# Studies on the Polyglutamate Specificity of Thymidylate Synthase from Fetal Pig Liver†

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ABSTRACT: Thymidylate synthase has been purified 1700-fold from fetal pig livers by using chromatography on Affigel-Blue, DEAE-52, and hydroxylapatite. Steady-state kinetic measurements indicate that catalysis proceeds via an ordered sequential mechanism. When 5,10-methylenetetrahydropteroylmonoglutamate ($CH_2$-$H_4$PteGlu$_1$) is used as the substrate, dUMP is bound prior to $CH_2$-$H_4$PTeGlu$_1$, and 7,8-dihydropteroylmonoglutamate ($H_2$PteGlu$_1$) is released prior to dTMP. Pteroylpolyglutamates (PteGlu$_n$) are inhibitors of thymidylate synthase activity and are competitive with respect to $CH_2$-$H_4$PteGlu$_1$ and uncompetitive with respect to dUMP. Inhibition constants ($K_i$ values), which correspond to dissociation constants for the dissocation of PteGlu$_n$ from the enzyme–dUMP–PteGlu$_n$ ternary complex, have been determined for PteGlu$_n$ derivatives with one to seven glutamyl residues: PteGlu$_1$, 10 $\mu$M; PteGlu$_2$, 0.3 $\mu$M; PteGlu$_3$, 0.2 $\mu$M; PteGlu$_4$, 0.06 $\mu$M; PteGlu$_5$, 0.10 $\mu$M; PteGlu$_6$, 0.12 $\mu$M; PteGlu$_7$, 0.15 $\mu$M. Thus, thymidylate synthase from fetal pig liver preferentially binds pteroylpolyglutamates with four glutamyl residues, but derivatives with two to seven glutamyl residues all bind at least 30-fold more tightly than the monoglutamate. When $CH_2$-$H_4$PteGlu$_4$ is used as the one carbon donor for thymidylate biosynthesis, the order of substrate binding and product release is reversed, with binding of $CH_2$-$H_4$PteGlu$_4$ preceding that of dUMP and release of dTMP preceding release of $H_2$PteGlu$_4$. $V_{max}$ and $K_m$ values for dUMP and $CH_2$-$H_4$PteGlu$_n$ show relatively little change as the polyglutamate chain length of the substrate is varied. Comparison of the kinetic data obtained in these studies with earlier studies on methylenetetrahydrofolate reductase from pig liver [Matthews, R. G., & Baugh, C. M. (1980) *Biochemistry 19*, 2040–2045] leads us to predict that the partitioning of limiting concentrations of $CH_2$-$H_4$PteGlu$_n$ between the reactions catalyzed by thymidylate synthase and methylenetetrahydrofolate reductase will vary with polyglutamate chain length, with hexaglutamyl substrates preferentially being reduced to methyltetrahydrofolate.

Intracellular folate derivatives are present mainly as pteroylpoly-$\gamma$-glutamates with 2–10 glutamyl residues (Brown et al., 1974; Eto & Krumdieck, 1981; Foo & Shane, 1982). There is evidence to suggest that the distribution of pteroylpolyglutamates is species dependent (Priest et al., 1981) and may differ with the nature of the folate derivative (Eto & Krumdieck, 1981) and with metabolic conditions inside the cell (Foo & Shane, 1982; Eto & Krumdieck, 1982). Synthesis of a polyglutamate "tail" requires considerable expenditure of cellular energy and presumably results in compensatory advantages to the cell. However, the rationale for formation of long-chain pteroylpolyglutamates remains unclear.

Our laboratory has been examining the binding properties of a series of folate-dependent enzymes for pteroylpolyglutamate derivatives. In addition to the present study on thymidylate synthase, we have examined methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), serine hydroxymethyltransferase (Matthews et al., 1982), and methylenetetrahydrofolate dehydrogenase (Ross et al., 1984). These studies have utilized pteroylpolyglutamate derivatives as *inhibitors* of these enzymes and have determined dissociation constants by kinetic or thermodynamic measurements for a series of inhibitors which differ only in the number of glutamyl residues. All studies have been performed with enzymes isolated from pig liver, to eliminate species differences in polyglutamate specificity. Once the specificity of each enzyme for the length of polyglutamate tail is known and the binding energies associated with the interaction of each glutamyl residue with the enzyme have been determined, we can also determine how significantly binding of the polyglutamate tail affects the kinetic parameters associated with catalysis utilizing pteroylpolyglutamate substrates.

Such studies provide base-line information which can be used to predict the flux of folate metabolites through competing pathways. They are also helpful in predicting the pharmacological and physiological effects that polyglutamylation will have on the inhibition of individual enzymes by folate and antifolate derivatives.

