Interactions of Pig Liver Serine Hydroxymethyltransferase with Methyltetrahydropteroylpolyglutamate Inhibitors and with Tetrahydropteroylpolyglutamate Substrates†

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ABSTRACT: The ternary complex of serine hydroxymethyltransferase, methyltetrahydrofolate, and glycine exhibits absorbance at 502 nm, which has been attributed to the formation of a quinoid structure following removal of the pro-*S* proton from the α carbon of glycine. Measurements of absorbance changes at 502 nm associated with the titration of pig liver serine hydroxymethyltransferase with methyltetrahydropteroylpolyglutamates (CH_3-H_4PteGlu$_n$) in the presence of 100 μM glycine have been used to determine the decrease in free energy associated with the binding of the residues of the polyglutamyl side chain to the enzyme. A decrease in free energy of about 1.0 kcal (4.18 kJ) per residue is associated with the binding of the second and third glutamyl residues. Binding of the fourth through seventh glutamyl residues contributes about 0.17 kcal (0.73 kJ) per residue to the decrease in free energy. The binding energy of the polyglutamyl side chain is expressed not only as an increased affinity of serine hydroxymethyltransferase for CH_3-H_4PteGlu$_n$ derivatives but also as an increased affinity of the enzyme–CH_3-H_4PteGlu$_n$ binary complex for glycine. The combined binding energy of the second and third glutamyl residues is expressed as a 33-fold increase in the affinity of the enzyme–CH_3-H_4PteGlu$_n$ complex for glycine, while the binding energy of additional glutamyl residues serves to increase the affinity of the enzyme for CH_3-H_4PteGlu$_n$. This enhanced two-ligand synergism is not seen in the interaction of serine hydroxymethyltransferase with tetrahydropteroylpolyglutamate derivatives (H_4PteGlu$_n$). Here, the free energy decrease associated with binding of the polyglutamate side chain is expressed entirely as increased affinity of the enzyme for H_4PteGlu$_n$, with no effect on the affinity of the binary complex for serine or glycine. We postulate that formation of inhibitory enzyme–glycine–CH_3-H_4PteGlu$_n$ complexes may serve to regulate serine hydroxymethyltransferase activity in response to cellular levels of CH_3-H_4PteGlu$_n$ and glycine. Inhibition of serine hydroxymethyltransferase by elevated levels of CH_3-H_4PteGlu$_n$ derivatives may also contribute to the depletion of methylenetetrahydrofolate derivatives which leads to depressed de novo thymidylate biosynthesis when methionine synthase activity is inhibited, e.g., in animals treated with nitrous oxide or in human pernicious anemia.

Intracellular folate derivatives are present mainly as polyglutamates with two to seven glutamyl residues linked through their γ-carboxyl groups (Leslie & Baugh, 1974; Brown et al., 1974; McBurney & Whitmore, 1974; Taylor & Hanna, 1977). Synthesis of a polyglutamyl side chain requires considerable expenditure of cellular energy and presumably results in compensatory advantages to the cell. However, the rationale for formation of long polyglutamyl side chains on intracellular folate derivatives remains unclear.

The mammalian enzymes serine hydroxymethyltransferase, thymidylate synthase, methylenetetrahydrofolate dehydrogenase, and methylenetetrahydrofolate reductase all catalyze reactions that generate or utilize CH_2-H_4folate,[1] and we have been interested in examining the specificities of these enzymes for the number of glutamyl residues in the polyglutamate side chain of their folate cosubstrates or inhibitors. Such studies should be useful for predicting what the flux through competing pathways will be under varying cellular conditions. They may also be useful in evaluating potential effects of inhibitors of these enzymes, particularly those which are substrate analogues, under intracellular conditions. The binding energy of the polyglutamate side chain of folate derivatives need not simply affect the affinity of an enzyme for the folate cosubstrate but may be expressed in V_{max} or as an enhanced affinity of the enzyme–folate binary complex for a second substrate. Since the kinetic parameters associated with inhibitors that affect folate-dependent enzymes have generally been assessed with monoglutamate folate derivatives as cosubstrates, a knowledge of the effect of the polyglutamate side chain on both V_{max} and binding parameters for the nonfolate substrate should provide useful additional information.

In comparing the specificities of enzymes that catalyze reactions involving CH_2-H_4folate, it is desirable to use enzymes that have all been obtained from the same source, since the distribution of folate polyglutamyl chain lengths may vary from one species to another and conceivably also from one organ to another. Our initial studies on pig liver methylenetetra-

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[1] Abbreviations: CH_3-H_4PteGlu$_n$, methyltetrahydropteroylpolyglutamate with *n* glutamyl residues; H_4PteGlu$_n$, tetrahydropteroylpolyglutamate with *n* glutamyl residues; H_2PteGlu$_n$, dihydropteroylpolyglutamate with *n* glutamyl residues; PteGlu$_n$, pteroylpolyglutamate with *n* glutamyl residues; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; H_4folate, tetrahydrofolate; AICAR, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

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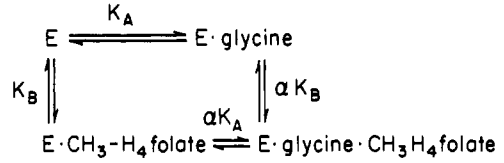
Scheme I



hydrofolate reductase established that this enzyme exhibits a marked preference for binding folate derivatives with a hexaglutamyl side chain (Matthews & Baugh, 1980). This specificity is expressed in the K_i values for H_2-PteGlu$_n$ inhibitors and in the values of V_{max}/K_m for CH_2-H_4PteGlu$_n$ substrates. Our present examination of the specificity of pig liver serine hydroxymethyltransferase for polyglutamate inhibitors indicates a different pattern, with the enzyme exhibiting a broader specificity for derivatives with between three and seven glutamyl residues in the polyglutamate side chain.

In order to measure the decrease in free energy associated with binding of each glutamyl residue of the polyglutamyl side chain, we have made use of the observation that serine hydroxymethyltransferase forms ternary complexes with glycine and H_4folate (Schirch & Mason, 1963) or glycine and CH_3-H_4folate (Schirch & Ropp, 1967) which exhibit long-wavelength absorbance. In the case of the ternary complex of enzyme with glycine and CH_3-H_4folate, the absorbance maximum lies at 502 nm. As discussed by Schirch & Ropp (1967), measurements of the absorbance changes at 502 nm at varying concentrations of glycine and CH_3-H_4folate may be used to calculate values for the dissocation constants for each step of the thermodynamic cycle shown in Scheme I.

The characteristic absorbance of the enzyme–glycine–H_4folate ternary complex has been attributed to the quinoid structure formed on abstraction of the pro-*S* proton from the α carbon of the imine formed from glycine and pyridoxal phosphate as shown in Scheme II. In agreement with this interpretation, Schirch & Jenkins (1964) showed that H_4folate stimulates the rate of exchange of the pro-*S* α hydrogen of glycine. More recently, Wang et al. (1979, 1981) have examined the reaction of the glycine analogue D-fluoroalanine with serine hydroxymethyltransferase. In this case, removal of the α hydrogen as a proton permits fluoride release, and H_4folate stimulates the rate of fluoride release from D-fluoroalanine several hundred-fold.

