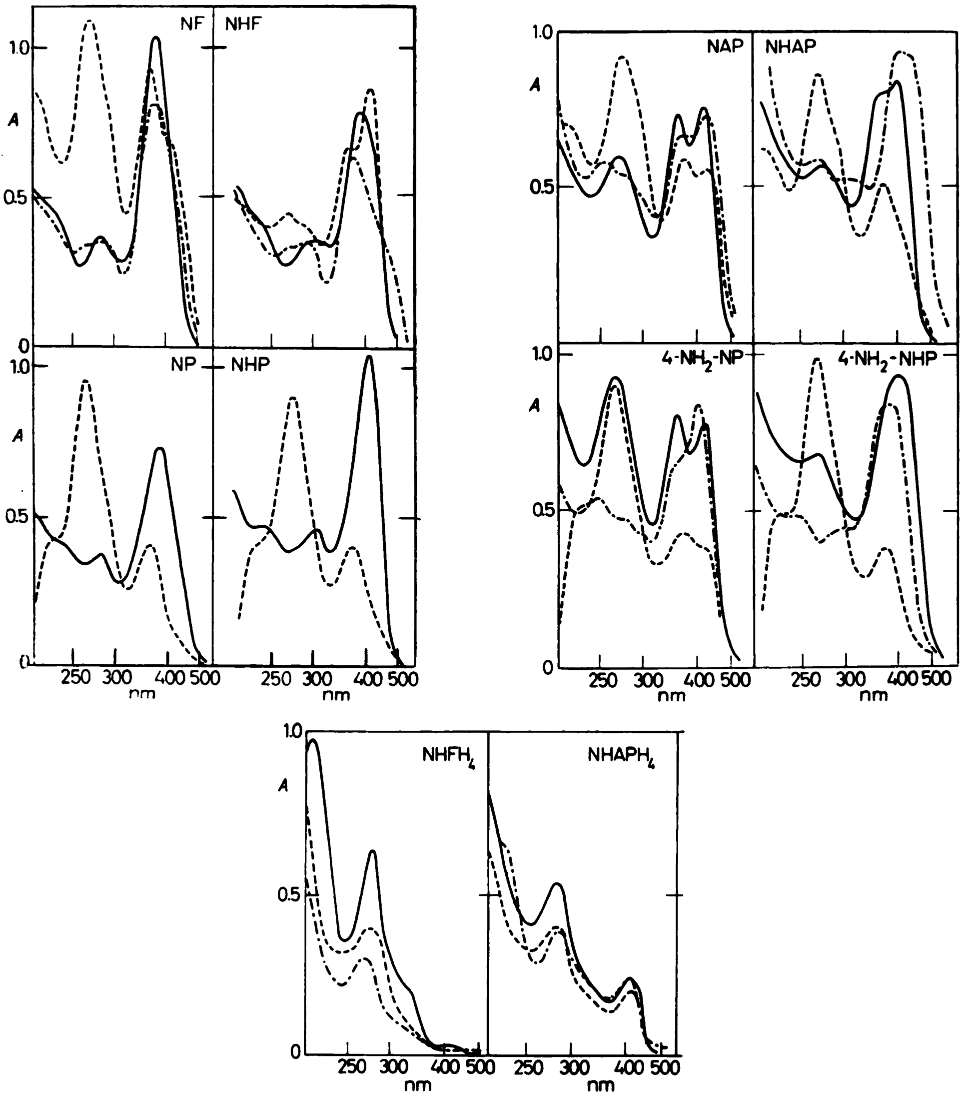

Fig. 4. *Absorption spectra of the azahomofolic acid derivatives in 0.1* M *HCl* (· — · —), *0.1* M *sodium phosphate, pH 7.0* (——), *and 0.1* M *NaOH* (- - - - -)

The abbreviations used in Figs. 4 and 5 are: NF, azahomofolic acid hydrazone; NHF, azahomofolic acid; NP, azahomopteroic acid hydrazone; NHP, azahomopteroic acid; NAP, azahomoaminopterin hydrazone; NHAP, azahomoaminopterin; 4-NH$_2$-NP, 4-aminoazahomopteroic acid hydrazone; 4-NH$_2$-NHP, 4-aminoazahomopteroic acid; NHFH$_4$, tetrahydroazahomofolic acid; NHAPH$_4$, tetrahydroazahomoaminopterin.

istered in the form of sodium salt suspensions, the suspensions could be found in the peritoneal cavity of mice even after 10 days. The toxic compounds, azahomoaminopterin and its hydrazone, if administered in high doses, appeared to cause the formation of yellow or orange infiltrates in the kidney tubules of dead mice. This phenomenon can be attributed to the precipitation of free acids in kidneys at the acidic pH of urine.