## Experimental Procedures

*Purification of Thymidylate Synthase.* Fetal pigs were obtained from a local slaughterhouse. The highest thymidylate synthase activity was associated with pigs weighing less than 225 g. Enzyme preparation was initiated within 6 h of slaughter, since enzyme activity in the pigs, dissected livers, or homogenates was not stable to storage at either 4 or −20 °C. The livers were dissected out and washed with 0.9% NaCl. They were homogenized in a small Waring blender in 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl, 50 mM 2-mercaptoethanol, and 100 $\mu$M dUMP.[1] The ratio of buffer to dissected livers in the homogenate was 1 mL of buffer/g of liver. The homogenate was centrifuged at 30000*g* for 1 h.

The supernatant was decanted and assayed for thymidylate synthase activity (see below). Affigel-Blue beads (Bio-Rad),

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[1] Abbreviations: $CH_2$-$H_4$PteGlu$_n$, 5,10-methylenetetrahydropteroylpolyglutamate with *n* glutamyl residues; $H_2$PteGlu$_n$, 7,8-dihydropteroylpolyglutamate with *n* glutamyl residues; $CH_3$-$H_4$PteGlu$_n$, 5-methyltetrahydropteroylpolyglutamate with *n* glutamyl residues; dTMP, thymidylate; dUMP, deoxyuridylate; HTP, hydroxylapatite; DTT, dithiothreitol; $CH_3$-$H_4$folate, 5-methyltetrahydrofolate; $CH_2$-$H_4$folate, 5,10-methylenetetrahydrofolate; $H_2$folate, 7,8-dihyrofolate; FdUMP, 5-fluorodeoxyuridylate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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previously equilibrated with the homogenizing buffer and then allowed to settle in the buffer, were added to the supernatant in the proportion 100 mL of settled beads per 0.22 unit of activity (in $\mu$mol min$^{-1}$). The suspension was stirred overnight at 4 °C. The beads were collected by filtration in a Buchner funnel, and the filtrate was discarded. The beads were resuspended in 50 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl, 50 mM 2-mercaptoethanol, and 100 $\mu$M dUMP and collected by filtration. Rinsing was repeated until the absorbance at 280 nm was less than 0.1 when measured vs. a buffer blank. A slurry of beads in the same buffer was poured into a 2.5-cm diameter column. The enzyme was eluted with 1 M NaCl in 50 mM phosphate, pH 7.5, containing 50 mM 2-mercaptoethanol and 100 $\mu$M dUMP. The elution was performed at 4 °C, and the column was pumped at a flow rate of 16 mL/h. Fractions, 4 mL, were collected and analyzed for thymidylate synthase activity and for differential absorbance at 280 nm. The active fractions (8–19) were pooled and concentrated in an Amicon concentrator with a PM30 membrane and were then dialyzed overnight in 10 mM phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol.

The dialyzed enzyme was applied to a 2.5 × 10 cm column of DEAE-52 (Whatman), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8, 20% glycerol, 10 mM 2-mercaptoethanol, and 1 mM EDTA.[1] The column was eluted with a linear gradient of 0–0.1 M KCl in the same buffer, and 5-mL fractions were collected. Active fractions (39–47) were collected, concentrated, and dialyzed overnight in 10 mM phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol.

The enzyme was then applied to a 1.5 × 7 cm Bio-Rad HTP column previously equilibrated with glass-distilled water. The adsorbed enzyme activity was eluted with a 250-mL linear gradient of 0–0.1 M phosphate buffer, pH 7.5, containing 5 mM DTT, and 4-mL fractions were collected. Active fractions (24–38) were pooled and concentrated to an activity of 0.026 $\mu$mol min$^{-1}$ (mL of enzyme solution)$^{-1}$. The enzyme solution was clarified by centrifugation, brought to 20% in glycerol, and then stored at −70 °C. Enzyme solutions prepared in this manner showed little or no loss of activity after storage for several weeks.

*Methods for Assay of Thymidylate Synthase Activity.* Enzyme activity was monitored during the purification of the protein by measuring tritium release from [5-$^3$H]dUMP in the presence of (6*RS*)-$CH_2$-$H_4$folate as initially described by Lomax & Greenberg (1967). Assay mixtures, 0.2 mL, contained 0.1 mL of enzyme and 0.1 mL of an assay cocktail [100 mM Tris-HCl buffer, pH 7.4/50 mM $MgCl_2$/30 mM formaldehyde/200 mM 2-mercaptoethanol/2 mM EDTA/1.25 mM (6*RS*)-$H_4$folate/0.2 M NaF/125 $\mu$M [5-$^3$H]dUMP (94 000 dpm/nmol)]. The mixture was incubated for 15 min at 37 °C and then quenched by addition of 1 mL of activated charcoal in 4% perchloric acid (20 g/100 mL). The quenched mixture was incubated for 5 min at 37 °C with frequent shaking and then centrifuged in an Eppendorf microfuge at 15600*g* for 3 min. An aliquot of the supernatant, 0.3 mL, was added to 5 mL of aqueous counting scintillant (Amersham). The dpm values detected in each sample were corrected for the dpm detected in a control assay, in which the charcoal/perchloric acid quench mixture was added to the enzyme prior to addition of the assay cocktail. Enzyme units are micromoles of $^3$H released per minute.