In principle, analyses similar to those performed by Schirch & Ropp (1967) should permit determination of the free energy of binding of each additional glutamyl residue of CH_3-H_4PteGlu$_n$ or H_4PteGlu$_n$ derivatives. Such studies can be used to obtain information on the specificity of serine hydroxymethyltransferase for polyglutamate forms of its H_4folate and CH_2-H_4folate cosubstrates and may also suggest how the binding energy associated with interactions between the enzyme and the polyglutamyl side chain is utilized in catalysis.

Experimental Procedures

Purification of Pig Liver Serine Hydroxymethyltransferase. The purification of the enzyme was carried out with potassium phosphate buffers of varying ionic strength, adjusted to pH 7.5, and containing in addition the following: (buffer I) 0.1 M sodium fluoride, 14 mM DL-serine, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 0.1 mM folate, and 2 mM 2-mercaptoethanol; (buffer II) 1 mM DL-serine, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 0.1 mM folate, and 2 mM 2-mercaptoethanol; (buffer III) same additions as buffer II except that folate was omitted; (buffer IV) 1 mM DL-serine, 1 mM EDTA, and 2 mM 2-mercaptoethanol. All operations were done at 4 °C unless otherwise specified.

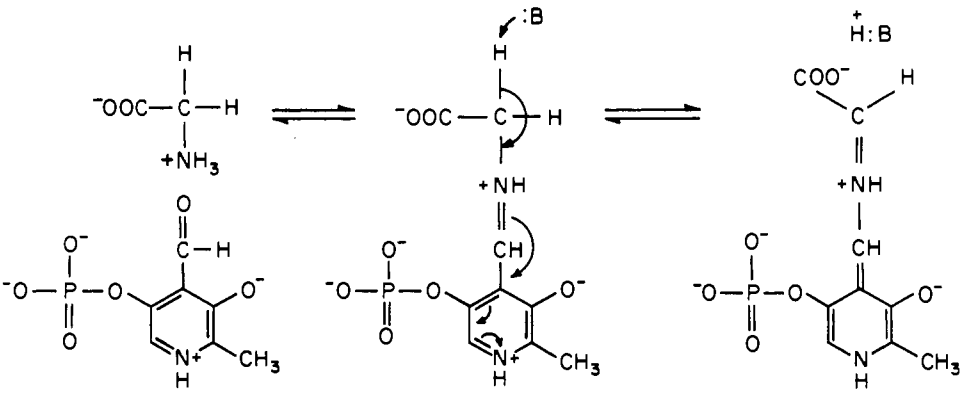
Step I: Homogenization. Frozen pig livers were chiseled into small pieces and minced in a manual meat grinder. The mince was homogenized in a blender for 1 min in 2 volumes of 0.05 M buffer I and then centrifuged for 20 min at 19600*g*.

Step II: Heat Treatment. Portions (250 mL) of supernatant from step I were heated in a boiling water bath to 70 °C with constant stirring. As soon as the temperature reached 70 °C the portions were removed from the bath, and crushed ice was added to initiate cooling. Cooling was continued in an ice bath until the aliquots reached 10 °C. The supernatant resulting from centrifugation at 19600*g* for 15 min was brought to 60% saturation with ammonium sulfate (390 g/L), the pH was readjusted to 7.5 with 2.5 M ammonium hydroxide, and the suspension was centrifuged for 10 min at 19600*g*. The precipitate was dissolved in 0.05 M buffer II and dialyzed against three changes of a 20-fold excess of 0.05 M buffer II.

Step III: Anion-Exchange Chromatography. The dialyzed enzyme solution was loaded onto a 5 × 25 cm column of DEAE-Sephadex A-50 which had previously been equilibrated with 0.05 M buffer IV. After the column was loaded, it was washed with 0.05 and then 0.1 M buffer IV, and the serine hydroxymethyltransferase activity was eluted with 0.3 M buffer IV. The active fractions were pooled, and the enzyme was precipitated with 60% ammonium sulfate. The precipitate was dissolved in 0.05 M buffer IV and dialyzed against the same buffer.

Step IV: Affinity Chromatography. The dialyzate was loaded onto a 2.5 × 20 cm column of Matrex Gel Red A equilibrated with 0.05 M buffer III. The column was washed with successive portions of 0.05, 0.1, and 0.2 M buffer III, and then enzyme was eluted with 0.3 M buffer III. The enzyme in the 0.3 M phosphate eluate was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had a subunit molecular weight of 53 000. This fraction of

Scheme II



enzyme was concentrated by ammonium sulfate precipitation, dialyzed, and brought to 20% with sucrose and then lyophilized. Lyophilized enzyme could be stored in the freezer for several months without loss of activity.

The purified enzyme had a specific activity of 13.8 units min^{-1} mg^{-1}, based on the rate of cleavage of *threo*-β-phenylserine at 30 °C. Prior to use for kinetic or spectrophotometric experiments, the enzyme was dissolved in 0.05 M phosphate buffer, pH 7.2 and 0.3 mM in EDTA, and dialyzed for 48 h against three changes of the same buffer.

Assays of Serine Hydroxymethyltransferase Activity. The rate of *threo*-β-phenylserine cleavage was measured by following the increase in absorbance at 279 nm, at 30 °C. The assay mixture contained 50 mM Hepes, 100 mM DL-*threo*-β-phenylserine, 25 mM sodium sulfate, and 1 mM Na_2EDTA and was adjusted to pH 7.5 with HCl prior to use. One unit of enzyme catalyzed a change in absorbance of 1.41/min in a 1-mL assay (1 μmol min^{-1}).

Serine hydroxymethyltransferase activity in the presence of $H_4PteGlu_n$ cosubstrates was measured by means of a coupled assay in which product CH_2-$H_4PteGlu_n$ was reduced to CH_3-$H_4PteGlu_n$ with NADPH and an excess of methylenetetrahydrofolate reductase. The reductase was purified from pig liver as described by Matthews & Haywood (1979). Reactions were measured at 25 °C. Each assay contained 100 μM NADPH, 0.3 unit of methylenetetrahydrofolate reductase (NADPH–menadione oxidoreductase activity in μmol min^{-1}), 50 μM to 5 mM serine, 10–200 μM $H_4PteGlu_n$, 50 mM 2-mercaptoethanol, and 0.3 mM EDTA in 0.05 M potassium phosphate buffer, pH 7.2. Allothreonine cleavage was measured by reducing the product acetaldehyde to ethanol with alcohol dehydrogenase and NADH (Schirch & Gross, 1968).

