The Metabolism of Methotrexate in *Lactobacillus casei* and Rat Liver and the Influence of Methotrexate on Metabolism of Folic Acid*

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SUMMARY

[1H]Methotrexate was metabolized mainly to 4-amino-4-deoxy-10-methylpteroyl acid in resting cells of *Lactobacillus casei* and a small amount of methotrexate remained inside the cells after 24 hours of incubation. Preincubation of *L. casei* with methotrexate inhibited the incorporation of [1H]folic acid completely and 1-5-formyl-[1H]tetrahydrofolic acid partially into pteroylglutamate and increased the amount of pteroylglutamate and lower polyglutamates.

In rats, methotrexate was metabolized partially to lower methotrexate polyglutamates; however, its uptake was much lower than folic acid and no pentaglutamate of methotrexate was found. Treatments of rats with 100 μg of methotrexate daily for 5 days decreased the uptake of [1H]folic acid into liver considerably (~20 to ~8%) and the radioactivity in liver was mainly reduced derivatives of pteroylpentaglutamate. With treatment with 300 μg of methotrexate per day for 6 days, however, not only the uptake of [1H]folic acid decreased to ~4%, but also pteroylpentaglutamate comprised only ~50% of total radioactivity in liver and the rest was lower polyglutamate and unmetabolized monoglutamate.

Column chromatography with Sephadex G-25 and DEAE-cellulose provides a good method for the identification of methotrexate-related compounds.

Previous studies from this laboratory have shown that reduced derivatives of pteroylglutamate are the major folate constituents in rat liver (1) and bacteria (2). *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043) convert [1H]folic acid almost quantitatively to its polyglutamate derivatives in both growing and resting cells. On the other hand, methotrexate, one of the most commonly used antineoplastic agents with a broad spectrum of antitumor activity (3, 4), has been understood to be metabolically inert and is excreted into urine unchanged in man and animals (5). Hydroxylation enzymes which metabolize methotrexate and its analog are present in guinea pig and rabbit (6). Recently Valerino (7) found another methotrexate metabolite, 4-amino-4-deoxy-10-methylpteroyl acid in rat urine and feces, and Baugh and co-workers (8) have characterized methotrexate as being a substrate for the polyglutamate synthetase in rats.

Griffin and Brown (9) reported that only tetrahydrofolic acid is the substrate for the polyglutamate synthetase in *Escherichia coli*. Spronk (10) and Sakami et al. (11) also found tetrahydrofolic acid is the substrate for the enzyme in rat liver and *Neurospora crassa*, respectively. However, Corrocher and Hoffbrand (12) made the observation that methotrexate-treated guinea pigs converted at least some folic acid into nonreduced pteroyl-, tri-, and polyglutamates and concluded reduction of pteroylglutamic acid is, therefore, not essential for the formation of polyglutamates.

In the present work we examined the metabolism of [1H]methotrexate in resting cells of *L. casei* and *S. faecalis* and in rat liver. Separation procedures of various metabolites of methotrexate and related compounds with Sephadex and DEAE-cellulose column chromatography were examined.

We also investigated folate metabolism in methotrexate pretreated rats and resting cells of *L. casei* to study whether reduction of the pterin moiety in folic acid is necessary for polyglutamate synthesis.

MATERIALS AND METHODS

Preparation of Bacteria—The organisms were grown in the medium used for microbiological assay (13) in the presence of 1 ng of folic acid per ml. After 24 hours of incubation at 37°, the cells were harvested by centrifugation (2000 × g, 4°) and washed with a buffer solution containing 20 g of glucose, 20 g of sodium acetate, 0.5 g of K₂HPO₄, 0.5 g of KH₂PO₄, 1.0 g of ascorbic acid, and 10 mg of L-glutamic acid in a volume of 1 liter.

Cells obtained from 100 ml of medium (~50 mg dry cell weight) were suspended in 10 ml of the buffer solution described above and incubated at 37° in the presence of 5 μCi of [1H]methotrexate (specific activity: 12.4 Ci per mmole, from Amersham-Searle purified over QAE-Sephadex column (2)). For the study of folic acid metabolism, cells were preincubated with various amounts of methotrexate for 10 min at 37° and then incubated with 10 μCi of [1H]folic acid (40 Ci per mmole, from Amersham-Searle Co. purified over QAE-Sephadex column).

