Inhibition of Glycine N-Methyltransferase by 5-Methyltetrahydrofolate Pentaglutamate*

(Glu5, folic acid pentaglutamate; H2PteGlu AdoMet, S

The enzyme was previously shown to be abundant in both the liver and pancreas of the rat, to consist of four identical monomers, and to contain tightly bound folate polyglutamates in vivo. We now report that the inhibition of glycine N-methyltransferase by (6S)-5-CH3-H4PteGlu5 is noncompetitive with regard to both S-adenosylmethionine and glycine. The enzyme exhibits strong positive cooperativity with respect to S-adenosylmethionine. Cooperativity increases with increasing concentrations of 5-CH3-H4PteGlu5 and is greater at physiological pH than at pH 9.0, the pH optimum. Under the same conditions, cooperativity is much greater for the pancreatic form of the enzyme. The Vmax for the liver form of the enzyme is approximately twice that of the pancreatic enzyme, while Km values for each substrate are similar in the liver and pancreatic enzymes. For the liver enzyme, at pH 7.0 half-maximal inhibition is seen at a concentration of about 0.2 mM (6S)-5-CH3-H4PteGlu5, while at pH 9.0 this value is increased to about 1 mM. For the liver form of the enzyme, 50% inhibition with respect to S-adenosylmethionine at pH 7.4 occurs at about 0.27 mM. The dissociation constant, Kd, obtained from binding data at pH 7.4 is 0.095. About 1 mol of (6S)-5-CH3-H4PteGlu5 was bound per tetramer at pH 7.0, and 1.6 mol were bound at pH 9.0. The degree of binding and inhibition were closely parallel at each pH. At equal concentrations of (6R,6S)- and (6S)-5-CH3-H4PteGlu5, the natural (6S) form was about twice as inhibitory. These studies indicate that glycine N-methyltransferase is a highly allosteric enzyme, which is consistent with its role as a regulator of methyl group metabolism in both the liver and the pancreas.

Glycine N-methyltransferase (EC 2.1.1.20) catalyzes the methylation of glycine by S-adenosylmethionine to form sarcosine and S-adenosylhomocysteine. The enzyme was previously shown to be abundant in both the liver and pancreas of the rat, to consist of four identical monomers, and to contain tightly bound folate polyglutamates in vivo. We now report that the inhibition of glycine N-methyltransferase by (6S)-5-CH3-H4PteGlu5 is noncompetitive with regard to both S-adenosylmethionine and glycine. The enzyme exhibits strong positive cooperativity with respect to S-adenosylmethionine. Cooperativity increases with increasing concentrations of 5-CH3-H4PteGlu5 and is greater at physiological pH than at pH 9.0, the pH optimum. Under the same conditions, cooperativity is much greater for the pancreatic form of the enzyme. The Vmax for the liver form of the enzyme is approximately twice that of the pancreatic enzyme, while Km values for each substrate are similar in the liver and pancreatic enzymes. For the liver enzyme, at pH 7.0 half-maximal inhibition is seen at a concentration of about 0.2 mM (6S)-5-CH3-H4PteGlu5, while at pH 9.0 this value is increased to about 1 mM. For the liver form of the enzyme, 50% inhibition with respect to S-adenosylmethionine at pH 7.4 occurs at about 0.27 mM. The dissociation constant, Kd, obtained from binding data at pH 7.4 is 0.095. About 1 mol of (6S)-5-CH3-H4PteGlu5 was bound per tetramer at pH 7.0, and 1.6 mol were bound at pH 9.0. The degree of binding and inhibition were closely parallel at each pH. At equal concentrations of (6R,6S)- and (6S)-5-CH3-H4PteGlu5, the natural (6S) form was about twice as inhibitory. These studies indicate that glycine N-methyltransferase is a highly allosteric enzyme, which is consistent with its role as a regulator of methyl group metabolism in both the liver and the pancreas.

