Polyglutamyl Derivatives of Folate as Substrates and Inhibitors of Thymidylate Synthetase*

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SUMMARY

(1)-Tetrahydropteroylglutamate and (1)-tetrahydropteroylhexaglutamate were prepared and tested as substrates for thymidylate synthetase (EC 2.1.1.6) (methylenetetrahydrofolate: deoxyuridylate C-methyltransferase) from Lactobacillus casei. Both tetrahydropteroylglutamates were more effective substrates than (1)-tetrahydropteroyglutamate, enhancing the reaction rate 3-fold when compared at 10-2 M.

Pteroylpolyglutamates and their corresponding dihydro and (d)-tetrahydro forms were inhibitors of the enzyme, the inhibitory potency increasing with the number of glutamyl residues. The concentration for 50% inhibition with pteroylglutamate was 1.5 x 10-4 M and for pteroylhexaglutamate 6 x 10-7 M. Inhibition by pteroylhexaglutarate, but not by pteroylglutamate, was abolished in the presence of 0.4 M NaCl. Inhibition obtained with dihydropteroylhexasglutamate and dihydropteroylglutamate (50% at 3.2 x 10-1 M) is sufficient to warrant consideration of these compounds as physiological regulators of thymidylate formation. Pteroylpolyglutamates on thymidylate synthetase and dihydrofolate reductase.

METHODS

The various resin-bound glutamyl derivatives of pteroic acid were prepared by the solid phase peptide-synthesis procedures as previously described (6, 7). Resin-bound p-aminobenzoylglutamate was prepared by coupling the mixed anhydride formed from N-trifluoroacetyl-p-aminobenzoic acid and isobutylchloroformate with resin-bound hexapeptide. To remove the resin the fully protected product was transferred to a 150-ml screw-cap centrifuge bottle. Fifty milliliters of 2.0 M sodium hydroxide, which had previously been degassed by boiling and cooling under N2, were then added followed by 50 ml of p-dioxane. The tube was tightly capped, and the suspension was shaken vigorously by hand for 1 hour. At the end of this period the reaction vessel (still capped) was placed in a 50° water bath for 15 min. This treatment provides a nearly quantitative cleavage of the product from the resin and completely removes the benzyl ester and trifluoracetyl-protecting groups. The product was separated from the resin by filtration. The filtrate was adjusted to pH 7.0, diluted 10-fold with water, and purified by DEAE-cellulose CI- ion exchange chromatography. The products so obtained were characterized after acid hydrolysis by quantitative ninhydrin assay. The glutamyl derivatives of pteroic acid were further characterized by ultraviolet spectra, their ability to serve as substrates for enzymically purified preparations of pteroylglutamyl-γ-(glutamyl)-glutamic acid carboxypeptidase from hog kidney (8), and finally by their ability to support growth of the folate-requiring bacteria Streptococcus faecalis (ATCC 8043) and Lactobacillus casei (ATCC 7469). Full folate acid equivalent activity was obtained for those products possessing more than 3 glutamyl residues after enzymic digestion with the enzyme preparation mentioned above.

Dihydropteroylglutamate derivatives were prepared by diethanolamine reduction in mercaptoethanol (9).
(dL)-Tetrahydropteroylglutamate derivatives were prepared by catalytic hydrogenation in neutral aqueous solution (10).

Km values calculated from abscissa intercepts of Lineweaver-Burk plots were 7.7 X 10^-7, 6.1 X 10^-5, and 2.5 X 10^-5 for (dL)-tetrahydropteroylglutamate, (dl)-tetrahydropteroylglutamate, and (l)-tetrahydropteroyltriglutamate, respectively, at 0.3 M NaCl. Since reaction rates vary considerably with pH as well as NaCl concentration, the Km values are given only as an estimate of relative affinities under comparable conditions. Vmax values calculated from ordinate intercepts under the same conditions were 0.015, 0.02, and 0.025 A units per min, respectively. The rate enhancement obtained with (l)-tetrahydropteroyltriglutamate or (l)-tetrahydropteroylhexaglutamate over (dL)-tetrahydropteroylglutamate varies from 180% at 2 x 10^-4 to 50% at 1.5 x 10^-4 M.

All of the tetrahydropteroylglutamate derivatives are assumed to be present as their 5,10-methylene derivatives.

Inhibition of Thymidylate Synthetase by Pteroylpolyglutamates—Pteroylpolyglutamates and their corresponding dihydro and (dL)-tetrahydro forms were inhibitors of thymidylate synthetase, the inhibitory potency increasing with the number of glutamyl residues, and dihydro derivatives with losses of activity or absolute configuration. In all cases, glutamyl residues have the L configuration.
synthetase reaction this inhibition is likely to be of physiological significance.

(d)-Tetrahydropteroylpolyglutamates (Fig. 3, Table I) are also quite inhibitory, and their presence cannot be ignored as is usually done in studies employing (dl)-tetrahydropteroylglutamate.

