THE SEPARATION AND DETERMINATION OF
PTEROYLGLUTAMIC ACID AND RELATED
COMPOUNDS*

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Metabolic alteration of certain antagonists of pteroylglutamic acid to biologically utilisable compounds has been postulated as an explanation of the apparent ability of these analogues to promote the growth of a strain of Tetrahymena (1) and an antagonist-resistant strain of Streptococcus faecalis (2). Direct investigation of the possibility that bacterial and leucemic cells, which have become resistant to potent antagonists of folic acid, are able to alter these compounds to metabolically active derivatives required the development of the procedures described herein for the separation of pteroylglutamic acid (PGA) from Aminopterin (4-amino-PGA) and A-methopterin (4-amino-10-methyl-PGA). During the use of a bioautographic technique to locate these compounds on paper chromatograms, the occurrence of considerable amounts of growth-promoting, as well as inhibitory, substances as contaminants in the antagonists was observed (3). PGA and pteroic acid were present in samples of Aminopterin and A-methopterin in sufficient amounts to account for the apparent “utilization” of these antagonists by a strain of S. faecalis (3, 4). The analysis of a sample of Aminopterin described herein indicated that the content of PGA was 24 per cent by microbial assay and 22 per cent by fluorometric assay.

Methods and Results

Bioautographic Technique—Growth-promoting and inhibitory compounds were located on paper chromatograms by appropriate modifications of the bioautographic techniques of Goodall and Levi (5) and Winsten and Eigen (6). The paper strips were placed for 10 minutes on the surface of the solid medium (folic acid assay medium (7) containing, per 100 ml., agar (1 gm.) and the washed cells from 5 ml. of a 20 hour culture of S. faecalis.

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1 The term pteroylglutamic acid refers to the synthetic compound.
The zones of growth or inhibition were visible after incubation for 6 hours at 37°, but readings were made after 18 hours. Clear zones, indicating the presence of inhibitory compounds, were distinct against a background of growth which occurred when PGA was included in the medium. When the amount of PGA (1 mg per ml.) supported only limited growth, both inhibitory and growth-promoting compounds could be readily located on a single bioautograph, although zones of growth were most distinct when PGA was omitted from the medium. The smallest amounts of PGA and Aminopterin causing visible zones were 0.5 and 5.0 mg per 1/2 inch strip, respectively. The zones became larger as the amounts of the compounds were increased until, at an amount of 1 γ of each compound per strip, the zones of Aminopterin and PGA began to overlap. The area actually occupied by the compounds on the paper chromatograms is smaller than the area of the zones observed on the bioautographs because of the diffusion of the compounds through the solid medium.

**Paper Chromatography**—Strips (1/2 inch in width) and sheets of Whatman No. 1 filter paper were developed by descending solvents, which were allowed to advance 45 to 50 cm. from the initial location of the compounds. None of the organic solvents which have been used for the chromatography of pteridines was satisfactory for the resolution of PGA and Aminopterin on paper. The systems tested were n-butanol-acetic acid-water, 8:1:1 (8), n-butanol-morpholine-water, 3:1:3 (8), 2,4,6-collidine saturated with water (6), isoamyl alcohol-5 per cent KH₂PO₄ or Na₂HPO₄ (9, 10). Aqueous solutions of 5 per cent KH₂PO₄ (10) and 3 per cent NaI₂Cl (8) gave some separation when the amounts of PGA and Aminopterin per strip were 50 mg or less of each. Solutions of Na₂HPO₄ gave better resolution of these compounds than solutions of KH₂PO₄. A more detailed comparison with various phosphate and acetate buffers (0.1 M) showed a definite relationship between the pH of the solvent and the movement of the compounds on the paper strips (Fig. 1). Good resolution of PGA and Aminopterin was obtained with neutral phosphate (0.1 M, pH 6.2 to 7.7) or acetate buffer (0.1 M, pH 6.3); as the pH of the acetate buffer was lowered, the relative positions of the two compounds tended to be reversed (Fig. 1). A greater mobility of the compounds was obtained with more dilute solvents. Aminopterin and PGA were located at Rₚ 0.22 and 0.41, respectively, when the strips were developed with a 0.05 M solution of neutral phosphate. When distilled water was used, Aminopterin moved to Rₚ 0.74 and PGA to Rₚ 0.82. The compounds were eluted readily from the paper by distilled water.