For kinetic studies utilizing the purified enzyme a spectrophotometric assay was used. The assay involves measurement of the absorbance changes at 340 nm accompanying the conversion of $CH_2$-$H_4$folate to $H_2$folate (Wahba & Friedkin, 1961). In calculating activities a molar extinction coefficient of 6152 M$^{-1}$ cm$^{-1}$ was used for the absorbance change at 340 nm associated with the conversion of $CH_2$-$H_4$folate to $H_2$folate. This molar extinction coefficient was calculated from the ratios of 340-nm absorbance to peak absorbance of our preparations of $CH_2$-$H_4$folate (340/297 = 0.045) and $H_2$folate (340/282 = 0.267) by using 32 000 M$^{-1}$ cm$^{-1}$ as the molar extinction coefficient for $CH_2$-$H_4$folate at 297 nm (Blakley, 1960a) and 28 400 M$^{-1}$ cm$^{-1}$ as the molar extinction coefficient for $H_2$folate at 282 nm (Blakley, 1960b). Assay mixtures, 1.0 mL, contained approximately 2.6 × 10$^{-3}$ unit of thymidylate synthase (as measured by the tritium release assay)/0.1 M phosphate buffer, pH 6.8/100 $\mu$M dUMP/20 $\mu$M (6*RS*)-$CH_2$-$H_4$folate. The $CH_2$-$H_4$folate was prepared as a mixture of 0.05 M $NaHCO_3$/2 mM (6*RS*)-$H_4$folate/1.3 mM formaldehyde/50 mM 2-mercaptoethanol and was stored at −20 °C under nitrogen prior to use. Such solutions were stable for several weeks. The assay mixture (with $CH_2$-$H_4$folate and enzyme omitted) was equilibrated with nitrogen in a 1-mL cuvette for several minutes, $CH_2$-$H_4$folate was added, and equilibration with nitrogen was continued for two more minutes. The cuvette was sealed with parafilm and equilibrated in a 25 °C bath for several minutes, and then the blank rate was measured at 340 nm in a spectrophotometer. The assay was initiated by addition of enzyme. Measurements were made on a recorder with an expanded scale (0.1 absorbance full scale) and a spectrophotometer with an optical offset.

Kinetic parameters were evaluated graphically from double-reciprocal plots by using linear regression analysis.

*Preparation of Folate Substrates and Inhibitors.* Pteroylpolyglutamates ($PteGlu_n$) were prepared by solid-phase synthesis (Krumdieck & Baugh, 1969, 1982), purchased from Dr. Charles M. Baugh, and used without further purification. The purity of the pteroylpolyglutamates was checked by high-pressure liquid chromatography using a modification of the procedure described by Schilsky et al. (1983) for the separation of polyglutamate analogues of methotrexate. Approximately 1.5 nmol of $PteGlu_n$ was applied to an Ultrasphere ODS column (0.46 × 25 cm) equilibrated with 30% acetonitrile/70% 5 mM tetrabutylammonium phosphate (Waters PIC A) in glass distilled water. The samples were eluted at 1 mL/min along gradients of 30–50% acetonitrile and 3.5–2.5 mM tetrabutylammonium phosphate in water over 30 min. The column was then eluted isocratically with 50% acetonitrile/50% 5 mM tetrabutylammonium phosphate in water for 15 min. The eluate was monitored at 254 nm. Under these conditions each $PteGlu_n$ derivative chromatographed as a single major peak, and this peak comprised 85–90% of the 254-nm-absorbing material eluting from the column. In particular, contamination by $PteGlu_{n-1}$ was always less than 10%, based on peak height ratios. Under these conditions the following retention times were observed: $PteGlu_n$, 7.0 min; $PteGlu_2$, 12.5 min; $PteGlu_3$, 18.6 min; $PteGlu_4$, 22.0 min; $PteGlu_5$, 24.5 min; $PteGlu_6$, 26.4 min; $PteGlu_7$, 27.9 min.

For studies with $CH_2$-$H_4PteGlu_1$, (6*RS*)-$H_4PteGlu_1$ was prepared by catalytic hydrogenation of a neutral aqueous solution of $PteGlu_1$ and purified as previously described (Ross et al., 1984). Alternatively, (6*S*)-$H_4PteGlu_1$ was prepared by enzymatic reduction of $PteGlu_1$ using dihydrofolate reductase from *L. casei* (Matthews et al., 1982). (6*S*)-$H_4PteGlu_n$ derivatives were prepared from $PteGlu_n$ derivatives in the same manner. $H_2PteGlu_n$ inhibitors were prepared by dithionite reduction of the corresponding $PteGlu_n$ derivatives as described

Table I. Purification of Thymidylate Synthase from Fetal Pig Liver[a]

| step | activity (unit)[b] | protein (mg)[c] | volume (mL) | sp act. (unit/mg) | yield (%) | purification (x-fold) |
|---|---|---|---|---|---|---|
| supernatant from 30000g centrifugation | 0.23 | 12420 | 180 | $1.85 \times 10^{-5}$ | 100 | 1 |
| eluate from AffiGel Blue | 0.045 | 325 | 50 | $1.38 \times 10^{-4}$ | 20 | 7.5 |
| eluate from DEAE-Sephadex | 0.066 | 45.2 | 58 | $1.46 \times 10^{-3}$ | 29 | 79 |
| eluate from Bio-Rad HTP | 0.066 | 2.1 | 38 | $3.14 \times 10^{-2}$ | 29 | 1700 |

[a] This preparation utilized 225 g of fetal pig liver, obtained from approximately 30 fetal pigs. [b] The units are micromoles of $^3H$ released per minute at 37 °C. [c] Protein determinations were made by using Bio-Rad protein assay, according to the manufacturer's directions, and using bovine serum albumin as a standard.

