Properties of Reduced Derivatives of Aminopterin*

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It has been reported that tetrahydroaminopterin1 is a more potent antimetabolite of folic acid in Streptococcus faecalis (ATCC 8043) and Pediococcus cerevisiae (ATCC 8081) than aminopterin (2). It seemed likely that tetrahydroaminopterin was competing with coenzyme forms of folate for sites on enzymes involved in the metabolism of single carbon units. In support of this view, certain enzyme systems have been reported to be inhibited by tetrahydroaminopterin under conditions where aminopterin is ineffective. They are the thymidylate synthetase (3), methionine methyl group-synthesizing system from Escherichia coli (2), and 5, 10-methylenetetrahydrofolate dehydrogenase of liver (4).

In the present work we examine in detail some of the chemical and biological properties of reduced aminopterin analogues.

EXPERIMENTAL PROEDURE

Purification of Aminopterin—Commercial aminopterin was purified by chromatography on DEAE-cellulose and elution with 10% Na2HPO4 according to the procedure employed by Keresztesy and Donaldson for the purification of 5-methyltetrahydrofolate (5). Fractions with a ratio of absorbance at 290 μm to 285 μm between 1.05 and 1.07 were combined and freed of Na2HPO4 as described by Keresztesy and Donaldson (5). Aminopterin was precipitated by adjusting the pH to 4.0 with HCl. It was then washed with alcohol and ether and dried in a vacuum. When 100 μg of the product were chromatographed on Whatman No. 1 filter paper with 0.01 M potassium phosphate, pH 7.5, as the solvent and the paper strip was examined under an ultraviolet lamp, no fluorescent spots were detected. Under these conditions untreated aminopterin showed five distinct fluorescent spots. Aminopterin (Rf, 0.5) itself appears as an ultraviolet-absorbing spot.

Preparation of Tetrahydroaminopterin—Tetrahydroaminopterin was prepared from purified aminopterin by catalytic hydrogenation in glacial acetic acid (6, 7). Analysis by DEAE chromatography (Fig. 3, Table I) revealed that it contained 15% dihydroaminopterin as the only major impurity.

\[
\text{C}_{49} \text{H}_{52} \text{N}_{15} \text{O}_{12} \cdot 2\text{H}_{2} \text{O}
\]
Calculated: C 47.6, H 5.4, N 23.4
Found: C 47.7, H 5.4, N 22.2

Its spectrum is shown in Fig. 1. At pH 7.0, the molecular extinction coefficient at 296 μm is 28,300.

Preparation of Dihydroaminopterin—Dihydroaminopterin was prepared from purified aminopterin by dithionite reduction (8, 9). Analysis by DEAE chromatography (Fig. 5, Table I) revealed that it contained 13% aminopterin as the only major impurity.

\[
\text{C}_{41} \text{H}_{42} \text{N}_{13} \text{O}_{9} \cdot 1\text{H}_{2} \text{O}
\]
Calculated: C 47.6, H 5.4, N 23.4
Found: C 47.7, H 5.4, N 22.2

Its spectrum is shown in Fig. 2. At pH 7.0, the molecular extinction coefficient at 289 μm is 27,000.

The reduced pteridines were stored in a vacuum in Thunberg tubes at room temperature. Under these conditions they are stable indefinitely.

DEAE Chromatography of Aminopterin Derivatives—This was carried out as described for the corresponding folate derivatives by Mathews and Huennekens (10) and modified by Wahba and Friedkin (3). A water-jacketed column kept at 0° was employed. The compounds were dissolved in a solution containing 1 M Tris, pH 7.0, and 0.2 M mercaptoethanol. Tetrahydroaminopterin is more readily soluble than dihydroaminopterin under these conditions. The solution was diluted 1:100 with 0.2 M mercaptoethanol and added to the column. A gradient elution with 200 ml of 0.005 M Tris in the mixing chamber and 400 ml of 1 M Tris in the reservoir was employed. Both solutions contained 0.2 M mercaptoethanol.

Growth of Pediococcus cerevisiae (ATCC 8081)—The medium employed was that described by Dawbarn, Hine, and Smith (11), except that 4 g of enzyme-hydrolyzed casein were added per liter of double strength medium. The reduced analogues diluted in potassium ascorbate (6 mg per ml, pH 6.0) were added septicly to the assay tubes after the medium had been autoclaved. The final concentration of ascorbate in the assay medium was 0.6 mg per ml. Potassium ascorbate is known to protect tetrahydrofolate from decomposition (12). Growth was estimated turbidimetrically with a 660-μm filter after incubation for 17 hours at 37°.