Analogues of PGA of the 4-amino and 10-methyl series were chromatographed on paper strips with 0.1 M phosphate buffer of pH 7.0 as the
developing solvent. The distribution of the compounds is shown in Table I. A-Methopterin and 10-methylpteroic acid, which had the same $R_F$ when developed by neutral phosphate, moved at different rates when de-

![Fig. 1. Effect of pH on the separation of pteroylglutamic acid (PGA) from Aminopterin (4-NH$_2$-PGA) on paper chromatograms.](image)

**TABLE I**  
*Distribution of Pteroylglutamic Acid, Pteroic Acid, and Their 4-Amino and 10-Methyl Analogues on Paper Chromatograms*

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_F$ Average</th>
<th>$R_F$ Limits of zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pteroic acid</td>
<td>0.04 (G.)</td>
<td>0.01-0.10</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>0.16 (I.)</td>
<td>0.12-0.20</td>
</tr>
<tr>
<td>4-Amino-10-methylpteroic acid</td>
<td>0.33 (&quot;&quot;)</td>
<td>0.27-0.39</td>
</tr>
<tr>
<td>PGA</td>
<td>0.37 (G.)</td>
<td>0.30-0.44</td>
</tr>
<tr>
<td>10-Methylpteroic acid</td>
<td>0.54* (I.)</td>
<td>0.47-0.62</td>
</tr>
<tr>
<td>A-Methopterin</td>
<td>0.55* (&quot;&quot;)</td>
<td>0.48-0.62</td>
</tr>
<tr>
<td>10-Methyl-PGA</td>
<td>0.75 (&quot;&quot;)</td>
<td>0.69-0.81</td>
</tr>
<tr>
<td>Citrovorum factor</td>
<td>0.74 (G.)</td>
<td>0.67-0.81</td>
</tr>
</tbody>
</table>

The letters in parentheses designate the nature of the zone, G. indicating growth and I. indicating inhibition.

* With acetate buffer of pH 5.0 as the developing solvent, 10-methylpteroic acid was located at $R_F$ 0.51 and A-methopterin at $R_F$ 0.68.
PTEROYLGLUTAMIC ACID

developed by acetate buffer of pH 5.0. Citrovorum factor (leucovorin) was readily separated from the 4-amino antagonists and from the other compounds which have activity for S. faecalis, namely PGA and pteroic acid (Table I). The movement of citrovorum factor on the paper is not influenced significantly by the pH of the developing solvent ($R_F$ 0.72 with 0.1 M K$_2$HPO$_4$ and $R_F$ 0.71 with 0.1 M KH$_2$PO$_4$).

Fluorometric Assay—The oxidation of pteroylglutamic acid to an intensely fluorescent derivative, pteroyl-6-carboxylic acid, was used by Allfrey et al. (11) as the basis of a quantitative assay of PGA. This method has been modified by prolonging the treatment with KMnO$_4$ to 1 hour in order to insure complete oxidation of PGA; constant readings were obtained with periods of from 1 to 4 hours. Readings were compared with a PGA standard within the range of 10 to 100 μg per ml. with a Farrand fluorometer (model A). The sensitivity of the instrument was adjusted so that a solution of quinine sulfate (2.4 μg per ml.) gave a galvanometer deflection of 70 units. The calculations are based on the assumption that 1 mole of pteroyl-6-carboxylic acid is derived from each mole of the pteridine compounds which were studied. Oxidation of citrovorum factor (leucovorin) resulted in the formation of a product which had little or no fluorescence.

Solutions of Aminopterin and A-methopterin gave lower fluorometric readings than PGA when assayed directly by this method (Fig. 2). After hydrolysis in 1 N NaOH for 15 minutes at 15 pounds pressure, Aminopterin and A-methopterin gave fluorometric readings which were identical to those of equimolar solutions of PGA (Fig. 2). Since the salt resulting from the neutralization of the alkaline solutions caused slight quenching of the fluorescence (11), the samples and pteroylglutamic acid which was used as a reference standard were subjected to identical treatment. In order to minimize the quenching effect of the salt, relatively concentrated solutions of the compounds were hydrolyzed, then diluted by 1:100 or more before oxidation with permanganate.

Microbial Determination of Pteroylglutamic Acid Activity—Turbidimetric readings of the growth of S. faecalis (ATCC 8043) after incubation for 20 hours in 10 ml. of the assay medium (7) were made in a Klett-Summerson colorimeter with filter No. 66. A sample of pteroylglutamic acid was used as a reference standard. The alkaline treatment described above did not alter the microbial potency of PGA (Fig. 3). The product of the alkaline treatment of Aminopterin had the same microbial potency as PGA (Fig. 3).

Analysis of Aminopterin—Several of the antagonists of PGA have been shown to contain growth-promoting compounds as contaminants (3, 4). The Aminopterin which is presently available contained both PGA and pteroic acid. A sample of Aminopterin was analyzed by the methods described above, with the results which are shown in Table II. Aminop-
pterin (100 γ) was chromatographed on each of two sheets (18 × 22\frac{1}{4} inches) of Whatman No. 1 filter paper. The compounds were located on

![Graph showing effect of alkaline treatment](http://www.jbc.org/)

**Fig. 2.** Effect of alkaline treatment of pteroylglutamic acid (●), Aminopterin (■), and A-methopterin (▲) on the fluorescence of their oxidation products. The solid symbols indicate the fluorometric readings of the solutions which were oxidized directly, the open symbols the fluorometric readings of the solutions oxidized after autoclaving the compounds at 15 pounds pressure for 15 minutes in 1 N NaOH. Fluorometric readings of equimolar amounts of the compounds are plotted against a scale indicating the concentration of PGA.