At the end of the incubation period, cells were collected by centrifugation, washed twice with ice-cold buffer, suspended in 30 ml of acetone-0.2 M 2-mercaptoethanol, and kept 1 hour at 37°. The radioactivity in the medium and washings was counted with Aquasol (New England Nuclear) as scintillation liquid. After removing the acetone by centrifugation (acetone extract), cell residues were washed several times with 0.2 M 2-mercaptoethanol (water extract) to extract metabolites of methotrexate and folic acid.
Animals—Male Sprague-Dawley rats weighing 300 to 350 g were maintained for 2 weeks on a commercial diet (Purina rat chow from Ralston Purina Co.), and thereafter treated by injection intraperitoneally of 20 μCi of methotrexate every day for 5 days. The animals were killed by decapitation 24 hours after the last injection and livers were removed immediately, cut into thin slices, and placed in boiling 0.1% ascrobate solution for 7 min to inactivate intracellular conjugase. The mixtures were cooled in ice and cell debris removed by centrifugation. In another experiment, two animals were injected with 100 μg of methotrexate daily for 5 days, and on the fifth day, in addition to methotrexate, 20 μCi of [3H]folate acid were given intraperitoneally. The animals were killed 24 hours thereafter and liver folates were extracted as described above.

Column Chromatography—The same chromatographic procedures for the separation of folic acid compounds was used for methotrexate and its metabolites according to the procedure employed in our laboratory (1, 14). DEAE-cellulose columns (0.9 × 30 cm) were eluted with an exponential gradient of 0.01 M potassium phosphate buffer (pH 6.0) to 0.5 M potassium phosphate buffer (pH 7.0). The eluant buffer for Sephadex column (0.75 × 200 cm) was 0.1 M potassium phosphate buffer (pH 7.0). All the buffers contained 0.2 M 2-mercaptoethanol.

4-Amino-4-deoxy-10-methylpteroyl acid was prepared according to the procedure of Levy and Goldman (18). 4-Amino-4-deoxy-10-methylpteroyl acid and 10-methylfolic acid were prepared by hydrolysis of 4-amino-4-deoxy-10-methylpteroyl acid and methotrexate, respectively (10). 1-5-Formylmethyl-[3H]PteGlu and 1-5-methyl-H[13H]PteGlu with specific activity of ~40 Ci per mmole were synthesized by incubating S. faecalis L. casei with [3H]PteGlu, respectively (2). [14C]Pteroyl polyglutamates (1 mCi per mmole) were synthesized by solid phase synthesis (17).

Conjugase Treatment and Microbiological Assay—Conjugase treatment of folic acid compounds was made according to Bird et al. (18) with crude hog kidney conjugase preparation (19). Microbiological assays with L. casei (ATCC 7469), S. faecalis (ATCC 8043), and Pediococcus cerevisiae (ATCC 8081) were carried out according to the method employed in this laboratory (13). The dilution of the sample and measurements of bacteria growth were done by the use of an automated microbiological assay machine.

RESULTS

Similar to folic acid, methotrexate was found to be strongly retarded on Sephadex G-25 and eluted after the total column volume (Table I). Substitution with an amino group at the C-4 position of folic acid, however, resulted in even stronger interaction with the gel matrix, i.e. methotrexate or aminopterin was eluted far later than folic acid, whereas introduction of a methyl group at the N-10 position of folic acid had little influence on its chromatographic behavior. 10-Methylfolic acid was eluted at about the same position as folic acid, and methotrexate at a similar position as aminopterin. Due to the low solubility of pteroyl acid at pH 7, this compound could not be eluted with 0.1 M phosphate buffer (pH 7.0), so its Kav value was determined by using 0.1 M glycine-NaOH buffer (pH 7.0).

Fig. 1 shows the removal of [3H]methotrexate from the medium by resting cells of Lactobacillus casei (L.C.) and Streptococcus faecalis (S.F.).

![Fig. 1. Removal of [3H]methotrexate from the medium by resting cells of Lactobacillus casei (L.C.) and Streptococcus faecalis (S.F.).](image)

Fig. 2 shows the chromatographic pattern of the water extract of L. casei cells after 24 hours of incubation with [3H]methotrexate (MTX). A, Sephadex G-25 column (0.75 × 200 cm) chromatography with 0.5 μmoles of aminodeoxymethyl pteroyl acid, 1.2 μmoles of methotrexate and 0.3 μmoles of 10-CH3PteGlu. B, DEAE-cellulose column (0.9 × 25 cm) chromatography with 1.2 μmoles of aminodeoxymethyl pteroyl acid, 2 μmoles of methotrexate, and 0.6 μmoles of 10-CH3PteGlu. Gradient, 230 ml of 0.01 M phosphate buffer (pH 6.0) to 0.6 M phosphate buffer (pH 6.0).