Preparation of Substrates and Inhibitors. Pteroylpolyglutamates were synthesized by the solid-phase method previously described (Krumdieck & Baugh, 1969, 1980). Methyltetrahydropteroylpolyglutamates were prepared by reduction of the corresponding pteroylpolyglutamates with NADPH, dihydrofolate reductase from *Lactobacillus casei* (purified as described in Liu & Dunlap, 1974), and methylenetetrahydrofolate reductase from pig liver (purified as described by Matthews & Haywood, 1979). $PteGlu_n$ (20 μmol) was dissolved in 100 mL of 10 mM Tris-HCl buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. After the buffer was equilibrated with nitrogen, 80 μmol of NADPH and 100 μmol of formaldehyde were added, followed by 5 units of dihydrofolate reductase and 5 units of methylenetetrahydrofolate reductase (NADPH–menadione oxidoreductase activity). The reaction was allowed to proceed overnight, and the flask was maintained at 25 °C, under nitrogen and shielded from light. The CH_3-$H_4PteGlu_n$ was purified by chromatography of the reaction mixture on a 1.5 × 20 cm column of DEAE-52, previously equilibrated with 0.05 M ammonium acetate, pH 7.2. Elution was effected with a 200-mL linear gradient of 0–1 M NaCl in 0.05 M ammonium acetate, pH 7.2, and 10 mM 2-mercaptoethanol. Fractions containing CH_3-$H_4PteGlu_n$ were identified by the presence of a symmetrical absorbance peak, maximal at 292 nm. Concentrations of stock solutions were determined with an ϵ_{292} of 31 700 M^{-1} cm^{-1}. The yields for each preparation and the glutamyl content per mole of each derivative are given in Table I.

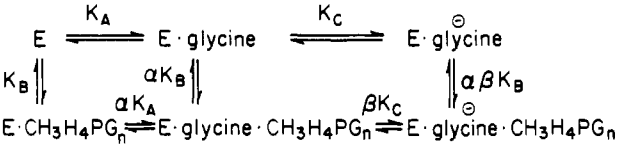
Tetrahydropteroylpolyglutamates were prepared in the same manner as methyltetrahydropteroylpolyglutamates, except that methylenetetrahydrofolate reductase and formaldehyde were omitted from the reaction mixtures and the 2-mercaptoethanol concentration was increased to 200 mM. The $H_4PteGlu_n$ product was purified by chromatography of the reaction mixture on a 1.5 × 20 cm column of DEAE-52, previously equilibrated with 5 mM Tris-HCl, pH 7.2, 0.2 M in 2-mercaptoethanol. Elution was effected with a 200-mL linear gradient of 0–1 M NaCl in the same buffer. Fractions containing $H_4PteGlu_n$ were identified by the presence of a symmetrical absorbance peak, maximal at 297 nm. Concentrations of stock solutions were determined with an ϵ_{297} of 29 400 M^{-1} cm^{-1}. Yields for these preparations were between 50 and 70% after purification by chromatography. The purified $H_4PteGlu_n$ could be stored under nitrogen at −15 °C for several weeks without loss of activity.

Table I: Preparation and Properties of Methyltetrahydropteroylpolyglutamates

starting pteroylpolyglutamate	yield of purified CH_3-$H_4PteGlu_n$ (%)	glutamyl residues per mol[a]
$PteGlu_1$	42	0.98
$PteGlu_2$	72	1.97
$PteGlu_3$	58	2.86
$PteGlu_4$	51	3.86
$PteGlu_5$	38	4.91
$PteGlu_6$	70	6.03
$PteGlu_7$	63	7.18

[a] Determined by amino acid analysis following 24-h hydrolysis. Aspartic acid (28 nmol) was added to each sample prior to hydrolysis as an internal standard for recovery of glutamic acid in the sample during transfer and loading on the analyzer.

Scheme III



Where enzyme concentrations or turnover numbers are given for serine hydroxymethyltransferase, they will be based on the concentration of the tetrameric protein.

Results

Scheme III represents the linked equilibria that we have examined in our spectrophotometric studies. Analysis of binding data for a system described by Scheme III is analogous to analysis of the kinetics of a rapid equilibrium bisubstrate kinetic mechanism, as pointed out previously by Schirch & Ropp (1967). Such analyses are discussed in a number of texts of enzyme kinetics, such as that by Segel (1975). The corresponding equations for Scheme III are

$$\frac{[\text{quinoid}]}{E_T} = \frac{[A][B]}{\left\{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[A][B]}{\alpha K_A K_B}\left(1 + \frac{1}{\beta K_C}\right)\right\}\alpha\beta K_A K_B K_C} \quad (1)$$

$$\frac{1}{[\text{quinoid}]} = \left\{\frac{\alpha\beta K_B K_C}{E_T}\left(1 + \frac{K_A}{[A]}\right)\right\}\frac{1}{[B]} + \frac{1}{E_T}\left(\beta K_C + 1 + \frac{\alpha\beta K_A K_C}{[A]}\right) \quad (2)$$

$$\frac{1}{[\text{quinoid}]} = \left\{\frac{\alpha\beta K_A K_C}{E_T}\left(1 + \frac{K_B}{[B]}\right)\right\}\frac{1}{[A]} + \frac{1}{E_T}\left(\beta K_C + 1 + \frac{\alpha\beta K_B K_C}{[B]}\right) \quad (3)$$

Of the species shown in Scheme III, only the quinoid forms at the extreme right absorb light in the 500-nm region of the spectrum. Figure 1 shows the results of a titration of serine hydroxymethyltransferase with glycine in the absence of added CH_3-H_4PteGlu$_n$. Since the absorbance changes associated with the formation of the enzyme–glycine complex are small, the experiment was performed as a difference titration. Note that formation of the enzyme–glycine complex is associated with very small increases in absorbance at 497 nm, presumably due to the formation of a small amount of quinoid. In the presence of saturating CH_3-H_4PteGlu$_n$, the absorbance changes in this region would be almost 100× greater. We conclude that the equilibrium shown by K_c in Scheme III strongly favors the E–glycine complex in which glycine is protonated and does not absorb light in the 500-nm region. A double-reciprocal plot of the absorbance changes at 450 nm seen in Figure 1 vs. glycine concentration is linear and can be used to calculate a value for K_A of 3.8 mM.

Figure 2 shows results obtained during an experiment in which serine hydroxymethyltransferase was titrated with a series of CH_3-H_4PteGlu$_n$ derivatives in the presence of 100 μM glycine. In the absence of added CH_3-H_4PteGlu$_n$ this concentration of glycine would lead to the formation of only about 3% of the enzyme–glycine complex. The data are displayed as double-reciprocal plots of the absorbance changes at 502 nm vs. the concentration of free CH_3-H_4PteGlu$_n$. It can be seen that increasing the number of glutamyl residues in CH_3-H_4PteGlu$_n$ affects both the slopes and the intercepts of the double-reciprocal plots. Inspection of eq 2 indicates that changes in the slopes at constant [A] (constant glycine) may be due to the effect of polyglutamate chain length on the affinity of free enzyme for CH_3-H_4PteGlu$_n$ (reflected in K_B) or on the affinity of the enzyme–CH_3-H_4PteGlu$_n$ complex for glycine (reflected in the parameter α) or on changes in the equilibrium between protonated and quinoid forms of the E–glycine–CH_3-H_4PteGlu$_n$ ternary complex (reflected in the parameter β). On the other hand, changes in the intercepts of the double-reciprocal plots as the polyglutamate chain length of CH_3-H_4PteGlu$_n$ is varied can only result from changes in the values of α and β, since K_B does not appear in the intercept term. Qualitatively, what we observe is that saturating levels of CH_3-H_4PteGlu$_n$ exert a profound effect on the affinity of the enzyme for glycine, with longer chain polyglutamate derivatives being more effective in this regard than the monoglutamate.