![Graph showing microbial activity](http://www.jbc.org/)

**Fig. 3.** Microbial activity of an alkaline hydrolysate of Aminopterin. X, turbidimetric readings of the growth of *S. faecalis* in response to pteroylglutamic acid; □, Aminopterin which was autoclaved at 15 pounds pressure for 15 minutes in 1 N NaOH; and O, pteroylglutamic acid which was treated similarly with alkali.

bioautographs of strips which were cut from one of the sheets. Eluates from sections of the other paper were treated with alkali (1 N) for 15 minutes at 15 pounds pressure and then assayed by the fluorometric and the
microbial procedures. The eluate from the Aminopterin zone of a duplicate sheet which was rechromatographed and assayed in the same manner contained considerably less PGA than the commercial material (Table II). Purification of Aminopterin was attempted with columns of cellulose powder (Whatman, standard grade). Aminopterin (3.0 mg.) was loaded on a cellulose column (3.5 X 50 cm.) which was developed with 0.05 M phosphate buffer of pH 7.5. Fractions of the effluent between 610 and 790 ml. contained PGA (about 20 per cent of the material). Fractions between

![Image](http://www.jbc.org/)

### Table II

**Analysis of Commercial and Chromatographed Aminopterin by Fluorometric and Microbial Assays**

<table>
<thead>
<tr>
<th>Compound eluted</th>
<th>Section eluted</th>
<th>Assay of eluates obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aminopterin, commercial grade</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorometric</td>
</tr>
<tr>
<td>Pteroic acid</td>
<td>0 - 0.12</td>
<td>1.1†</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>0.12 - 0.30</td>
<td>64†</td>
</tr>
<tr>
<td>PGA</td>
<td>0.30 - 0.53</td>
<td>24†</td>
</tr>
<tr>
<td>Inactive microbially</td>
<td>0.53 - 1.0</td>
<td>2.6†</td>
</tr>
</tbody>
</table>

* Identification based upon the $R_F$ of the individual compounds.
† These amounts are calculated from low fluorometric readings and therefore are somewhat less accurate than the other tabulated values.

1010 and 1620 ml. contained Aminopterin, which was shown by microbial assay to contain 1.2 per cent PGA.

### Discussion

None of the 4-amino antagonists of PGA which are presently available is suitable as a reference standard for microbial or fluorometric assays, since they have been shown to contain several components (3). PGA is the main contaminant in Aminopterin, although pteroic acid is present as well. The main contaminant in A-methopterin is 10-methyl-PGA; it also contains small amounts of 4-amino-10-methylpteroic acid, Aminopterin, and PGA (3). The 4-amino compounds are oxidized by KMnO$_4$ to a product which has a lower intensity of fluorescence than the product derived from PGA by similar treatment (Fig. 2). Seeger et al. described a procedure for the alkaline hydrolysis of Aminopterin to PGA; similar treat-
ment of A-methopterin yielded 10-methyl-PGA (12). The hydrolysis of Aminopterin described here yielded a material which had the same microbial potency as PGA. The methyl group present in the 10 position of hydrolyzed A-methopterin did not prevent the oxidation of this compound to a product which had the same intensity of fluorescence as the product derived from PGA (Fig. 2). This procedure is applicable to PGA, pteroic acid, and their 4-amino and 10-methyl analogues, which can be separated clearly on paper. The method is discussed in detail with respect to PGA and Aminopterin, since the separation of the 4-hydroxy and 4-amino compounds is a more critical test of the procedure than is the separation of compounds of less similarity in structure.

The presence of a large amount of PGA in the commercial grade of Aminopterin obviously complicates studies of possible mechanisms of resistance to the antagonist. Considerable purification of the compound was effected by chromatography on paper sheets or cellulose columns, but small amounts of PGA remained following such procedures. Sufficient amounts of PGA and pteroic acid have been demonstrated to be present in a number of antagonists (3, 4), for which deamination and demethylation steps were postulated, to account for the apparent utilization of these antagonists by a resistant strain of S. faecalis (2) and by Tetrahymena gelii (1). The methods described here are being applied to studies of the mechanisms involved in the development of resistance to folic acid analogues by bacterial and leucemic cells.

SUMMARY

Pteroylglutamic acid and its 4-amino analogue (Aminopterin) can be separated by paper chromatography, depending upon the pH of the aqueous solvent. A fluorometric assay of Aminopterin and A-methopterin involves (a) alkaline hydrolysis, which replaces the amino group in position 4 with a hydroxyl group, and (b) oxidation with KMnO₄ to a product, presumably pteroyl-6-carboxylic acid, which has the same intensity of fluorescence as the product derived from PGA by similar treatment. This procedure is applicable to PGA, pteroic acid, and their 4-amino and 10-methyl analogues.

Analysis of a sample of Aminopterin showed that it contained PGA to the extent of 24 per cent, by fluorometric assay, and 22 per cent by microbial assay. The significance of the presence of growth-promoting compounds as contaminants in the antagonists is discussed with respect to concepts of the biological utilization of these antagonists.

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BIBLIOGRAPHY

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