Table II: Product Inhibition of the Thymidylate Synthase Reaction With $CH_2$-$H_4$PteGlu$_1$[a] as Folate Substrate[b]

| variable substrate | fixed substrate ($\mu$M) | inhibitor | inhibition pattern | $K_i$ ($\mu$M) |
|---|---|---|---|---|
| dUMP | $CH_2$-$H_4$PteGlu$_1$ (7.2) | dTMP | competitive | $K_{iq} = 20$ |
| $CH_2$-$H_4$PteGlu$_1$ | dUMP (5) | dTMP | noncompetitive | $K_i$(slope) = 60<br>$K_{iq} = 20$ |
| $CH_2$-$H_4$PteGlu$_1$ | dUMP (100) | dTMP | noncompetitive | $K_i$(slope) = 1100<br>$K_{iq} = 20$ |
| $CH_2$-$H_4$PteGlu$_1$ | dUMP (10) | $H_2$PteGlu$_1$ | noncompetitive | $K_i$(intercept) = 135<br>$K_{ip} = 115$ |
| $CH_2$-$H_4$PteGlu$_1$ | dUMP (100) | $H_2$PteGlu$_1$ | noncompetitive | $K_i$(intercept) = 97<br>$K_{ip} = 95$ |
| dUMP | $CH_2$-$H_4$PteGlu$_1$ (7.2) | $H_2$PteGlu$_1$ | noncompetitive | $K_i$(intercept) = 13 |
| dUMP | $CH_2$-$H_4$PteGlu$_1$ (72) | $H_2$PteGlu$_1$ | uncompetitive | $K_{ip} = 90$ |

[a] Enzymatically reduced (6*R*)-$CH_2$-$H_4$PteGlu$_1$ was used for these experiments. With (6*R*)-$CH_2$-$H_4$PteGlu$_1$ the $K_m$ for dUMP is 1.7 $\mu$M, and the $K_m$ for $CH_2$-$H_4$PteGlu$_1$ is 5.2 $\mu$M, while when racemic (6*RS*)-$CH_2$-$H_4$PteGlu$_1$ is used, the $K_m$ for dUMP is 8 $\mu$M, and the $K_m$ for (6*R*)-$CH_2H_4$PteGlu$_1$ is 8 $\mu$M. We infer that (6*S*)-$CH_2$-$H_4$PteGlu$_1$ may have some affinity for the dUMP binding site. [b] Plots of the primary data are available to the interested reader on request.

by Matthews & Baugh (1980).

*Source and Preparation of Other Reagents.* [5-$^3$H]dUMP was purchased from Amersham and purified on Dowex AG 1-X8 formate columns as described by Lomax & Greenberg (1967). Purified dihydrofolate reductase from *L. casei* was the generous gift of Professor Bruce Dunlap, University of South Carolina. dUMP and dTMP were purchased from Sigma and used without further purification.

## Results

*Purification of Thymidylate Synthase.* The purification procedure utilized to prepare enzyme for kinetic studies is shown in Table I. Certain aspects of this procedure deserve comment. The thymidylate synthase activity in the initial homogenate was very labile if 0.1 M NaCl was omitted from the homogenizing buffer and disappeared with a half-time of about 1 h. With 0.1 M NaCl in the homogenizing buffer, activity in the homogenate was considerably stabilized, with a half-time for inactivation of about 4 h. Addition of phenylmethanesulfonyl fluoride to the homogenate did not protect the enzyme against inactivation. Once the enzyme had been purified by adsorption on Affigel Blue, its lability was greatly decreased and NaCl could be omitted without loss of activity. However, activity disappeared on storage at 4 °C for 4 days unless 5 mM DTT and 20% glycerol were present in the buffer. Inclusion of these agents permitted storage of the enzyme for several weeks at this stage of purification without loss of activity at either 4 or −70 °C. Chromatography on DEAE-52 could be carried out conveniently in 20% glycerol at 4 °C provided that wide, short columns were used. However, reasonable flow rates during chromatography on Bio-Rad HPT at 4 °C could be achieved only if glycerol was removed from the protein prior to application to the column. At this stage of the purification, glycerol was only required to protect the enzyme against inactivation due to freezing.