As discussed above, the presence of CH_3-H_4PteGlu$_n$ alters the equilibrium between quinoid and protonated forms of the enzyme–glycine complex. If K_c represents the ratio of protonated to quinoid complex in the absence of a folate derivative, then β represents the degree to which this equilibrium is altered in the presence of CH_3-H_4PteGlu$_n$. If we examine the absorbance at 502 nm associated with fully formed ternary complex in the presence of saturating concentrations of glycine and CH_3-H_4PteGlu$_n$, we can determine whether the number of glutamyl residues on CH_3-H_4PteGlu$_n$ affects β. Such an alteration would have the effect of changing the apparent

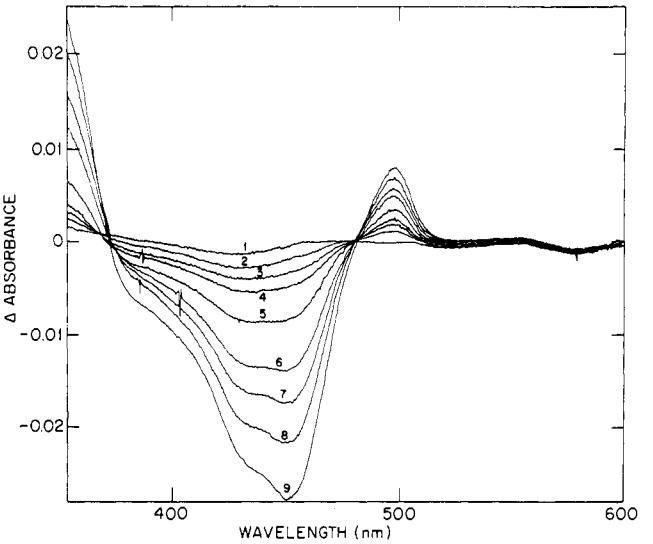


FIGURE 1: Titration of serine hydroxymethyltransferase with glycine. Serine hydroxymethyltransferase solutions (6 μM) in 0.05 M phosphate buffer, pH 7.2, containing 0.3 mM EDTA were placed in both the front and rear cuvettes in a double-beam spectrophotometer. A difference titration was performed in which aliquots of 0.1 or 1.0 M glycine (pH 7.2) were added to the front cuvette, while aliquots of buffer were added to the rear cuvette. The spectra are not corrected for dilution, which was approximately 6.4% by the end of the titration. The concentration of added glycine associated with each spectrum was (1) no glycine, (2) 0.2, (3) 0.4, (4) 0.7, (5) 1.5, (6) 2.94, (7) 4.89, (8) 9.73, and (9) 46.8 mM.

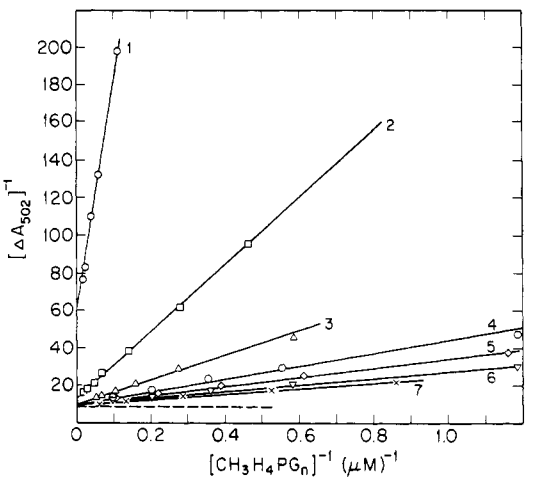


FIGURE 2: Titration of serine hydroxymethyltransferase with methyltetrahydropteroylpolyglutamates in the presence of 100 μM glycine. Solutions of serine hydroxymethyltransferase (0.8 μM) in 0.05 M phosphate buffer, pH 7.2, containing 0.3 mM EDTA and 100 μM glycine were titrated with CH_3-H_4PteGlu$_n$ derivatives at 25 °C. The numbers next to the double-reciprocal plots indicate the number of glutamyl residues, n, on the CH_3-H_4PteGlu$_n$ derivative used in that titration. The concentration of glycine carbanion or quinoid was determined by measurement of the absorbance changes at 502 nm as a function of the concentration of added CH_3-H_4PteGlu$_n$. Although not all data points are shown, each titration included at least four points where the final concentration of CH_3-H_4PteGlu$_n$ was between 10 and 50 μM. The value shown by the dashed line represents the fully formed ternary complex and was obtained from the experiments shown in Figure 3 and discussed in the text. As discussed in the text, we have assumed that "100% bound" corresponds to saturation of about 2 pyridoxal phosphate sites/tetramer or in this case to 1.6 μM ternary complex. All the CH_3-H_4PteGlu$_n$ concentrations shown here represent the concentration of free ligand and have been corrected for bound ligand on the basis of the absorbance at 502 nm at that point in the titration.

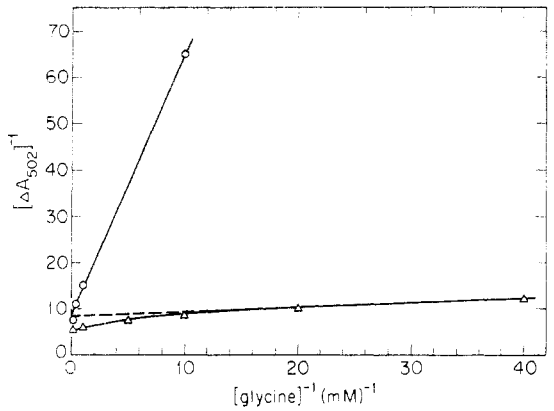


FIGURE 3: Variation in the formation of the quinoid ternary complex with glycine concentration. Each point on the graph represents the extrapolated maximal absorbance change at 502 nm obtained from a titration of serine hydroxymethyltransferase with CH_3-H_4PteGlu$_1$ (○) or CH_3-H_4PteGlu$_6$ (△) in the presence of the indicated glycine concentration. In all cases serine hydroxymethyltransferase (0.8 μM) was in 0.05 M phosphate buffer, pH 7.2, containing 0.3 mM EDTA, and the titrations were performed at 25 °C. This experiment was performed with the same enzyme stock as was used in Figure 2 and on the same day as that experiment.

extinction coefficient at 502 nm associated with complete formation of the ternary complex. Our studies indicate that the extinction coefficient of the fully formed ternary complex increases about 15% on going from a monoglutamyl to a diglutamyl CH_3-H_4PteGlu$_n$ derivative but thereafter remains constant as the polyglutamate chain length is increased. We conclude that the primary effect of the polyglutamate side chain of CH_3-H_4PteGlu$_n$ in altering the intercepts in Figure 2 must result from changes in α, the ligand synergism parameter, as the polyglutamate side chain is varied.