![Fig. 2. The elution pattern of radioactivity in the water extract of Lactobacillus casei cells after 24 hour of incubation with 5 μCi of [3H]methotrexate (MTX). A, Sephadex G-25 column (0.75 × 200 cm) chromatography with 0.5 μmoles of aminodeoxymethyl pteroyl acid, 1.2 μmoles of methotrexate and 0.3 μmoles of 10-CH3PteGlu. B, DEAE-cellulose column (0.9 × 25 cm) chromatography with 1.2 μmoles of aminodeoxymethyl pteroyl acid, 2 μmoles of methotrexate, and 0.6 μmoles of 10-CH3PteGlu. Gradient, 230 ml of 0.01 M phosphate buffer (pH 6.0) to 0.6 M phosphate buffer (pH 6.0).](image)

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kav&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Kav&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>10-Methylfolic acid</td>
<td>1.38</td>
<td>1.63</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.76</td>
<td>2.86</td>
</tr>
<tr>
<td>10-Methylpteroyl acid</td>
<td>2.95</td>
<td>2.56</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>2.95</td>
<td>2.56</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>2.95</td>
<td>2.56</td>
</tr>
<tr>
<td>Pteroyl acid</td>
<td>2.95</td>
<td>2.56</td>
</tr>
<tr>
<td>4-Amino-4-deoxy-10-methylpteroyl acid</td>
<td>4.25</td>
<td>2.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> Column size, 0.75 × 200 cm.

<sup>b</sup> Eluant, 0.1 M potassium phosphate buffer (pH 7.0) with 2-mercaptoethanol. Kav = Vx - Vs/Vx + Vt, where Vx is the exclusion volume, Vs the elution volume, and Vt the volume of the gel bed.

<sup>c</sup> Eluant, 0.1 M glycine-NaOH buffer (pH 10.0).

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<sup>1</sup> Gift from Dr. J. R. Bertino, Yale University.

<sup>2</sup> The abbreviations used are: PteGlu, folic acid; 1-5-formylmethyl-HPteGlu, 1-5-methyl-HPteGlu, 1-5-formyltetrahydrofolic acid; 1-5-methyl-HPteGlu, 1-5-methyltetrahydrofolic acid.

<sup>3</sup> J. E. Watson and E. L. R. Stokstad, manuscript in preparation.
FIG. 3. Sephadex G-25 column chromatography of acetone extract of *Lactobacillus casei* with 1 μ mole of aminodeoxymethylpteroic acid (A) and medium radioactivity with 0.7 μ mole of aminodeoxymethylpteroic acid (B). Acetone was removed under reduced pressure and the residue dissolved in 0.1 M potassium phosphate buffer (pH 7.0); column size, 0.75 × 200 cm; flow rate, 14 ml per hour. Peaks I and II are unidentified products, Peak III is 10-methylpteroyl acid, and Peak IV is aminodeoxymethylpteroic acid. MTX, methotrexate.

**TABLE II**

<table>
<thead>
<tr>
<th>Metabolites of methotrexate in <em>Lactobacillus casei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>10-Methylfolic acid</td>
</tr>
<tr>
<td>Methotrexate</td>
</tr>
<tr>
<td>10-Methylpteroyl acid</td>
</tr>
<tr>
<td>4-Amino-4-deoxy-10-methylpteroyl acid</td>
</tr>
<tr>
<td>Unidentified products</td>
</tr>
</tbody>
</table>

* L. casei (50 mg dry cell weight) was incubated in the presence of 5 μCi of [3H]methotrexate (specific activity: 12.4 Ci per mmole) at 37° for 24 hours. Results are given as percentage of the initial radioactivity.

Radioactivity in the acetone washings was almost completely accounted for by aminodeoxymethylpteroic acid, which, contrary to methotrexate and folic acid, is quite soluble in acetone, and it was also the major metabolite in the buffer medium, comprising more than 50% of the total radioactivity (Table II and Fig. 3B). The concentration of methotrexate inside the cell after 24 hours of incubation was far greater than in the buffer medium, suggesting that intracellular methotrexate was tightly bound, presumably in part by dihydrofolate reductase.