Glycine N-methyltransferase (GNMT1; EC 2.1.1.20) catalyzes the methylation of glycine by S-adenosylmethionine to form sarcosine and S-adenosylhomocysteine. The enzyme was previously shown to be abundant in both the liver and pancreas of the rat, to consist of four identical monomers, and to contain tightly bound folate polyglutamates in vivo. We now report that the inhibition of glycine N-methyltransferase by (6S)-5-CH3-H4PteGlu5 is noncompetitive with regard to both S-adenosylmethionine and glycine. The enzyme exhibits strong positive cooperativity with respect to S-adenosylmethionine. Cooperativity increases with increasing concentrations of 5-CH3-H4PteGlu5 and is greater at physiological pH than at pH 9.0, the pH optimum. Under the same conditions, cooperativity is much greater for the pancreatic form of the enzyme. The Vmax for the liver form of the enzyme is approximately twice that of the pancreatic enzyme, while Km values for each substrate are similar in the liver and pancreatic enzymes. For the liver enzyme, at pH 7.0 half-maximal inhibition is seen at a concentration of about 0.2 mM (6S)-5-CH3-H4PteGlu5, while at pH 9.0 this value is increased to about 1 mM. For the liver form of the enzyme, 50% inhibition with respect to S-adenosylmethionine at pH 7.4 occurs at about 0.27 mM. The dissociation constant, Kd, obtained from binding data at pH 7.4 is 0.095. About 1 mol of (6S)-5-CH3-H4PteGlu5 was bound per tetramer at pH 7.0, and 1.6 mol were bound at pH 9.0. The degree of binding and inhibition were closely parallel at each pH. At equal concentrations of (6R,6S)- and (6S)-5-CH3-H4PteGlu5, the natural (6S) form was about twice as inhibitory. These studies indicate that glycine N-methyltransferase is a highly allosteric enzyme, which is consistent with its role as a regulator of methyl group metabolism in both the liver and the pancreas.

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Materials—Rat liver GNMT was purified from rat liver extract by a modification of the method described previously by Suzuki and Wagner (5). Rat pancreatic GNMT was purified from fresh pancreas as described by Yeo and Wagner (9). Synthetic folic acid pentaglutamate (PteGlu5) was provided by B. Schircks Laboratories, Jona, Switzerland. Synthetic (6R,6S)-5-CH3-H4PteGlu5 was prepared from the corresponding folate polyglutamates as described by Yeo and Wagner (9). Synthetic (6S)-5-CH3-H4PteGlu5 was prepared as described below. Bio-Safe II scintillation fluid was from Research Products International Corp. AdoMet was obtained from Sigma, and S-adenosyl-L-[methyl-3H]methionine (73.5 Ci/mmole) was from NEN Life Science Products. The labeled and unlabeled AdoMet were repurified by a modification of the procedure by Zappia et al. (11).

Synthesis of (6S)-5-CH3-H4PteGlu5—(6R)-5-CH3-H4PteGlu5 was prepared by a modification of the method used for the preparation of radioactive (6S)-5-CH3-H4PteGlu5 from folic acid (12). The procedure consisted of direct reduction of PteGlu5 to H4PteGlu5 by using dihydrofolate reductase. The H4PteGlu5 reacted with formaldehyde to give 5,10-methylene-H4PteGlu5, which was reduced to 5-CH3-H4PteGlu5 with sodium borohydride. The details are described below.

About 0.25 μmol of PteGlu5 (100 μl) was added to the reaction mixture containing 0.5 mg of NADPH (100 μl), 0.5 ml of 0.05 mM sodium acetate buffer, pH 4.8, 9.9 mg of solid sodium ascorbate, 10 μl of 0.37% (v/v) formaldehyde, and 0.3 ml of distilled water. The solution was bubbled with N2 for 2 min, and then 1 mg of solid dihydrofolate reductase from Lactobacillus casei (0.075 unit/mg, dry weight) was added. It
is important to use the enzyme from \textit{L. casei}, because dihydrofolate reductase from many other sources does not readily react with \textit{P. gingivalis} derivatives but only with 7, 8-\textit{H}4PteGlu5 derivatives. The mixture was incubated under nitrogen in the dark for 1 h, at 37 °C. Additional formaldehyde (250 μl of 0.37%) was added and incubated for an additional 30 min at 37 °C. To the reaction mixture, 0.5 ml of cold deionized water and 1 ml of 0.2 M Tris-HCl, pH 7.5, was added. Then 10 mg of sodium borohydride in 100 μl of the Tris buffer was added in four portions over a period of 5 min. The tube was flushed with nitrogen and incubated at 37 °C for 1 h. After the incubation, the tube was cooled in an ice bath, and 10 μl of 14 mM 2-mercaptoethanol was added to protect folates from oxidation. The pH was reduced to 5.0 with 5 N acetic acid to destroy excess borohydride, and then the pH was adjusted back to 7.2 with 1 N NaOH. The reaction mixture \((6S)-5-\text{CH}_3-\text{H}_4\text{PteGlu5}) was purified by fast protein liquid chromatography with a Mono Q column, which was equilibrated with 10 mM 2-mercaptoethanol and was eluted with a gradient of 0–2 M ammonium acetate, pH 7.2, in 10 mM 2-mercaptoethanol. The product was lyophilized and taken up in 5 ml of 0.5 M potassium phosphate, pH 7.0, and 40 mM 2-mercaptoethanol. Its identity was confirmed by treatment with conjugal high performance liquid chromatography analysis as described by Wilson and Horne (13).