A double-reciprocal plot of the variation in extent of ternary complex formation in the presence of saturating CH_3-H_4PteGlu$_n$ and varied glycine concentrations is shown in Figure 3 for the mono- and hexaglutamyl derivatives. These double-reciprocal plots are nonlinear, as is especially evident in the case of the hexaglutamyl derivative. Such nonlinearity may be due to negative cooperativity associated with quinoid formation (the native enzyme consists of four apparently identical subunits) or to site heterogeneity. Regardless of its origin, such apparent negative cooperativity could complicate the interpretation of the data shown in Figure 2 if the degree of saturation of the enzyme varied with polyglutamate chain length. However, the data shown in Figure 3 suggest that at glycine concentrations at or below 100 μM only the high-affinity sites for glycine are saturated (these correspond roughly to 2 sites/tetramer) and that the extrapolated values for the fully formed ternary complex based on saturation of only the high-affinity sites are the same for the mono- and hexaglutamyl derivatives. Similar results were obtained for analogous experiments with the heptaglutamyl derivative. Figure 3 also confirms that E–CH_3-H_4PteGlu$_6$ binary complexes bind glycine more tightly than E–CH_3-H_4PteGlu$_1$ complexes, as implied by the data in Figure 2.

In Table II we present calculated values for the dissociation constants associated with Scheme III. K_A was determined from the data shown in Figure 1. We are unable to obtain a direct measurement of α from the data shown in Figure 2. This is because saturating concentrations of CH_3-H_4PteGlu$_n$ convert all the enzyme to a mixture of the species shown at the bottom of Scheme III. We do not have a means of determining an absolute extinction coefficient for fully formed quinoid and thus do not have an independent measurement

Table II: Dissociation Constants[a] Associated with E-Glycine-CH_3-H_4PteGlu$_n$ Formation

derivative	K_A (μM)	K_B (μM)	α^*K_A (μM)	α^*K_B (μM)
CH_3-H_4PteGlu$_1$	3800	22	650	3.8
CH_3-H_4PteGlu$_2$	3800	36	57	0.54
CH_3-H_4PteGlu$_3$	3800	38	20	0.21
CH_3-H_4PteGlu$_4$	3800	20	20	0.11
CH_3-H_4PteGlu$_5$	3800	14	20	0.08
CH_3-H_4PteGlu$_6$	3800	10	20	0.06
CH_3-H_4PteGlu$_7$	3800	8	20	0.04

[a] The dissociation constants are defined in Scheme III and $\alpha^* = \alpha/[1 + 1/(\beta K_C)]$.

of βK_c. What we actually measure at saturating CH_3-H_4PteGlu$_n$ is the equilibrium defined by eq 4. Similarly α^*K_B

$$\alpha^*K_A = \frac{[\text{E–CH}_3\text{-H}_4\text{PteGlu}_n][\text{glycine}]}{[\text{quinoid}] + [\text{E–glycine–CH}_3\text{-H}_4\text{PteGlu}_n]} = \frac{\alpha K_A}{1 + 1/(\beta K_c)} \quad (4)$$

is defined by eq 5 and represents an apparent dissociation

$$\alpha^*K_B = \frac{[\text{E–glycine}][\text{CH}_3\text{-H}_4\text{PteGlu}_n]}{[\text{quinoid}] + [\text{E–glycine–CH}_3\text{-H}_4\text{PteGlu}_n]} = \frac{\alpha K_B}{1 + 1/(\beta K_c)} \quad (5)$$

constant for the dissociation of CH_3-H_4PteGlu$_n$ from the ternary complex. Values for α^*K_A are calculated from the ratio of the observed intercepts of the double-reciprocal plots shown in Figure 2 to the intercept value obtained when glycine is saturating (dashed line in Figure 2). Values for α^*K_B are then calculated from the concentration of B at which the absorbance changes at 502 nm are half-maximal. At this concentration of CH_3-H_4PteGlu$_n$ half the enzyme contains bound CH_3-H_4PteGlu$_n$ and is present as a binary or ternary complex. The ratio between the binary E–CH_3-H_4PteGlu$_n$ complex and ternary complex can be calculated from α^*K_A and the ratio between E and E–glycine can be calculated from K_A and the concentration of A. Thus values for K_B can be calculated directly, and then values for α^*K_B can be calculated from them. It will be noted that K_B actually increases a little on going from mono- to triglutamyl CH_3-H_4PteGlu$_n$, while values for α^*K_B decrease 33-fold. This change is largely due to a decrease in the value of α (the ligand synerigism parameter) as the polyglutmate chain length is increased.

Since the free energy decrease associated with binding of the glutamyl residues of the polyglutamate side chain of CH_3-H_4PteGlu$_n$ to enzyme in the presence of glycine may affect α, β, or K_B, we have chosen to calculate this free energy decrease for the binding of CH_3-H_4PteGlu$_n$ to the quinoid ternary complex. As shown in Scheme III, this equilibrium is given by eq 6. We can obtain estimates of the *relative*

$$K_{eq} = \frac{[\text{E–glycine}][\text{CH}_3\text{-H}_4\text{PteGlu}_n]}{[\text{E–glycine–CH}_3\text{-H}_4\text{PteGlu}_n]} = \alpha\beta K_B \quad (6)$$

values for K_{eq} by comparing the slopes of the double-reciprocal plots of Figure 2. Differences in slope as the polyglutamate chain length is varied reflect changes in $\alpha\beta K_B$. These data are shown in Figure 4 and can be used to calculate the free energy decreases associated with the binding of each of the six terminal glutamyl residues of CH_3-H_4PteGlu$_7$. Binding of the second and third glutamyl residues is associated with

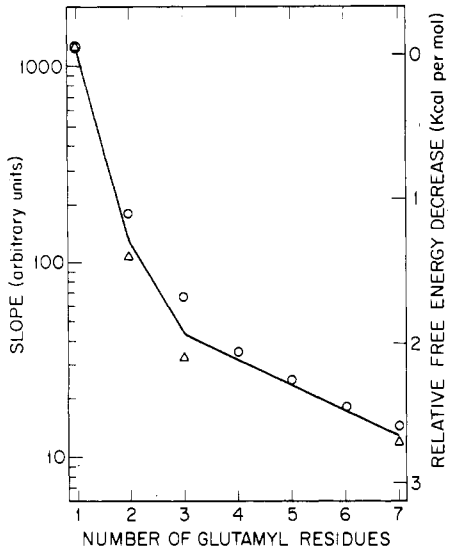


FIGURE 4: Variation of the slopes of the double-reciprocal plots shown in Figures 2 and 5 with the number of glutamyl residues on CH_3-$H_4PteGlu_n$ (○) or $H_4PteGlu_n$ (△). To facilitate comparisons, we normalized the slope obtained with $H_4PteGlu_1$ (Figure 5) to coincide with the slope obtained with CH_3-$H_4PteGlu_1$, and all slopes obtained with $H_4PteGlu_n$ have been multiplied by the same factor.

a free energy decrease of about 1 kcal (4.18 kJ) per residue, while binding of additional glutamyl residues is associated with a free energy decrease of about 0.17 kcal (0.73 kJ) per residue.

Schirch et al. (1977) have shown that while CH_3-$H_4PteGlu_1$ inhibits the cleavage of allothreonine and leads to accumulation of the quinoid enzyme–glycine–CH_3-$H_4PteGlu_1$ complex in the steady state, $H_4PteGlu_1$ is much less inhibitory and does not lead to the accumulation of a quinoid complex. Thus it seemed of interest to compare the effect of the length of the polyglutamate side chain on the affinity of the E–$H_4PteGlu_n$ complex for glycine. Figure 5 shows titrations of serine hydroxymethyltransferase with $H_4PteGlu_n$ derivatives with one to seven glutamyl residues, in the presence of 100 μM glycine. The experiments are analogous to those shown in Figure 2. However, comparison of the two figures reveals that increasing the chain length of $H_4PteGlu_n$ leads primarily to slope effects with very little change in the intercepts of the double-reciprocal plots, in contrast to the data obtained with CH_3-$H_4PteGlu_n$. Thus the binding energy of the polyglutamate side chain of $H_4PteGlu_n$ derivatives is expressed primarily as an enhanced affinity of enzyme for $H_4PteGlu_n$ (a decrease in K_B).