Although it was impossible to determine the exact LD$_{50}$ of all the compounds tested, it can be concluded that their toxicity is lower than that of the "classical" antifolic aminopterin (in excess of 100 mg/kg).

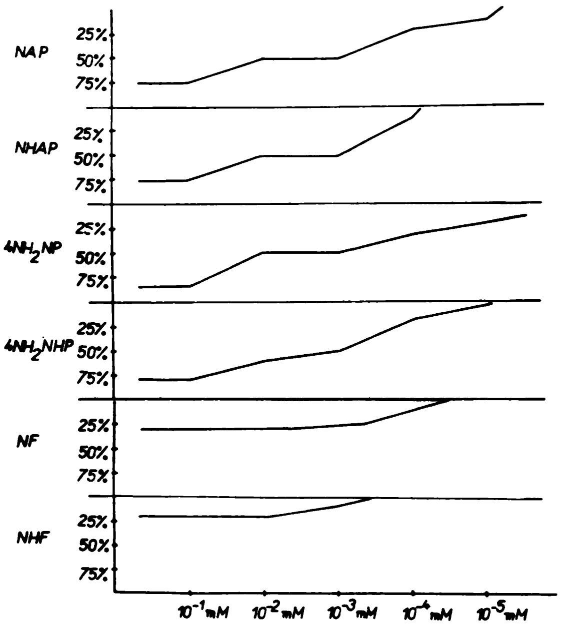

FIG. 5. *The growth-inhibitory effect of azahomoaminopterin and related substances on HeLa cells in tissue culture*

The ordinate shows percentage of inhibition; the abscissa, concentration of antimetabolite. Cells grown in medium without inhibitor served as the basis of calculation. Abbreviations are defined in the legend to Fig. 4.

Figure 5 represents the growth-inhibitory effects of the folic acid analogues, determined by a screening test on HeLa cells grown in tissue culture. Azahomofolic acid showed only a negligible cytostatic effect on HeLa cells. In contrast, azahomoaminopterin and its parent hydrazone showed a cytostatic effect down to concentrations of 10^{-7}–10^{-8} M, which is comparable to the effect of dibromoaminopterin or amethopterin. 4-Aminoazahomopteroic acid and its parent hydrazone showed nearly the same cytostatic effect as the 4-amino analogues containing the glutamate moiety.

The cytological characteristics of the pteroic and folic acid analogues are quite different (Fig. 6). If azahomoaminopterin or its parent hydrazone was added to the tissue culture, individual cells or groups of a few cells with pycnotic nuclei appeared. The same effect was also exerted by aminopterin and amethopterin. 4-Azahomopteroic acid and its parent hydrazone, although acting as inhibitors in the same concentration as azahomoaminopterin, exerted a different effect on the division of HeLa cells, consisting of granulation of the cell nucleus and, finally, complete destruction of the nucleus.

Preliminary experiments on enzyme inhibition. All compounds described in this communication were tested as potential inhibitors of enzymes converting folic and tetrahydrofolic acids to their coenzyme forms. Besides these reactions, their effects on purine biosynthesis and thymidylate synthetase were investigated. Azahomofolic acid, azahomopteroic acid, and their parent