**Kinetic Studies**—Preliminary studies had shown that inhibition of GNMT activity by 5-\textit{CH}3-H4PteGlu5 was variable and tended to be influenced by the order of addition of substrate or inhibitor. When the enzyme was preincubated with 5-\textit{CH}3-H4PteGlu5, inhibition was consistently observed. Kinetic measurements were carried out using the stopped reaction method for GNMT activity as described below (6) at pH 7.4 and at pH 9.0, the pH optimum. To examine the effects of varying glycine on GNMT activity, the reaction mixture contained about 1.5 μg of GNMT; either 0.2 mM Heps, pH 7.4, or 0.2 mM Tris, pH 9.0; 0.5 mM \[^{[3]}\text{H}-\text{CH}_3\]AdoMet (5,000 dpm/μmol); and 5 mM DTT. To examine the effects of varying AdoMet, 8 mM glycine was included. Inhibition of activity was determined by including (6S)-5-\textit{CH}3-H4PteGlu5 at varying concentrations. The 5-\textit{CH}3-H4PteGlu5 was made up in 40 mM 2-mercaptoethanol, and the same amount of 2-mercaptoethanol was added to the samples without inhibitor. Measurement of inhibition was carried out by preincubating the enzyme, buffer, DTT, and either 5-\textit{CH}3-H4PteGlu5 or 2-mercaptoethanol for 15 min at 25 °C. The reaction was started by the addition of cold 10% trichloroacetic acid followed by 250 μl of a charcoal suspension (38 mg/ml in 0.1 M acetic acid). After incubation for an additional 15 min at 0 °C, the sample was centrifuged for 4 min in a microcentrifuge. Two hundred μl of the supernatant was added to 5 ml of Bio-Safe II scintillation fluid, and the radioactivity incorporated into sarcosine was counted in a BetaTrac supernatant was added to 5 ml of Bio-Safe II scintillation fluid, and the radioactivity incorporated into sarcosine was counted in a BetaTrac counter. Activity was compared with control samples preincubated in the absence of (6S)-5-\textit{CH}3-H4PteGlu5. Inhibition of liver GNMT by (6S)-5-\textit{CH}3-H4PteGlu5, at pH 7.0 was also examined. This was carried out in a 60-μl incubation mixture with 0.1 mM potassium phosphate buffer, pH 7.0, containing 4.5 μg of enzyme. After a 15-min preincubation at 25 °C, the substrates were added, and the volume was brought to 100 μl. The reaction was continued for 1 h. The kinetic reaction was started by the addition of \[^{[3]}\text{H}\]AdoMet and varying amounts of glycine, so the final concentration of AdoMet was 0.5 mM and varying amounts of glycine in a final volume of 0.1 ml. Incubation was carried out at 25 °C for 15 min. The reaction was stopped, and activity was measured as described above.

**RESULTS**

Effect of 5-\textit{CH}3-H4PteGlu5 on Kinetic Parameters—GNMT is a highly allosteric enzyme. Fig. 1 shows extensive cooperativity with respect to AdoMet as substrate for the rat liver enzyme at pH 7.4. Cooperativity appears to be increased with increasing concentrations of the inhibitory ligand, 5-\textit{CH}3-H4PteGlu5. The pH optimum for GNMT is pH 9.0 (4). At this pH, GNMT shows minimal cooperativity, but in the presence of 5-\textit{CH}3-H4PteGlu5 sigmoid kinetics is easily seen (Fig. 2). In contrast, hyperbolic kinetics is seen when bound folate is added to the second substrate, glycine, at pH 7.4 and pH 9.0 (data not shown). 5-\textit{CH}3-H4PteGlu5 behaves as a noncompetitive inhibitor with respect to AdoMet. This is more clearly seen at pH 9.0 (Fig. 3). Here, the data are presented in a Hanes-Woolf plot to avoid the undue weighting of the lowest concentration of substrate inherent in the double reciprocal Lineweaver-Burk plot. It can be seen that three of...
the lines clearly intersect on the x axis, while the fourth line is only slightly below the others, indicating that the inhibition is noncompetitive. The greater sigmoidicity at pH 7.4 results in plots that are nonlinear and are difficult to interpret (data not shown). In contrast, there is no cooperativity with respect to glycine as a substrate, and a double reciprocal plot shows that 5-CH3-H4PteGlu5 is clearly a noncompetitive inhibitor with respect to glycine (Fig. 4). Pancreatic GNMT is also inhibited by 5-CH3-H4PteGlu5. The pancreatic enzyme appears to be even more allosteric than the liver enzyme, although there is no significant cooperativity at pH 9.0 in the absence of 5-CH3-H4PteGlu5 (Fig. 5). The effects of 5-CH3-H4PteGlu5 on the cooperativity of the liver and pancreatic forms of GNMT are summarized as apparent Hill coefficients in Table I. Values for V_max(app) and K_a(app) are shown in Table II. No data were collected for the pancreatic enzyme at pH 7.4. The pancreatic enzyme is about half as active as the liver enzyme under similar conditions.