Less than 5% of the labeled methotrexate was taken up by resting cells of *S. faecalis* in 24 hours and more than 90% of the radioactivity inside the cell represented unchanged methotrexate, the amount of other metabolites being too small for identification. The radioactivity in the medium represented exclusively methotrexate. In logarithmically growing cell cultures of *S. faecalis*, the uptake of [3H]methotrexate was somewhat higher (5 to 7% of the applied radioactivity), but still much smaller than uptake of [3H]folic acid (~40 to 50% of the applied radioactivity after 20 min of incubation) under the same conditions. Methotrexate itself at concentrations 20 times higher than [3H]folic acid did not inhibit the uptake of folic acid, suggesting that folic acid and methotrexate may not share the same uptake system or that the affinity of folic acid for the transport system was much greater than for methotrexate. The lower uptake of methotrexate by *S. faecalis* compared to *L. casei* may also explain its higher toxicity for *L. casei*. Although methotrexate at a level of 0.1 ng per ml inhibited the growth of *L. casei* completely (Fig. 4A), almost 100 times more was required for complete growth inhibition of *S. faecalis* (Fig. 4B).

Fig. 5 shows the influence of various amounts of methotrexate on the conversion of folic acid to its polyglutamates in resting *Lactobacillus casei* cells. Cells grown in 50 ml of medium (~25 mg dry weight) were incubated with ~330,000 cpm (~1 ng per ml) [3H]folic acid with different levels of methotrexate preincubation. O—O, with no methotrexate, Δ--Δ, with 1 ng, and □——□, with 10 ng of methotrexate per ml of medium, respectively. a, the elution position of PteGlu; b, of PteGlu, c, of PteGlu under comparable conditions. Sephadex C-15 columns, 0.75 × 200 cm; eluant, 0.1 M phosphate buffer (pH 7.0).
The metabolism of reduced derivatives of folic acid in L. casei in the presence of methotrexate was studied using 1-5-formyl-[3H]PteGlu as substrate. Whereas methotrexate at a level of 1 µg per ml completely inhibited the incorporation of [3H]folic acid (1 ng per ml) into the resting cell, more than 55% of the initial radioactivity was found inside the cell when 5-formyl-H₄PteGlu (1 ng per ml) was used as substrate under the same conditions (2 hours of incubation at 37°C, ~5 mg dry cell weight per ml). Methotrexate at a concentration of 100 ng per ml, 100 times higher than the concentration of 5-formyl-H₄[3H]PteGlu, inhibited the formation of higher polyglutamates (Fig. 6), but to a lesser extent than when folic acid was used as a substrate (Fig. 5). Even in the presence of 1 µg per ml of methotrexate there was still formation of higher polyglutamate forms of folic acid from 5-formyl-H₄PteGlu.

The metabolism of methotrexate in rat liver was investigated after intraperitoneal injection of [3H]methotrexate for 5 days. By this time, 70% of the radioactivity incorporated into the liver consisted of unmetabolized methotrexate (Fig. 7). Approximately 30% of the radioactive material appeared before methotrexate with Sephadex G-25 and after methotrexate with DEAE-cellulose columns (Fig. 7). After treatment of this material with hog kidney conjugase it was identified as methotrexate by co-chromatography with the authentic compound, indicating that Peaks I and II in Fig. 7A are polyglutamate forms of methotrexate. The incorporation of [3H]methotrexate into liver and brain was considerably lower than that of folic acid under comparable conditions (Table III).

To study the influence of methotrexate on folate metabolism, rats after receiving 100 µg of methotrexate daily for 5 days were given an injection of [3H]PteGlu on the fifth day. The uptake of [3H]PteGlu into the liver was lower than in rats without methotrexate treatment (Table III); however, in both cases the major folate derivatives were derivatives of PteGlu. The fractions eluted at the position of PteGlu from the Sephadex column were combined, and subjected to the treatment with hog kidney conjugase. After treatment with conjugase only small amounts of the radioactive material eluted at the position of PteGlu and H₄PteGlu, the major forms were 5-methyl-H₄PteGlu, H₂PteGlu, and formyl forms of H₄PteGlu (Fig. 9), which are similar to the forms found in the liver of animals not treated with methotrexate. Whether the small amount of radioactivity, which eluted at the position of PteGlu, originated from PteGlu or was formed by oxidative degradation of H₄PteGlu was not examined. When rats were treated with 300 µg of methotrexate daily for 6 days, the uptake of [3H]folic acid was decreased to 4% and pentaglutamate fraction in liver to 50% of total liver radioactivity (Table III).

**DISCUSSION**

The metabolism of methotrexate in L. casei was similar to that described by Levy and Goldman (15) in Pseudomonas. However, in contrast to the *Pseudomonas* and *Flavobacterium* (20),...
which can use the glutamic acid portion of folic acid as the sole source of energy and carbon, *L. casei* was not able to convert folic acid to pteroic acid, even in the presence of a large excess of methotrexate.