Thymidylate Synthetase—Partially purified thymidylate synthetase from E. coli B (Fraction II described by Friedkin et al. (14)) was kindly supplied by Professor M. Friedkin. The assay conditions are described in Table II.

Dihydrofolate Reductase—This enzyme was prepared from a mouse tumor (L1210-C95) resistant to amethopterin, 6-mercaptopurine, and cytoxan kindly provided by Dr. A. Goldin and Mr. S. R. Humphreys and maintained in this laboratory as described (15). Acetone-dried powders were prepared from the local tumors. Two hundred milligrams of acetone-dried powder were extracted with 5 ml of 0.05 M potassium phosphate, pH 7.5, containing 0.01 M mercaptoethanol and centrifuged at 80,000 × g.
RESULTS

Stability of Tetrahydroaminopterin under Conditions of Microbiological Assay—In order to study the decomposition of tetrahydroaminopterin during incubation, 4.1 mg (plus 0.14 ml of 0.1 N NaOH to maintain neutrality) were incubated with ascorbate in the assay medium for 17 hours at 37°. Column chromatography of the mixture (Fig. 4, Table I) revealed that a large proportion of the tetrahydroaminopterin added decomposed. Dihydroaminopterin was not observed as a breakdown product of tetrahydroaminopterin under these conditions. p-Aminobenzoylglutamate was recovered, but the pteridine fragment was not. It must have either broken down to nonultraviolet-absorbing material or remained on the DEAE column. No material absorbing at 295 mp could be recovered on further
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FIG. 5. Chromatography of dihydroaminopterin prepared by dithionite reduction. Aminopterin was identified spectrally.

FIG. 6 (left). ---, chromatography of dihydroaminopterin incubated under conditions of microbiological assay. ---, constituent of microbiological assay medium.

FIG. 7 (right). Chromatography of dihydroaminopterin incubated for 48 hours in 0.2 M 2-mercaptoethanol. The peak at 300 ml represents a pteridine breakdown product of dihydroaminopterin which elutes from the column at the same point as does aminopterin.

Stability of Dihydroaminopterin under Conditions of Microbiological Assay—Dihydroaminopterin is somewhat more stable than tetrahydroaminopterin (Table I). As described in the previous paragraph, 2.3 mg were incubated (plus 0.07 ml of 0.1 N NaOH to maintain neutrality). The dihydroaminopterin decomposed to p-aminobenzoylglutamate and an unidentified fragment that emerged from the column at the same point as does aminopterin (peak at 295 ml, effluent volume; Fig. 6). We believe that the quantitative data (Table I) discussed below rule out the possibility that the unidentified fragment is aminopterin.

Stability of Reduced Pteridines in Mercaptoethanol—As 2-mercaptoethanol is commonly used to protect reduced pteridines from decomposition, the stability of both reduced compounds was tested in the presence of this substance. Solutions of the

TABLE I

Recovery of products after chromatography of reduced aminopterin derivatives

<table>
<thead>
<tr>
<th>Recovered product</th>
<th>Tetrahydroaminopterin</th>
<th>Dihydroaminopterin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Incubated in assay</td>
</tr>
<tr>
<td>Tetrahydroaminopterin</td>
<td>74</td>
<td>18</td>
</tr>
<tr>
<td>Dihydroaminopterin</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-Aminobenzoylglutamate</td>
<td>Negligible</td>
<td>76</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>102</td>
</tr>
</tbody>
</table>

* Based on absorbance of original solution and recovered fractions (Figs. 3 to 7). Values used for the molecular extinction coefficient at 290 μm were: aminopterin, 25,000, and p-aminobenzoylglutamate, 11,000.

† One of the breakdown products of dihydroaminopterin was eluted from the DEAE column at the same point as aminopterin (Figs. 6 and 7). It was assumed that the aminopterin present initially did not decompose in either incubation.
reduced pteridines were allowed to stand in 0.2 m 2-mercapto-ethanol-1 m Tris, pH 7.0, for 48 hours at room temperature. The chromatographic pattern obtained from tetrahydroaminopterin is shown in Fig. 3. Appreciable dihydroaminopterin was formed. Dihydroaminopterin under these conditions breaks down to p-aminobenzoylglutamate and a fragment emerging from the DEAE chromatogram at the same point as aminopterin (Fig. 7). Dihydroaminopterin therefore appears to break down to the same products, whether it is incubated in the assay medium or in mercaptoethanol.