*Characterization of the Kinetic Mechanism Associated with Use of $CH_2$-$H_4$PteGlu$_1$.* Figure 1 shows a kinetic analysis of the thymidylate synthase reaction using (6*RS*)-$CH_2$-$H_4$PteGlu$_1$ as the one-carbon donor. It can be seen that the

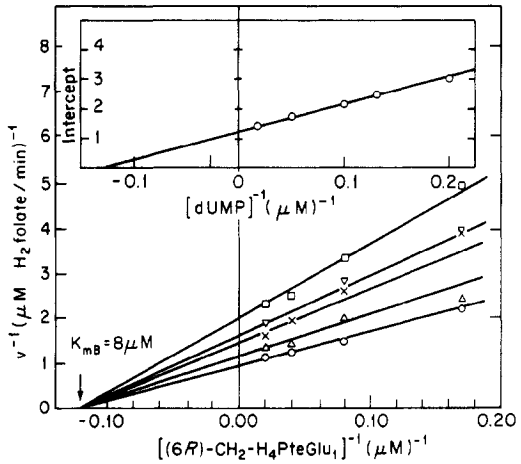
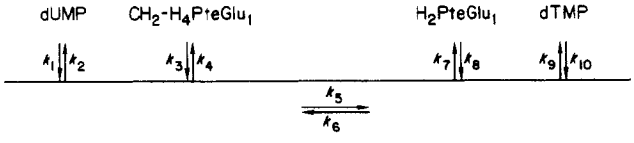



FIGURE 1: Steady-state kinetic measurements with $CH_2$-$H_4$PteGlu$_1$ as substrate. A double-reciprocal plot of velocity vs. [(6*R*)-$CH_2$-$H_4$PteGlu$_1$] is shown. The folate substrate was added as the racemic mixture, (6*RS*)-$CH_2$-$H_4$PteGlu$_1$. The dUMP concentrations were (□) 5, (△) 7.5, (×) 10, (▽) 20, and (○) 60 $\mu$M. Velocities were determined by using the spectrophotometric assay described under Experimental Procedures.

double-reciprocal plots converge, indicative of a sequential mechanism. The $K_m$ for dUMP is 8 $\mu$M, as is the $K_m$ for (6*R*)-$CH_2$-$H_4$PteGlu$_1$. The order of addition of substrates and of release of products was determined by product inhibition studies, and these experiments are summarized in Table II. The product inhibition studies are indicative of the following kinetic pathway:




*Measurement of the Inhibition Constants Associated with PteGlu$_n$ Inhibitors.* The $K_i$ values associated with PteGlu$_n$ inhibitors were measured by using (6*RS*)-$CH_2$-$H_4$PteGlu$_1$ as

Table III: Inhibition Constants for $PteGlu_n$ Inhibitors[a]

| inhibitor | $K_i$ ($\mu$M) | inhibitor | $K_i$ ($\mu$M) |
|---|---|---|---|
| $PteGlu_1$ | 15 | $PteGlu_5$ | 0.100 |
| | 6 | | 0.099 |
| $PteGlu_2$ | 0.38 | $PteGlu_6$ | 0.12 |
| | 0.24 | | 0.12 |
| $PteGlu_3$ | 0.30 | $PteGlu_7$ | 0.19 |
| | 0.13 | | 0.10 |
| $PteGlu_4$ | 0.060 | | |
| | 0.060 | | |

[a] All assays were conducted with (6*RS*)-$CH_2$-$H_4PteGlu_1$ as the folate substrate, under the assay conditions described under Experimental Procedures and employing 100 $\mu$M dUMP.

Table IV: Kinetic Parameters Associated with $CH_2$-$H_4PteGlu_n$ Substrates[a]

| substrate | rel $V_{max}$ | $K_m$ (dUMP) ($\mu$M) | $K_m$ ($CH_2$-$H_4PteGlu_n$) ($\mu$M) |
|---|---|---|---|
| $CH_2$-$H_4PteGlu_1$ | 1.00 | 1.7 | 5.2 |
| $CH_2$-$H_4PteGlu_2$ | 0.56 | 1.7 | 2.0 |
| $CH_2$-$H_4PteGlu_3$ | 0.39 | 1.7 | 1.9 |
| $CH_2$-$H_4PteGlu_4$ | 0.36 | 1.7 | 1.9 |
| $CH_2$-$H_4PteGlu_5$ | 0.38 | 2.3 | 1.6 |
| $CH_2$-$H_4PteGlu_6$ | 0.43 | 2.5 | 1.6 |
| $CH_2$-$H_4PteGlu_7$ | 0.37 | 2.6 | 2.1 |

[a] All assays utilized enzymatically prepared (6*R*)-$CH_2$-$H_4PteGlu_n$ derivatives as the folate substrates. As noted in the footnote to Table II, somewhat different $K_m$ values for dUMP and for $CH_2$-$H_4PteGlu_1$ are obtained when racemic (6*RS*)-$CH_2$-$H_4PteGlu_1$ is used as the substrate. Assay conditions are described under Experimental Procedures. Double-reciprocal plots of velocity vs. [$CH_2$-$PteGlu_n$] and of velocity vs. [dUMP] intersected on the $x$ axis. Thus in all cases, $K_{is} = K_{ms}$ for the first substrate bound.