(dl)-Tetrahydropteroylxegalglutamate (Fig. 3, Table I) at $10^{-4}$ M causes greater inhibition of the standard assay system than $10^{-4}$ M (dl)-tetrahydropteroylxegalglutamate. A reasonable explanation for this result would be that since (l)-tetrahydropteroylxegalglutamate has a higher affinity for the enzyme than the (l)-tetrahydropteroylxegalglutamate in the standard assay system, dihydropteroylxegalglutamate would be formed early in the reaction and contribute to the inhibition. Dihydropteroylxegalglutamate at $10^{-4}$ M inhibits the reaction by 30%. A similar situation obtains with inhibitions noted with (dl) versus (d)-tetrahydropteroylxegalglutamate at low concentrations (Fig. 3). As the concentrations of (dl)-tetrahydropteroylxegalglutamate and (d)-tetrahydropteroylxegalglutamate are increased opposing effects are seen. The (d)-tetrahydropteroylxegalglutamates plus dihydro-

**Table I**

Concentration (m) of pteroylglutamates required for 50% inhibition of thymidylate synthetase

<table>
<thead>
<tr>
<th>Total glutamate residues</th>
<th>Pteroyl</th>
<th>Dihydropteryl</th>
<th>(d)-Tetrahydropteryl</th>
<th>(d)-Tetrahydropteryl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.5 \times 10^{-4}$</td>
<td>$3 \times 10^{-4}$</td>
<td>$&gt;10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>$3 \times 10^{-4}$</td>
<td>$2 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$8 \times 10^{-7}$</td>
<td>$3.2 \times 10^{-4}$</td>
<td>$3 \times 10^{-4}$</td>
<td>$&gt;10^{-4}$</td>
</tr>
<tr>
<td>6</td>
<td>$6 \times 10^{-7}$</td>
<td>$3.2 \times 10^{-4}$</td>
<td>$1.8 \times 10^{-4}$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

a Standard assay conditions.

pteroylpolyglutamates formed during the reaction are inhibitory, whereas the (l)-tetrahydropteroylpolyglutamates derivatives are effective substrates. These opposing effects serve to flatten the inhibition curves.

**Abolition of Pteroylglutamate Inhibition of Thymidylate Synthetase by NaCl**—If the assay system is brought to 0.4 M NaCl pteroylhexaglutamates is no longer inhibitory (Table II). Inhibition by pteroylglutamate was not abolished by salt. This suggests that the polyglutamate moiety or its corresponding site on the enzyme (or both) are altered by high salt concentration. KCl was just as effective as NaCl. The over-all enzyme activity was inhibited 40% by 0.4 M NaCl.

**Dihydropteroylpolyglutamates as Substrates for Dihydrofolate Reductase—Dihydropteroylpolyglutamates and dihydropteroylhexaglutamate were reduced at the same rate as dihydropteroylglutamate when tested at $5 \times 10^{-3}$ M in the presence of $8 \times 10^{-5}$ M NADPH and L. casei dihydrofolate reductase.** The amount of enzyme chosen gave a $\Delta A_{450}$ of 0.02 per min.

**DISCUSSION**

It has been shown that as the number of glutamate residues attached to folate is increased, the polarity of NaCl required for elution from a DEAE-cellulose column increases (1). This was to be expected because the more acid the compound the greater would be its attraction to the tertiary amine groups of the DEAE-cellulose. As shown in Fig. 1 the elution of tetrahydrofolate-polyglutamate also follows this pattern. However, dihydropteroyl derivatives, which bind more tightly than tetrahydropteroyl derivatives, elute at about the same molarity of NaCl regardless of the number of glutamate residues. For example, adding 5 glutamyl residues to tetrahydropteroylglutamate does not change its affinity for DEAE-cellulose as much as converting it to dihydropteroylglutamate. These elution patterns illustrate a possible pitfall in determining the number of glutamate residues bound to pteroylg glutamate in natural materials by their position of elution from a DEAE-cellulose column (14).

Friedkin et al. reported that polyglutamyl derivatives of 5-formyltetrahydropteroylglutamate are potent inhibitors of *Escherichia coli* thymidylate synthetase (15). The pattern of inhibition obtained as the number of glutamyl residues was increased is similar to that reported in the present studies (Table I).

Homopteroylpolyglutimate derivatives also become more inhibitory to *L. casei* thymidylate synthetase as the length of the polyglutamate chain increases (16). Tetrahydrohomopteroyl polyglutamates are substrates for thymidylate synthetase from both *E. coli* (17) and *L. casei* (16), but their relative effectiveness has not been critically assessed because inhibition by the (d)-diastereoisomers was not considered.
The formation of dihydropteroylpolyglutamyl derivatives during the course of the thymidylate synthetase reaction appears to be a situation where product inhibition plays a role in metabolic regulation. As the ratio of dihydropteroylpolyglutamate to tetrahydropteroylpolyglutamate increases, it would become more difficult to utilize the remaining tetrahydro derivative for thymidylate formation. The tetrahydro derivative might thereby be spared to catalyze other pteroylglutamate-requiring reactions.

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REFERENCES
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