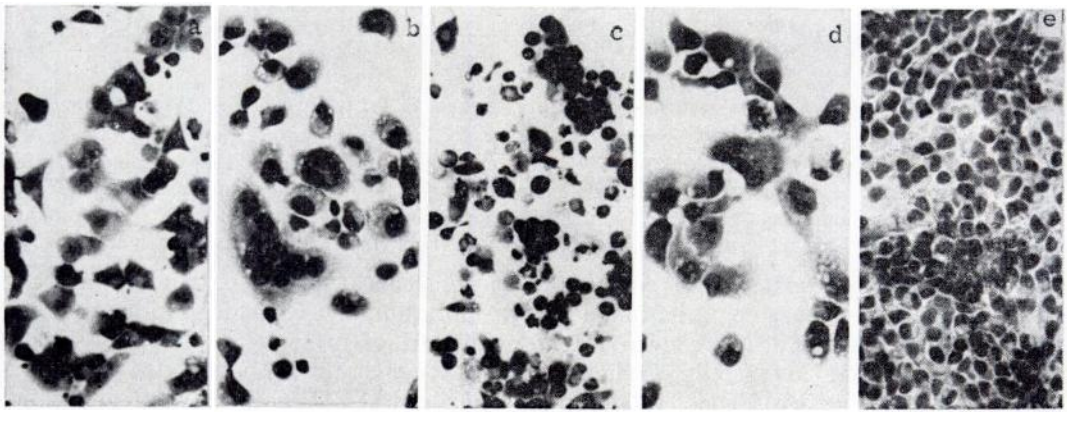

FIG. 6. *Cytostatic effect of azahomoaminopterin* (10^{-5} M) *(a), azahomoaminopterin hydrazone* (10^{-5} M) *(b), 4-aminoazahomopteroic acid hydrazone* (10^{-5} M) *(c), and aminopterin* (10^{-8} M) *(d); the last panel (e) is a culture without inhibitor (native, unstained preparations)*

hydrazones, as well as azahomotetrahydrofolate, were found to be ineffective at concentrations of 10^{-4} M in all the enzyme systems tested. Azahomoaminopterin and its tetrahydro derivative, at the same concentrations, were also ineffective as inhibitors of serine hydroxymethylase, methylenetetrahydrofolate dehydrogenase, tetrahydrofolate formylase, and formiminotransferase. Nor did they show any inhibitory or substrate activity in the system of purine biosynthesis.

Only two enzymes appeared to be inhibited by azahomoaminopterin and its tetrahydro derivative: folate reductase and thymidylate synthetase. With calf thymus thymidylate synthetase, azahomoaminopterin exerted a very weak inhibitory effect (only 18% inhibition at a concentration of 4×10^{-4} M). The tetrahydro derivative appeared to be a stronger inhibitor of thymidylate synthetase. The inhibition appeared on a Lineweaver-Burk plot to be noncompetitive with tetrahydrofolate. The pteroic acid analogues were completely ineffective; none of the derivatives tested showed any substrate activity (Fig. 7).

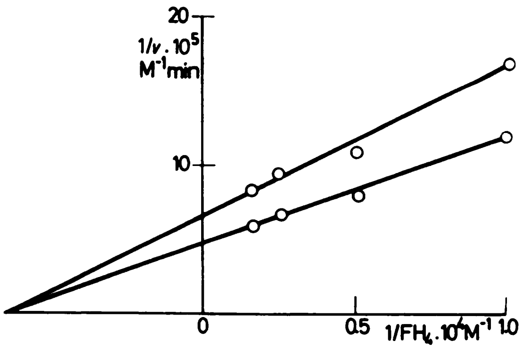

Fig. 7. *The noncompetitive character of inhibition of calf thymus thymidylate synthetase by tetrahydroazahomoaminopterin*

The concentration of inhibitor used was 2.4×10^{-4} M. The incubation mixture contained 0.83 μmole of tetrahydrofolic acid (FH_4), 4.5 μmoles of formaldehyde, 48.3 μmoles of Tris-HCl buffer (pH 7.4), 13.5 μmoles of mercaptoethanol, and 1 μmole of deoxyuridylic acid in a total volume of 1.1 ml. The reaction was started by the addition of deoxyuridylate and was continued for 15 min at 20°.