Control experiments established that the extinction coefficient at 490 nm for the E–glycine–$H_4PteGlu_n$ complex did not change appreciably as a function of the number of glutamyl residues on $H_4PteGlu_n$. Double-reciprocal plots of absorbance changes at 490 nm vs. the glycine concentration at saturating $H_4PteGlu_n$ concentrations (analogous to the plots shown in Figure 3) are again nonlinear, but the degree of saturation of the enzyme is independent of the polyglutamate chain length in the glycine concentration range from 25 to 200 μM. Table III shows the calculated values for the dissociation constants associated with the E–$H_4PteGlu_n$–glycine ternary complex. In Figure 4, the free energy decreases associated with the binding of the glutamyl residues of $H_4PteGlu_n$ to the quinoid enzyme–glycine complex are shown as triangles. As expected, if CH_3-$H_4PteGlu_n$ and $H_4PteGlu_n$ are bound at the same site on the enzyme, the free energy decreases are approximately the same for both derivatives. However, they are expressed entirely in K_B for $H_4PteGlu_n$, while the binding energy of the polyglutamate side chain of CH_3-$H_4PteGlu_n$ is expressed primarily as enhanced two-ligand synergism (decrease in α).

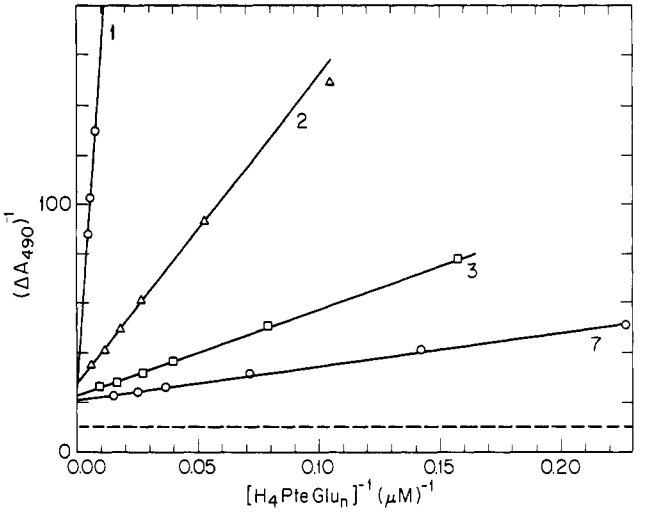
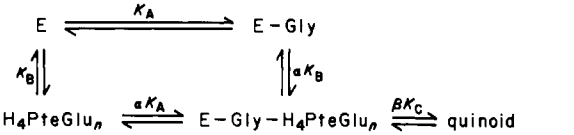


FIGURE 5: Titration of serine hydroxymethyltransferase with tetrahydropteroylpolyglutamates in the presence of 100 μM glycine. Solutions of 0.05 M phosphate buffer, pH 7.2, containing 0.3 mM EDTA, 50 mM 2-mercaptoethanol, and 100 μM glycine were equilibrated with nitrogen, the cuvette was sealed with parafilm, and then 50 μL of concentrated serine hydroxymethyltransferase was added with a Hamilton syringe to yield a final enzyme concentration of 0.9 μM. Aliquots of $H_4PteGlu_n$ were added with a Hamilton syringe, and the absorbance at 490 nm, where the E–glycine–$H_4PteGlu_n$ complex exhibits maximal absorbance, was measured after each addition. The value shown by the dashed line represents the absorbance changes associated with "fully formed" complex and corresponds to the saturation of approximately 2 pyridoxal phosphate sites/tetramer. This value was obtained by an experiment analogous to the one shown for CH_3-$H_4PteGlu_n$ in Figure 3.

Table III: Dissociation Constants Associated with E–Glycine–$H_4PteGlu_n$ Formation[a]



derivative	K_A (μM)	$\alpha^* K_A$ (μM)	K_B (μM)	$\alpha^* K_B$ (μM)
$H_4PteGlu_1$	3800	100	195	5.2
$H_4PteGlu_2$	3800	170	7.2	0.33
$H_4PteGlu_3$	3800	120	3.1	0.10
$H_4PteGlu_7$	3800	100	2.8	0.07

[a] $\alpha^* = \alpha/[1 + 1/(\beta K_C)]$.

We have also examined the effect of the polyglutamate side chain on the kinetic parameters associated with the conversion of serine and $H_4PteGlu_n$ to glycine and CH_2-$H_4PteGlu_n$. The studies of Schirch et al. (1977) on the enzyme from rabbit liver provide evidence for a random sequential mechanism with $H_4PteGlu_1$ as substrate. Using our coupled assay method and the pig liver enzyme, we also see intersecting line patterns for initial velocity measurements using both $H_4PteGlu_1$ and $H_4PteGlu_2$ as the varied cosubstrates and serine as the fixed varied substrate. We wished to determine whether the decrease in free energy associated with the binding of the polyglutamate side chain of $H_4PteGlu_n$ would affect the K_m for serine or the K_m for H_4folate or both. Since the enzyme shows evidence of apparent negative cooperativity in the formation of E–glycine–$H_4PteGlu_n$ complexes, we were concerned that cooperativity might also characterize the formation of catalytically competent E–serine–$H_4PteGlu_n$ complexes, especially with polyglutamate substrates. In such case, the degree of formation of the catalytically competent ternary complex

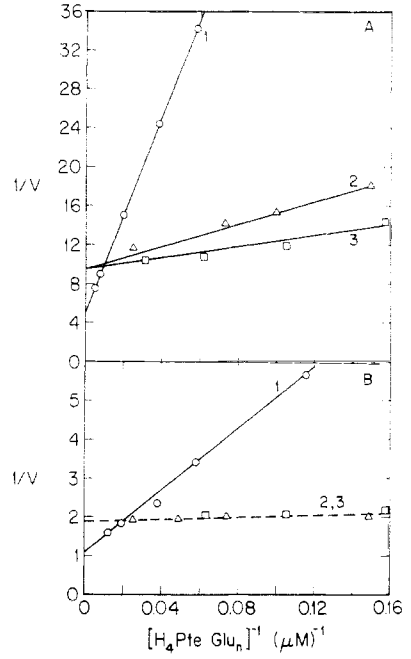


FIGURE 6: Initial velocity measurements of serine hydroxymethyltransferase activity at low (A) and high (B) serine concentration. (A) Initial velocities were measured with the coupled assay described under Experimental Procedures in the presence of 47.6 μM serine and 1.34 μg of serine hydroxymethyltransferase. The assay buffer (1.0 mL) was equilibrated with nitrogen in the cuvette, the cuvette was sealed with parafilm, and then $H_4PteGlu_n$, methylenetetrahydrofolate reductase, and finally serine hydroxymethyltransferase were added to the reaction mixture with Hamilton syringes. (B) Initial velocities were measured as above except that the serine concentration was 4.76 mM. The cosubstrate was $H_4PteGlu_1$ (○), $H_4PteGlu_2$ (△), or $H_4PteGlu_3$ (□). The units for velocity are arbitrary, and a velocity of 1 corresponds to the consumption of 16 nmol of NADPH/min.