the folate substrate in each experiment. A typical experiment is shown in Figure 2, with $PteGlu_4$ as the inhibitor. The inhibitor pattern is linearly competitive with respect to $CH_2$-$H_4PteGlu_1$ and linearly uncompetitive with respect to dUMP. Such inhibition patterns are expected if $PteGlu_4$ competes with $CH_2$-$H_4PteGlu_1$ for the folate binding site on the enzyme–dUMP binary complex. Similar patterns were observed for each $PteGlu_n$ inhibitor, and the $K_i$ values that were calculated from replots such as is shown in the inset to Figure 2A are listed in Table III. In each case, the $K_i$ values correspond to dissociation constants for dissociation of $PteGlu_n$ inhibitor from enzyme–dUMP–$PteGlu_n$ ternary complexes.

*Measurement of the Kinetic Parameters Associated with $CH_2$-$H_4PteGlu_n$ Substrates.* Table IV summarizes the relative values for $V_{max}$ and the $K_m$ values for dUMP and $CH_2$-$H_4PteGlu_n$ obtained from kinetic analyses of the series of (6*R*)-$CH_2$-$H_4PteGlu_n$ substrates. These experiments were all run on the same day with the same preparation of enzyme. Despite the very large differences in affinity of the enzyme

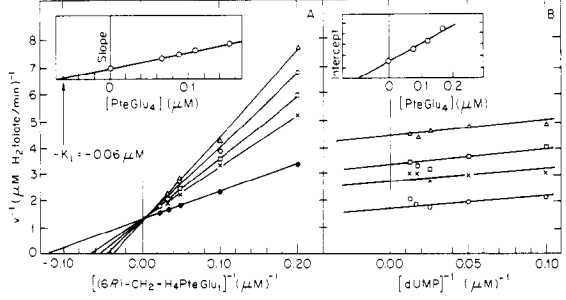
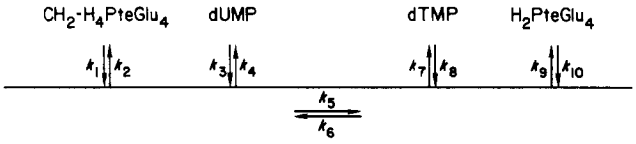



FIGURE 2: Inhibition of thymidylate synthase by $PteGlu_4$. (A) Double-reciprocal plots of velocity vs. [(6*R*)-$CH_2$-$H_4PteGlu_1$], in the presence of 100 $\mu$M dUMP. The folate substrate was added as the racemic mixture (6*RS*)-$CH_2$-$H_4PteGlu_1$. Concentrations of $PteGlu_4$ inhibitor present were (●) 0, (×) 0.065, (□) 0.087, (○) 0.108, and (△) 0.152 $\mu$M. (B) Double-reciprocal plots of velocity vs. [dUMP], in the presence of 10 $\mu$M (6*R*)-$CH_2$-$H_4PteGlu_1$ added as the racemic mixture (6*RS*)-$CH_2$-$H_4PteGlu_1$. Concentrations of $PteGlu_4$ inhibitor were (○) 0, (×) 0.07, (□) 0.12, and (△) 0.17 $\mu$M.

for $PteGlu_n$ inhibitors, the variation in $V/K$ with polyglutamate chain length is very small. One possible explanation for these discrepancies would be that longer chain polyglutamate substrates result in a change in order of addition of substrates or of release of products so that the rate constants contained in these kinetic parameters are not comparable for all substrates. In order to look for changes in order of addition of substrates and/or of product release, we have performed product inhibition studies using $CH_2$-$H_4PteGlu_4$ as the folate substrate and $H_2PteGlu_4$ and dTMP as product inhibitors. The results of these studies are summarized in Table V. These inhibition studies are consistent with the following mechanism:




Comparison with the kinetic scheme obtained by using $CH_2$-$H_4PteGlu_1$ as the folate substrate indicates that use of the polyglutamate substrate has resulted in changes in the order of addition of substrates and of release of products. Similar product inhibition studies with $CH_2$-$H_4PteGlu_2$ as substrate using $H_2PteGlu_2$ and dTMP as product inhibitors suggest that with this substrate also the folate substrate is bound first and the folate product is released last.