Quantitative data on the decomposition products of the reduced pteridines is given in Table I. In each case the amount of p-aminobenzoylglutamate formed is roughly equivalent to the disappearance of the reduced aminopterin derivatives. Aminopterin itself is thus excluded as a major breakdown product of the reduced forms.

**Inhibition of Growth of Pediococcus cerevisiae.** The reduced analogues, which were freed of impurities by the column chromatographic procedure described, were tested for their ability to inhibit the growth of *Pediococcus cerevisiae*. As shown in Fig. 8, dihydroaminopterin is about twice as inhibitory as tetrahydroaminopterin. Inhibition by both derivatives is reversed by 8 × 10⁻⁵ m thymidine.

Our finding that dihydroaminopterin is 100 times as inhibitory as aminopterin agrees with the data of Zakrzewski, Hakala, and Nichol (9).

**Inhibition of Dihydrofolate Reductase and Thymidylate Synthetase—**Wahba and Friedkin (3) reported that tetrahydroaminopterin was a significantly more potent inhibitor of *E. coli* thymidylate synthetase than aminopterin. We have confirmed this observation with material purified by column chromatography, and in addition, we have tested dihydroaminopterin. We have also compared the ability of the reduced analogues to inhibit dihydrofolate reductase derived from mouse tumors resistant to antifolate. The results are shown in Table II.

Dihydroaminopterin is as potent as aminopterin as an inhibitor of dihydrofolate reductase. At the same time its ability to inhibit thymidylate synthetase is 20 times as great as that of aminopterin. Purified tetrahydroaminopterin, although it is only 0.025 as effective as aminopterin as an inhibitor of the reductase, is 10 times as effective an inhibitor of thymidylate synthetase.

**TABLE II**

<table>
<thead>
<tr>
<th>Aminopterin derivative</th>
<th>Concentration required for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>5 × 10⁻⁴</td>
</tr>
<tr>
<td>Dihydroaminopterin</td>
<td>5 × 10⁻⁴</td>
</tr>
<tr>
<td>Tetrahydroaminopterin</td>
<td>2 × 10⁻⁴</td>
</tr>
</tbody>
</table>

* This assay system (17) consists of 0.04 mM dUMP, 0.3 mM dl,L-tetrahydrofolate, 12 mM formaldehyde, 21 mM MgCl₂, 110 mM 2-mercaptoethanol, 0.75 mM EDTA, 37 mM Tris, and 20 μg of enzyme per ml, pH 7.4. The assay is read at 340 μm against a blank cuvette identical with the assay mixture except that dUMP is omitted. The change at 340 μm (about 0.1 in 10 minutes) depends on the conversion of tetrahydrofolate to dihydrofolate concomitant with thymidylate formation.

No decomposition of tetrahydroaminopterin is observed spectrophotometrically when it is incubated with the assay system minus tetrahydrofolate. However tetrahydroaminopterin must be included in the blank cuvette because, in the presence of 0.3 mM tetrahydrofolate, 0.01 mM tetrahydroaminopterin causes an absorbance increase of 0.027 during the 10-minute assay period (0.02 mM tetrahydroaminopterin causes an increase of 0.050). This increase is observed consistently and is dependent only on the presence of the two tetrahydropteridine derivatives in a solution containing 110 mM mercaptoethanol, 37 mM Tris at pH 7.4, and 0.75 mM EDTA as the only other solutes.

The inhibition of thymidylate synthetase cannot be due to inhibition of the formation of 5,10-methylenetetrahydrofolate because this material is present in the initial reaction mixture (Table II). Excess formaldehyde, which can react spontaneously with tetrahydroaminopterin, as it does with tetrahydrofolate (7), is also present. It is therefore difficult to determine whether the inhibiting compound in the mixture is tetrahydroaminopterin or its 5,10-methylene derivative.

**DISCUSSION**

The enhanced ability of dihydroaminopterin and tetrahydroaminopterin to inhibit thymidylate synthetase while retaining potent antireductase activity leads to the suggestion that a sequential block of thymidylate formation may explain the increased potency of the reduced forms. Both enzymes are required for the net formation of thymidylate under conditions where folate is present in catalytic quantities. This is true because dihydrofolate is an obligatory product of the thymidylate synthetase reaction and must be reduced to tetrahydrofolate before being reutilized (17).