Because reciprocal plots of velocities obtained with GNMT under different concentrations of 5-CH3-H4PteGlu5 at pH 7.4 are not linear, as with all enzymes exhibiting positive cooperativity, it was impossible to determine the K_i from a Dixon plot. Therefore, the concentration of 5-CH3-H4PteGlu5 that results in 50% inhibition was determined at saturating (0.70 and 0.35 mM) concentrations of AdoMet. A value of about 0.27 μM was obtained (Fig. 6). This value is comparable with the dissociation constant, K_i, obtained from binding data (see below).

The inhibitory effects of (6R,6S)- or (6S)-5-CH3-H4PteGlu5 on liver GNMT are shown in Fig. 7. When equimolar amounts (1 μM) of either the (R,S) or the (S) form were added, the (S) form was approximately twice as inhibitory, suggesting that the unnatural (R) form is a much less potent inhibitor.

Inhibition and Binding by (6S)-5-CH3-H4PteGlu5—When purified liver GNMT was incubated with increasing amounts of (6S)-CH3-H4PteGlu5 for 1 h, complete inhibition was obtained at about 9 μM at pH 7.0 (Fig. 8A). Half-maximal inhibition occurred at about 0.2 μM. A similar experiment was carried out at pH 9.0 (Fig. 8B), but in this case the enzymatic reaction was continued for only 30 min. In contrast, at pH 9.0, a maximum of 80% inhibition was obtained, and half-maximal inhibition

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**Table I**

| Concentration of 5-CH3-H4PteGlu5 | Liver, pH 7.4 | Liver, pH 9.0 | Pancreas, pH 9.0 |
|----------------------------------|--------------|--------------|-----------------|
| μM                               |              |              |                 |
| 0.00                             | 1.53         | 1.08         | 1.04            |
| 0.05                             | 1.82         |              | ND†             |
| 0.10                             | 1.82         | 1.01         | 1.79            |
| 0.20                             | 2.00         | 1.52         | 1.93            |

*ND, not determined.

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FIG. 2. Double reciprocal plot of the effect of glycine concentration on liver GNMT activity at pH 9.0 in the absence and presence of 0.3 μM (6R,6S)-5-CH3-H4PteGlu5. AdoMet was maintained at 0.5 mM, and glycine was varied from 0.1 to 4.0 mM. All other conditions were the same as for Fig. 2.

FIG. 4. Double reciprocal plot of the effect of AdoMet concentration on liver GNMT activity at pH 9.0 in the absence and presence of (6S)-5-CH3-H4PteGlu5. Conditions were the same as for Fig. 2.