Table 1 compares the inhibitory effects of azahomoaminopterin and its derivatives on folate reductase from mouse liver. The strongest inhibitor, although two orders of magnitude weaker than aminopterin, was azahomoaminopterin. Its parent hydrazone showed about 10 times less inhibitory activity. 4-Aminoazahomopteroic acid and its hydrazone appeared to be weak inhibitors of folate reductase; the degree of

Table 1

Comparison of the inhibitory effect of azahomoaminopterin and its derivatives on folate reductase from mouse liver

The folate reductase incubation mixture contained 0.3 μmole of folic acid, 0.25 μmole of NADPH, 10 μmoles of $MnCl_2$, 40 μmoles of sodium citrate buffer (pH 5.5), and 0.2 ml of enzyme preparation in a total volume of 1.7 ml. Incubation was carried out at 37° for 2 hr. The dihydrofolate reductase incubation mixture contained 0.2 μmole of dihydrofolic acid, 0.5 μmole of NADPH, 10 μmoles of $(NH_4)_2SO_4$, 10 μmoles of cysteine, 250 μmoles of sodium citrate buffer (pH 7.4), and 0.2 ml of enzyme preparation in a total volume of 3.4 ml The reaction was followed at room temperature for 10 min.

Substance	Concentration causing 50% inhibition with Folate at pH 5.5	Dihydrofolate at pH 7.5
	M	M
Azahomoaminopterin	1.47×10^{-6}	7.35×10^{-7}
Azahomoaminopterin hydrazone	1.47×10^{-5}	4.7×10^{-5}
4-Aminoazahomopteroic acid	5.8×10^{-5}	1.4×10^{-4}
4-Aminoazahomopteroic acid hydrazone	2.9×10^{-5}	1.4×10^{-4}
Amethopterin	2.3×10^{-8}	2.9×10^{-8}
2,4-Diaminopteridine	1.47×10^{-4}	1.4×10^{-4}

inhibition was comparable to the inhibitory effect of 2,4-diaminopteridines or 2,4-diaminopyrimidines.

None of the compounds investigated showed any substrate activity for the folate reductase of mice.

DISCUSSION

In preliminary experiments azahomofolic acid and related substances containing a hydroxyl group in position 4 of the pyrimidine ring proved to be ineffective both as enzyme inhibitors and as cytostatic agents. In contrast, azahomoaminopterin and its derivatives showed a rather strong cytostatic effect on HeLa cells and also proved to be inhibitors of folate reductase and thymidylate synthetase, respectively. Their cytostatic effect contrasts with the low toxicity of this group of compounds for mice and also does not correspond to the relatively low inhibitory effect on the enzymes mentioned.

Of the compounds studied, 4-aminoazahomopteroic acid and its parent hydrazone showed the strongest discrepancy in this respect. Although no experimental evidence to explain these findings has been discovered as yet, several hypothetical considerations should be mentioned. In the preliminary enzyme inhibition studies, folate reductase from mouse liver and thymidylate synthetase from calf thymus were employed. Although the inhibition of both enzymes was relatively weak, it is not certain whether enzymes obtained from different, tumor-bearing species might not be inhibited much more strongly than the enzymes from healthy tissues employed here. This will be investigated in future experiments.

Two enzyme systems in which tetrahydrofolate acts as a coenzyme were not investigated in this study: the system synthesizing methionine (14) and the methionyl-sRNA transformylase (16). Possible inhibition of the methionine-synthesizing system does not seem to be the growth-limiting factor, because enough methionine was present in the tissue culture medium. Inhibition of the methionyl-sRNA-formylating system could be considered as growth-limiting because of the specific role of formylmethionyl-sRNA in the initiation of peptide chain biosynthesis (17). Some evidence for this idea has been obtained by Lengyel and Eisenstadt (18), in experiments with pteridine inhibitors of folate reductase. Their data, however, were obtained in bacterial systems, and there is no evidence of an analogous function in animal cells.

The interaction of azahomoaminopterin and related substances with methionyl-sRNA transformylase will be investigated in future experiments. Many other factors could also influence the effect of azahomoaminopterin and derivatives on living cells, such as detoxification or penetration of the cell wall, but they cannot be discussed here without further experimental evidence. Nevertheless, our preliminary results warrant additional study of the chemotherapeutic effect on some experimental tumors, which will be reported later.

Considerable differences have been found in the degree and nature of inhibition of mouse liver folate reductase by folic acid antimetabolites. This finding has stimulated a more detailed study, in which these compounds have been used as tools for investigating the inhibitory specificity of folate reductase. The results of this study will be presented later.

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