**TABLE III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage of doses injected</th>
<th>Liver$^a$</th>
<th>Brain$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate$^c$</td>
<td>0.7 ± 0.21</td>
<td>0.012 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>PteGlu$^d$</td>
<td>21.3 ± 3.77</td>
<td>0.220 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>PteGlu$^e$</td>
<td>7.6 ± 1.47</td>
<td>0.070 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>PteGlu$^f$</td>
<td>3.9 ± 1.40</td>
<td>0.063 ± 0.010</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D. from three animals.

$^b$ Injected with 20 μCi of methotrexate (12.4 Ci per mmole) 24 hours before being killed.

$^c$ Injected with 20 μCi of PteGlu (40 Ci per mmole) 24 hours before being killed.

$^d$ PteGlu, 20 μCi (40 Ci per mmole), was injected after the treatment with 100 μg of methotrexate daily for 5 days.

$^e$ PteGlu, 20 μCi (37 Ci per mmole), was injected after the treatment with 500 μg of methotrexate daily for 5 days.

The conversion of methotrexate to aminodeoxymethylpterinic acid was relatively slow, unlike its uptake and the uptake and metabolism of folic acid (2). Even after 24 hours of incubation, considerable amounts of methotrexate were present inside the cell. This may have been bound to dihydrofolate reductase and thus may have been protected from enzymatic conversion to aminodeoxymethylpterinic acid.

Methotrexate at a level of 10 ng per ml of buffer medium in resting cells inhibited the conversion of PteGlu to polyglutamate forms of folic acid with more than 5 glutamic acid residues. Unreduced polyglutamate forms of folic acid with less than 5 glutamic acid residues were formed instead. This result may be interpreted that the formation of higher polyglutamates may require prior reduction of folic acid to the tetrahydro level, as was demonstrated by Griffin and Brown (9) in *Escherichia coli* and by Sakami et al. (11) in *Neurospora crassa*. Our results further suggest that there might be two different enzymes for the conversion of folic acid to its polyglutamate forms. One enzyme would be capable of converting H$_4$PteGlu as well as PteGlu to folic acid polyglutamates with less than 5 glutamic acid residues, whereas the other would require reduced forms of folic acid for the formation of higher polyglutamates. A two-enzyme system for the synthesis of folic acid polyglutamates was recently described by Sakami et al. (11).

The inhibition of the conversion of 5-formyl-H$_4$PteGlu to higher polyglutamate forms by methotrexate may have been an indirect effect resulting from the inhibition of the reduction of dihydrofolate acid to tetrahydrofolate acid, which may be the major cofactor for polyglutamate synthetase. 5-Formyl-H$_4$PteGlu may be metabolized to 5,10-methylene-H$_4$PteGlu and subsequently converted partially to dihydrofolate acid by thymidylate synthetase. However, the other possibility, that methotrexate acted as an inhibitor of the enzyme or enzymes responsible for the formation of polyglutamates, cannot be eliminated.

In rat liver, methotrexate was partially metabolized to polyglutamate forms of methotrexate. These findings are in agreement with the results previously obtained by Baugh et al. (8). However, its uptake into liver and its conversion to polyglutamates were not significant at all compared to folic acid. Methotrexate itself may not be the substrate for the polyglutamate synthetase in liver which converts derivatives of PteGlu to PteGlu forms almost quantitatively. When rats were treated with 100 μg of methotrexate daily for 5 days, the proportion of different forms of folic acid in the liver did not differ from those found in untreated rats (Fig. 8), although the uptake of folic acid...
has decreased significantly (Table III). In contrast, a preliminary study from our laboratory shows that polyglutamate fractions in rat brain are decreased significantly after treatment with the same doses of methotrexate. This observation suggests that either dihydrofolate reductase in liver is regenerating very fast or being induced, or the block of dihydrofolate reductase in liver was not complete with 100 μg of methotrexate, which was less than half of the median lethal dose (LD₅₀) (21). However, when the methotrexate dose was increased to 300 μg daily, inhibition of conversion of [³H]folate acid to pentaglutamate did occur in rat liver. If only monoglutamate is permeable to the cell membrane and polyglutamates are not freely exchangeable, then low net uptake of folic acid into liver in methotrexate pre-treated rats would be a consequence of any partial inhibition of polyglutamate formation by methotrexate. In this case, a larger proportion of monoglutamate would be expected, but not observed, as they may be transported out of the liver into serum and in turn excreted into urine.

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