When the inhibition patterns associated with inhibition by $PteGlu_4$ are examined with $CH_2$-$H_4PteGlu_4$ as the folate substrate, $PteGlu_4$ is linearly competitive with respect to $CH_2$-$H_4PteGlu_4$ but exhibits a noncompetitive pattern with respect to dUMP. These inhibition patterns are also consistent with $CH_2$-$H_4PteGlu_4$ binding prior to dUMP. Furthermore,

Table V: Product Inhibition of the Thymidylate Synthase Reaction with $CH_2$-$H_4PteGlu_4$ as Folate Substrate[a,b]

| variable substrate | fixed substrate ($\mu$M) | inhibitor | inhibitor pattern | $K_i$ ($\mu$M) |
|---|---|---|---|---|
| $CH_2$-$H_4PteGlu_4$ | dUMP (10) | $H_2PteGlu_4$ | competitive | $K_{iq}$ = 3.3 |
| dUMP | $CH_2$-$H_4PteGlu_4$ (7.2) | $H_2PteGlu_4$ | noncompetitive | $K_i$(slope) = 15<br>$K_{iq}$ = 3.1 |
| dUMP | $CH_2$-$H_4PteGlu_4$ (72) | $H_2PteGlu_4$ | noncompetitive | $K_i$(slope) = 45<br>$K_{iq}$ = 1.2 |
| dUMP | $CH_2$-$H_4PteGlu_4$ (7.2) | dTMP | noncompetitive | $K_i$(intercept) = 400<br>$K_{ip}$ = 320 |
| dUMP | $CH_2$-$H_4PteGlu_4$ (64) | dTMP | noncompetitive | $K_i$(intercept) = 222<br>$K_{ip}$ = 220 |
| $CH_2$-$H_4PteGlu_4$ | dUMP (10) | dTMP | noncompetitive | $K_i$(intercept) = 50 |
| $CH_2$-$H_4PteGlu_4$ | dUMP (100) | dTMP | uncompetitive | $K_{ip}$ = 320 |

[a] Enzymatically reduced (6*R*)-$CH_2$-$H_4PteGlu_4$ was used for these experiments. [b] Plots of the primary data are available to the interested reader on request.

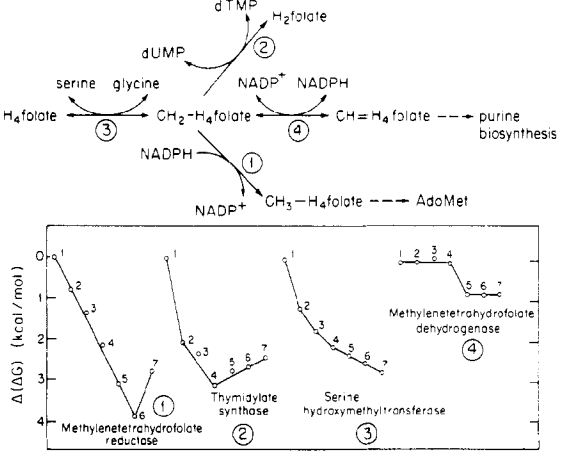



FIGURE 3: Semilogarithmic plots of $\Delta(\Delta G)$ associated with the binding of folate inhibitors vs. the number of glutamyl residues on the folate inhibitor. (1) For methylenetetrahydrofolate reductase, $\Delta(\Delta G)$ values were calculated from $K_i$ values determined kinetically for inhibition of enzyme by $H_2PteGlu_n$ inhibitors in the presence of saturating NADPH and varied $CH_2$-$H_4PteGlu_1$ (Matthews & Baugh, 1980). (2) For thymidylate synthase, $\Delta(\Delta G)$ values were calculated from the data in Table III. (3) For serine hydroxymethyltransferase, $\Delta(\Delta G)$ values were calculated from spectrophotometric determinations of the $K_d$ values for dissociation of $CH_3$-$H_4PteGlu_n$ from E–$CH_3$-$H_4PteGlu_n$–glycine ternary complexes (Matthews et al., 1982). (4) For methylenetetrahydrofolate dehydrogenase, $\Delta(\Delta G)$ values were calculated from $K_i$ values determined kinetically for inhibition of the enzyme by $PteGlu_n$ inhibitors in the presence of saturating $NADP^+$ and varied $CH_2$-$H_4PteGlu_1$ (Ross et al., 1984).

the measured $K_i$ for $PteGlu_4$ binding to the free enzyme (measured with $CH_2$-$H_4PteGlu_4$ as substrate and 100 $\mu$M dUMP) is 0.35 $\mu$M, which is substantially higher than the $K_i$ associated with $PteGlu_4$ binding to the enzyme–dUMP binary complex (0.06 $\mu$M, measured with $CH_2$-$H_4PteGlu_1$ as substrate and 100 $\mu$M dUMP). Clearly $PteGlu_4$ binds both free enzyme and the enzyme–dUMP binary complex. However, the relatively simple inhibition patterns we observe with this inhibitor suggest that such inhibition studies are relatively insensitive to a small amount (less than 20%) of inhibitor binding to an alternate enzyme form.