might be different with different polyglutamate chain lengths and yet not be due to intrinsic differences in the kinetic parameters but rather to different degrees of occupancy of the lower affinity population of sites. Therefore we have used an approach similar to the one taken in the measurement of the thermodynamic parameters associated with formation of the ternary E–glycine–CH_3-$H_4PteGlu_n$ complex. That is, we have determined V_{max} values at saturating levels of serine and $H_4PteGlu_n$ and then measured initial velocities at a serine concentration (50 μM) that is low relative to the dissociation constant for the enzyme–serine complex (600 μM, measured by difference titration) and relative to the K_m for serine (210 μM, measured with $H_4PteGlu_1$). Under these conditions, only the high-affinity sites should be saturated. The results of such experiments are shown in Figure 6. The kinetic parameters that can be calculated from the data shown in Figure 6 are as follows: turnover number under V_{max} conditions, 2530 min^{-1} with $H_4PteGlu_1$ and 1440 min^{-1} with $H_4PteGlu_2$ and $H_4PteGlu_3$; K_m for serine, 210 μM ($H_4PteGlu_{1,2,3}$); K_m for $H_4PteGlu_n$, 56 μM ($H_4PteGlu_1$), 3.9 μM ($H_4PteGlu_2$), and 1.7 μM ($H_4PteGlu_3$). It should be noted that we have not performed product inhibition studies or equilibrium exchange studies for the pig liver enzyme with each $H_4PteGlu_n$ derivative, and therefore we do not know the order of addition of the substrates nor can we assume that a rapid equilibrium sequential mechanism is followed. Therefore, the actual dissociation constants for the substrates, K_{iA} and K_{iB}, cannot be determined from our data. However, our results establish that the polyglutamate side chains of $H_4PteGlu_2$ and $H_4PteGlu_3$ do not lead to decreases in the values for the K_m of serine (which would appear as decreases in the intercept values of the double-reciprocal plots at low serine concentrations). Instead the free energy decreases associated with binding of the residues of the polyglutamate side chain appear to be expressed only as decreases in the K_m for $H_4PteGlu_n$ (decreases in the slopes of the double-reciprocal plots).

Schirch et al. (1977) have shown that CH_3-$H_4PteGlu_1$ inhibits catalysis of allothreonine cleavage. CH_3-$H_4PteGlu_1$ was shown to be uncompetitive with respect to allothreonine and to give rise to hyperbolic plots of velocity vs. inhibitor concentration at fixed levels of allothreonine. The rate of turnover of serine hydroxymethyltransferase in the presence of saturating concentrations of CH_3-$H_4PteGlu_1$ and allothreonine was shown to be equal to the off constant for the dissociation of glycine from the ternary complex. We have examined the inhibition of allothreonine cleavage by CH_3-$H_4PteGlu_n$ derivatives of varying side chain length at 25 °C. While the extent of inhibition at saturating concentrations of allothreonine and CH_3-$H_4PteGlu_n$ was independent of the number of glutamyl residues (in all cases the inhibited turnover was 20% of the uninhibited turnover), the K_i associated with inhibition of allothreonine cleavage by CH_3-$H_4PteGlu_n$ decreased with increasing numbers of glutamyl residues. Since the extent of maximal inhibition reflects the rate constant for dissociation of glycine from the enzyme, k_{off}, we conclude that the effect of the chain length of CH_3-$H_4PteGlu_n$ on the K_D for dissociation of glycine from the enzyme–glycine–CH_3-$H_4PteGlu_n$ ternary complex is exerted primarily on k_{on} rather than k_{off}. In all cases the value for k_{off} is about 30 min^{-1}, which is 12× slower than the turnover number under V_{max} conditions for serine hydroxymethyltransferase activity in the presence of polyglutamate derivatives of H_4folate. (The latter turnover number is 360 mol of substrate consumed min^{-1} (mol of pyridoxal phosphate^{-1}).)

Discussion

The enzyme used in these studies was homogeneous as assessed by the presence of a single band (53 000 daltons) on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. We do not know definitely whether this is the mitochondrial or the cytoplasmic form of pig liver serine hydroxymethyltransferase,[2] since these two forms have been shown to coisolate from rabbit liver (Schirch & Peterson, 1980). However the pig liver enzyme exhibits several of the properties that distinguish the cytoplasmic rabbit liver enzyme from its mitochondrial counterpart, e.g., a high turnover number in the allothreonine cleavage assay (1440 min^{-1} at 37 °C) and the absence of significant ionization of the α carbon of glycine in the enzyme–glycine complex at pH values below 9.3.

The specificity of pig liver serine hydroxymethyltransferase for the polyglutamate side chain of folate cosubstrates differs considerably from the specificity of pig liver methylenetetrahydrofolate reductase (Matthews & Baugh, 1980). The latter enzyme binds the hexaglutamyl forms of $H_2PteGlu_n$ and CH_2-$H_4PteGlu_n$ more tightly than derivatives of longer or shorter polyglutamate chain length and shows a decrease in free energy of about 0.75 kcal mol^{-1} for the binding of each of the five terminal residues on $H_4PteGlu_6$. Serine hydroxymethyltransferase binds CH_3-$H_4PteGlu_7$ more tightly than shorter chain length derivatives, and the decreases in free energy associated with the binding of the second and third glutamyl residues are much larger than the decreases in free

[2] However, when mitochondria from fresh pig liver were isolated, they were found to contain only 5% of the total serine hydroxymethyltransferase activity (J. D. Cook and L. Davis, unpublished results).

energy associated with binding of the fourth through seventh residues. Thus serine hydroxymethyltransferase exhibits a much broader specificity for polyglutamate chain length than does methylenetetrahydrofolate reductase.

The observation of a broader specificity in the enzyme responsible for generation of CH_2-H_4folate than in one of the enzymes that utilize this metabolite suggests that the polyglutamate chain length of CH_2-H_4folate might play a role in directing the flux of these metabolites through the competing pathways of thymidylate biosynthesis, purine biosynthesis, and the remethylation of homocysteine. A similar proposal has been made by Baggott & Krumdieck (1979). It will now be of interest to examine the specificities of pig liver thymidylate synthase and methylenetetrahydrofolate dehydrogenase for the chain length of polyglutamate substrates and/or inhibitors.

Our results demonstrate that the binding energy of the polyglutamate side chain of H_4folate cosubstrates serves to lower the K_m values for these folate cosubstrates (K_{mB}) and does not appear to affect the K_m for serine (K_{mA}). A 43% decrease in V_{max} is seen with polyglutamate substrates relative to V_{max} when $H_4PteGlu_1$ is used as substrate. Such results are quite similar to the effects of polyglutamate cosubstrates on the reactions catalyzed by methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), thymidylate synthase from bacterial (Kisliuk et al., 1981) and human (Dolnick & Cheng, 1978) sources, avian AICAR transformylase (Baggott & Krumdieck, 1979), clostridial formyltetrahydrofolate synthetase (Curthoys & Rabinowitz, 1972), and methionine synthase from bovine brain (Coward et al., 1975).