While data obtained with *E. coli* and mouse tumor enzymes need not necessarily apply to the corresponding *Pediococcus* systems, evidence is available that dihydrofolate reductases from a variety of sources are inhibited by aminopterin derivatives in an analogous manner, as discussed below. Data on the inhibition of thymidylate synthetase from different sources are not yet available.

1 R. L. Kisliuk, unpublished observation.
The above suggestion of a sequential block is supported by the earlier studies of Elion, Singer, and Hitchings (18) on the inhibition of microbial growth. These authors reported that a synergistic relationship exists between agents that block the reduction of folate and the antithymines, 5-bromouracil and 6-azathymine. They did not observe such a synergistic relationship in Lactobacillus casei (ATCC 7469). It is of interest that tetrahydroaminopterin is no more potent than aminopterin in this organism.

In both the Pediococcus and thymidylate synthetase systems, dihydroaminopterin is twice as inhibitory as tetrahydroaminopterin. These observations may be in part explained by the fact that tetrahydroaminopterin is a mixture of two diastereoisomers. Conceivably only one of these is inhibitory.

The striking inhibition of P. cerevisiae by dihydroaminopterin (Fig. 8), first observed by Zakrzewski et al. (9), is noteworthy when one considers that dihydrofolate (purified by column chromatography as described under "Experimental Procedure"), due to impermeability to it (19), is only about 0.0002 as active as 5-formyltetrahydrofolate in supporting growth of this organism. Two possibilities may be considered to explain the potency of dihydroaminopterin: (a) it is taken up by the cells even though its structure is similar to dihydrofolate, which does not penetrate the cells; or (b) it is an effective inhibitor of the permease system of P. cerevisiae for tetrahydrofolate, and the enzymatic inhibitions mentioned are of secondary importance. Possibility a is favored by the observation that dihydrofolate, 1.0 µg per ml, did not block the utilization of 5-formyltetrahydrofolate present at 0.0005 of this concentration.

The possibility that the enzymatic and microbial inhibitions observed are due to decomposition products of the reduced aminopterin analogues formed during incubation must be considered. Such decomposition is not observed during the 10-minute enzymatic assays that are carried out in the presence of mercaptoethanol. In the Pediococcus incubation, it is likely that the reduced aminopterin derivatives cause a phenomenon similar to "thymineless death" (20) early in the incubation before extensive decomposition has taken place.

The results reported here on the inhibition of the growth of P. cerevisiae by tetrahydroaminopterin clearly demonstrate that tetrahydroaminopterin does not owe its inhibitory properties to contamination with dihydroaminopterin as has been suggested (9). This conclusion is supported by the fact that media incubated with purified tetrahydroaminopterin become less inhibitory with time. One would expect them to become more inhibitory if the more stable dihydroaminopterin were formed in appreciable amounts. However, contamination of tetrahydroaminopterin with dihydroaminopterin is significant in solutions stored in mercaptoethanol.

Evidence has been obtained by Ozols (cited by Huenekeens (21)) that aminopterin and dihydroaminopterin are equivalent in their ability to inhibit chicken liver dihydrofolate reductase. Tetrahydroaminopterin was about 0.02 as active. These findings are similar to those presented in Table II, except that the mouse tumor enzyme is 10 times as sensitive for each analogue. Zakrzewski et al. (9) also found aminopterin and dihydroaminopterin to give equivalent inhibition of folate acid reductase in S. faecalis and chicken liver.

The absorption spectra of dihydroaminopterin and tetrahydroaminopterin reported here are quite similar to those reported for the corresponding folate derivatives (22). However dihydroaminopterin does not have the pronounced shoulder at 310 µm observed with dihydrofolate. The molecular extinction value reported here for dihydroaminopterin is similar to that reported by Blakley for dihydrofolate (molecular extinction at 282 µm, 28,000) (23). The values given by Zakrzewski et al. (9) for dihydrofolate and dihydroaminopterin are somewhat lower (molecular extinction at 289 µm, 21,000).

**SUMMARY**

The spectra and molecular extinctions of dihydroaminopterin and dl-L-tetrahydroaminopterin have been determined. Dihydroaminopterin is twice as potent as dl-L-tetrahydroaminopterin and 100 times as potent as aminopterin in inhibiting the growth of *Pediococcus cerevisiae*. The inhibitory action of dl-L-tetrahydroaminopterin for *P. cerevisiae* cannot be ascribed to contamination with dihydroaminopterin. On the basis of enzyme studies, it is proposed that reduced analogues of aminopterin owe their increased potency to a sequential inhibition of dihydrofolate reductase and thymidylate synthetase.

**Acknowledgment**

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