## Discussion

The values for inhibition constants of $PteGlu_n$ inhibitors shown in Table III can be used to calculate the change in free energy $\Delta(\Delta G)$ associated with the binding of each of a series of polyglutamate inhibitors. In Figure 3, these free energy changes associated with the binding of folylpolyglutamates to thymidylate synthase are compared with similar profiles obtained for other folate-dependent enzymes from pig liver in earlier studies from our laboratory. We note that the folate-dependent enzymes differ markedly in their affinity for folylpolyglutamates as compared to folylmonoglutamates, and they differ also in the chain length of folylpolyglutamate which is preferentially bound. Such comparisons suggest that the chain length of folylpolyglutamate derivatives may indeed play a role in determining how these derivatives will be metabolized, as originally suggested by Baggott & Krumdieck (1979). Two of the enzymes that have been studied, serine hydroxymethyltransferase and methylenetetrahydrofolate dehydrogenase, catalyze reactions that are thought to be maintained at equilibrium in the cytoplasm and presumably play little role in the direction of one-carbon units to various metabolic pathways. The other two enzymes, thymidylate synthase and methylenetetrahydrofolate reductase, determine the flux of one-carbon units through their respective pathways, e.g., into thymidylate biosynthesis or regeneration of AdoMet

Table VI: Partitioning of Limiting Concentrations of $CH_2$-$H_4PteGlu_n$ Substrates between the Reactions Catalyzed by Methylenetetrahydrofolate Reductase

| substrate | relative flux: methylenetetrahydrofolate reductase[a] | relative flux: thymidylate synthase[b] | flux ratio |
|---|---|---|---|
| $CH_2$-$H_4PteGlu_1$ | 1 | 1 | 1 |
| $CH_2$-$H_4PteGlu_2$ | 2.4 | 1.4 | 1.7 |
| $CH_2$-$H_4PteGlu_3$ | 7.2 | 1.1 | 6.5 |
| $CH_2$-$H_4PteGlu_4$ | 15.5 | 1.0 | 15.5 |
| $CH_2$-$H_4PteGlu_5$ | 11.6 | 1.2 | 9.7 |
| $CH_2$-$H_4PteGlu_6$ | 27.1 | 1.3 | 20.8 |
| $CH_2$-$H_4PteGlu_7$ | 5.5 | 0.9 | 6.1 |

[a] Data calculated from Matthews & Baugh (1979) assuming a cytoplasmic NADPH concentration of 200 $\mu$M. [b] Data calculated from Table IV of this paper assuming a cytoplasmic dUMP concentration of 60 $\mu$M (Jackson, 1978).

via $CH_3$-$H_4$folate and methionine, and catalyze reactions that are essentially irreversible in vivo. In Table VI we have attempted to calculate the effect of the polyglutamate chain length of $CH_2$-$H_4$folate on the relative rates of incorporation of the methylene group into dTMP or into $CH_3$-$H_4$folate. These calculations assume a cytoplasmic concentration of NADPH of about 200 $\mu$M (Conway et al., 1983) and a cytoplasmic dUMP concentration of about 60 $\mu$M [calculated from the data of Jackson (1978) assuming 0.68 mL of water/$10^9$ cells]. The calculations further assume limiting concentrations of $CH_2$-$H_4$folate ($V/K$ conditions). Under these conditions, it can be seen that the ratio of fluxes through these two competing pathways is quite dependent on the polyglutamate chain length of $CH_2$-$H_4PteGlu_n$, so that if the flux ratio through the two pathways is set equal to 1 for the monoglutamate, it increases to 20.8 for the hexaglutamate, with polyglutamates being preferentially reduced to $CH_3$-$H_4$folate. Thus, under conditions of limiting $CH_2$-$H_4$folate, chain length is expected to be an important determinant of flux.

Our studies also suggest that one should be very cautious about drawing inferences about substrate *affinities* from comparisons of $V_{max}/K_m$ with folate substrates of varying polyglutamate chain length, particularly for ordered sequential mechanisms. Our observation that increased affinity of thymidylate synthase for folylpolyglutamates leads to reversals in the order of substrate binding and product release means that the kinetic constants contained in $V_{max}/K_m$ change with polyglutamate chain length and hence that these values can not be directly compared.

Previous studies of thymidylate synthase from a number of sources have suggested an ordered mechanism in which dUMP binding precedes $CH_2$-$H_4PteGlu_1$ binding (Lorenson et al., 1967; Dolnick & Cheng, 1977; Danenberg & Danenberg, 1978; Daron & Aull, 1978; Bisson & Thorner, 1981). Evidence in support of an ordered mechanism in which dUMP binding precedes $CH_2$-$H_4PteGlu_1$ binding was also obtained from equilibrium dialysis measurements with the enzyme from *L. casei* (Galivan et al., 1976b). Studies have also been performed with enzyme from a variety of sources that examined the effect of substrate polyglutamate chain length on $V_{max}$ and the $K_m$ values for $CH_2$-$H_4PteGlu_n$. Thus, the yeast enzyme exhibits a 10-fold decrease in $K_m$ when $CH_2$-$H_4PteGlu_5$ is compared with $CH_2$-$H_4PteGlu_1$ (Bisson & Thorner, 1981); the enzyme from human blast cells shows a 15-fold decrease in $K_m$ when the same two substrates are compared (Dolnick & Cheng, 1978), and the enzyme from *L. casei* shows decreases in both $V_{max}$ (3-fold) and the $K_m$ for $CH_2$-$H_4$folate (15-fold) (Kisliuk et al., 1981). The enzyme from calf thymus

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