In contrast to the lack of effect of the polyglutamate derivatives of H_4folate on the K_m for serine or on the affinity of the enzyme–H_4folate complex for glycine is our observation that enzyme–CH_3-H_4folate binary complexes involving polyglutamate derivatives show enhanced affinities for glycine in comparison to the E–CH_3-$H_4PteGlu_1$ complex. In the presence of glycine concentrations in excess of 20 μM, CH_3-$H_4PteGlu_n$ derivatives with three or more glutamyl residues should be inhibitors of serine hydroxymethyltransferase activity, while glycine concentrations in excess of 100 μM will result in inhibition by both CH_3-H_4folate and H_4folate derivatives due to the formation of dead-end ternary complexes. We believe that the conjoint concentrations of glycine and CH_3-H_4folate derivatives may play an important role in the regulation of serine hydroxymethyltransferase activity in vivo. Such a role of CH_3-H_4folate was initially suggested by Schirch et al. (1977) on the basis of their studies with CH_3-$H_4PteGlu_1$. Inhibition of serine hydroxymethyltransferase activity in the presence of elevated intracellular levels of CH_3-H_4folate polyglutamates may also occur when methionine synthase activity is lowered, as in pernicious anemia or following administration of nitrous oxide. In the latter situation, de novo pyrimidine biosynthesis is rapidly depressed (as assessed by deoxyuridine suppression of the incorporation of [^{3}H]thymidine into DNA of marrow cells) in both animals and man, and megaloblastic changes occur rapidly in man but not in animals (Scott et al., 1979; Deacon et al., 1980). Under these conditions cellular levels of CH_3-H_4folate polyglutamates are elevated at least transiently. Thus inhibition of serine hydroxymethyltransferase activity may contribute to the depletion of CH_2-H_4folate derivatives, which leads to the rapid onset of impaired de novo pyrimidine biosynthesis. Perry, Chanarin, and co-workers (Perry et al., 1979; Deacon et al., 1980) have observed impaired uptake of H_4folate following treatment of rats with nitrous oxide and also found that in vitro addition of H_4folate did not ameliorate the abnormal deoxyuridine suppression seen in marrow cells from the nitrous oxide treated rats.

Tikerpae & Chanarin (1978) have reported that the incorporation of [^{14}C]formate into serine by lymphocytes was impaired approximately 5-fold in all of 16 patients with untreated pernicious anemia who were studied. Normal incorporation was not restored by administration of $PteGlu_1$, although this led to normoblastic haemopoiesis, but normal incorporation of labeled formate into serine could be restored by administration of vitamin B_{12}. Since the conversion of formate into serine requires functional serine hydroxymethyltransferase as well as the availability of methylenetetrahydrofolate, the failure to convert formate to serine in patients who were treated with $PteGlu_1$ may suggest that serine hydroxymethyltransferase activity is impaired in these patients.

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Purification and Biosynthesis of Quench Spot, a Drosopterin Precursor in *Drosophila melanogaster*†

Dale Dorsett‡ and K. Bruce Jacobson*

ABSTRACT: Pteridine biosynthesis has been examined in extracts of the heads of *Drosophila melanogaster* by measuring the conversion of dihydroneopterin triphosphate to sepiapterin and the "drosopterins" (six eye pigments that are dipterin derivatives). These two products share a common first step in the production of an intermediate that is a branch point from which both products are formed. This first step can be catalyzed by sepiapterin synthase or by an enzyme found in particles that sediment at 600*g*. A substance named "quench spot" was found earlier to be at low levels in the purple mutants that were defective in drosopterin synthesis and to be restored to normal when a suppressor mutant, *su(s)*^{2}, restored drosopterins in purple to normal levels. The sepia mutant is also deficient in the levels of both quench spot and drosopterins. In this report we propose that quench spot is a precursor of drosopterins, but not sepiapterin, and that it is formed from the sepiapterin synthase intermediate mentioned above. An additional precursor that is formed independently of the sepiapterin synthase pathway is also proposed that would react with quench spot to form drosopterins. These proposals are based on the following: (1) quench spot biosynthesis is observed in extracts of *Drosophila* heads in which [U-^{14}C]dihydroneopterin triphosphate is the substrate; (2) Mg^{2+} is required for the synthesis of quench spot but either NADH or NADPH causes diminished incorporation of the label; (3) extracts from heads of a purple mutant (*pr*^{bw}*cn*) contain only 30% of the quench spot biosynthetic activity as compared to heads from wild type (Oregon-R); (4) quench spot has been purified from heads of wild-type *Drosophila*; (5) addition of quench spot stimulates the biosynthesis of drosopterins in an enzyme preparation from Oregon-R.

In a previous report we described the in vitro enzymatic conversion of the 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)pterin [H_2-neopterin-$(P)_3$][1] to the six dipterin-derivative red accessory eye pigments of *Drosophila* known collectively as the "drosopterins" [neodrosopterin, fraction e, aurodrosopterins I and II, drosopterin, and isodrosopterin] (Dorsett et al., 1979). It was concluded that sepiapterin synthase, which is responsible for the conversion of H_2-neopterin-$(P)_3$ to sepiapterin, consists of two enzymes, the first of which also occurs in pellets obtained at 600*g* from head homogenates. This first enzyme utilizes Mg^{2+} as a cofactor and produces a product that can be converted to sepiapterin by the second enzyme in the presence of NADPH (Krivi & Brown, 1979; Tanaka et al., 1981). In the presence of either NADPH or NADH, the sepiapterin synthase intermediate can be converted by another set of enzymes to drosopterins. The intermediate is nonphosphorylated, but it is not H_2-neopterin (Dorsett et al., 1979; Krivi & Brown, 1979; Tanaka et al., 1981). The separation of the two activities of sepiapterin synthase from *Drosophila* (Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1981) has been reported. The purple (*pr*) eye color mutant of *Drosophila* is thought to be a lesion in the structural locus coding for the first enzyme of sepiapterin synthase (Yim et al., 1977; Dorsett et al., 1979).

Quench spot (QS) is an unidentified compound that has been found and quantitated in wild-type, *pr*, sepia (*se*), and Henna-recessive-3 (*Hn*^{r3}) flies (Wilson & Jacobson, 1977b). Purple, which contains low levels of sepiapterin and drosopterins, also has low levels of QS. Sepia contains very high amounts of sepiapterin but neither QS nor drosopterins. Henna-recessive-3 has a high level of both sepiapterin and QS but low drosopterins. These considerations lead us to hypothesize that QS is an intermediate in drosopterin biosynthesis. The data presented in this report support this hypothesis

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; H_2-neopterin-$(P)_3$, 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)-pterin; sepiapterin, 7,8-dihydro-6-lactoylpterin; pterin, 2-amino-4-hydroxypteridine; isoxanthopterin, 7-hydroxypterin; Pipes, 1,4-piperazinediethanesulfonic acid; QS, quench spot; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; HPLC, high-performance liquid chromatography; PFE, pteridine-free extract; TE, Triton extract; TLC, thin-layer chromatography.

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