FIG. 5. Effect of AdoMet concentration on pancreatic GNMT activity at pH 9.0 in the absence and presence of (6S)-5-CH3-H4PteGlu5. Conditions were the same as for Fig. 2.
occurred at about 1.1 m (Fig. 8). The increased reaction time at pH 7.0 was required in order to obtain accurate measurements, because GNMT is less active at this pH. Binding studies were carried out in a similar manner but in the absence of substrates. Following the incubation period, a portion of the incubation mixture was rapidly filtered as described under “Experimental Procedures” to separate bound and unbound folate. The amount of 5-CH₃-H₄PteGlu₅ was measured by fluorescence after acidification as described under “Experimental Procedures.” Scatchard plots of the binding data (Fig. 9) show that binding is concave upward. Analysis of the data is complicated by the nonlinear nature of the binding process, but extrapolation of the curves to the x axis provides an estimate of the maximum concentrations of ligand bound that are about 0.36 m at pH 7.0 and 0.57 m at pH 9.0. Since each experiment used 0.35 m enzyme, this calculates to a stoichiometry of 1 mol of 5-CH₃-H₄PteGlu₅ per tetramer at pH 7.0. At pH 9.0, the corresponding stoichiometry is 1.6. The binding data were recalculated as percentage of maximum binding (% Bmax) at each concentration of 5-CH₃-H₄PteGlu₅, and these values were replotted in Fig. 8, A and B. There is a very close correspondence between the amount of inhibition of GNMT activity and the amount of ligand bound, despite the fact that only 1 mol of bound 5-CH₃-H₄PteGlu₅ causes complete inhibition of the tetrameric enzyme at pH 7.0. At pH 9.0, the binding of 1 mol of ligand produced about 80% inhibition. Hill plots of the binding of 5-CH₃-H₄PteGlu₅ at pH 9.0 and 7.0 gave n values of 0.65 and 1.0, respectively (data not shown). Values of n less than 1.0 and Scatchard plots that are concave upward are characteristic of

| Enzyme source | pH | Substrate | Vmax/app (nmol/min/mg) | Km(app) (mM) |
|---------------|----|-----------|------------------------|--------------|
| Liver         | 7.4| AdoMet    | 258 ± 32               | 0.39 ± 0.05  |
|               | 7.4| Glycine   | 203 ± 12               | 0.91 ± 0.02  |
| Liver         | 9.0| AdoMet    | 371 ± 28               | 0.10 ± 0.10  |
|               | 9.0| Glycine   | 400 ± 17               | 0.99 ± 0.12  |
| Pancreas      | 7.4| AdoMet    | ND                     | ND           |
| Pancreas      | 9.0| AdoMet    | 234 ± 13               | 0.13 ± 0.01  |
|               | 9.0| Glycine   | 244 ± 11               | 0.82 ± 0.22  |

* ND, not determined.
either a heterogeneity of binding sites or negative cooperativity between binding sites in which the first ligand has a higher apparent affinity than subsequent ligands (16). Calculation of the dissociation constant from the slopes of the extrapolated linear portions of the Scatchard plots in Fig. 9, A and B, gave values of 0.095 \( \mu M \) at pH 7.4 and 1.9 \( \mu M \) at pH 9.0.

**DISCUSSION**

GNMT is believed to play a central role in the regulation of methyl group metabolism of the liver (2). It is present in large amounts, and the product of methyl transfer in the reaction is sarcosine, which has no known physiologic role in mammals. For this reason it has been suggested (2) that GNMT serves as a mechanism for converting excess AdoMet, arising from methionine, to AdoHcy in order to stabilize the ratio of AdoMet/AdoHcy. Since most methylation reactions are product-inhibited by AdoHcy, this ratio is believed to reflect the overall methylaing ability of the cell (17). The fact that GNMT is also inhibited by 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) links the availability of preformed methyl groups, as manifested by the level of AdoMet, to the \textit{de novo} synthesis of methyl groups via the one-carbon folate pool (7). The abundance of GNMT in the pancreas as well as the liver of the rabbit and the rat was first noted by Kerr (2). Ogawa \textit{et al.} (18) showed that mRNA for GNMT is almost as abundant in pancreas as in liver. We have shown that pancreatic GNMT contains tightly bound folate in vivo (9). The present study shows that the patterns of inhibition of both liver and pancreatic forms of GNMT are very similar.

The inhibition by 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) has unusual features. It behaves in many respects as a slow, tightly binding inhibitor. Preliminary studies indicated that inhibition was consistent when the enzyme was preincubated with 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) for 15 min before the addition of substrate. Because only a discontinuous assay for GNMT activity is available, it has been impossible to describe the decrease in reaction velocity as a function of time. Ogawa \textit{et al.} (19) have developed a continuous assay for GNMT activity that involves coupling the production of S-adenosylhomocysteine to S-adenosylhomocysteine hydrolase and adenosine deaminase. The adenosine formed by S-adenosylhomocysteine hydrolase is then converted to inosine with a decrease in absorbance at 265 nm. Unfortunately, S-adenosylhomocysteine hydrolase is inhibited by S-adenosylmethionine, and the coupled reaction cannot be carried at concentrations of S-adenosylmethionine greater than 0.1 \( \mu \)M. 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) also binds very tightly to GNMT. GNMT was first purified as a liver cytosolic folate-binding protein. Radioactive folate was injected into rats, and 24 h later the liver cytosol contained a protein of about 130 kDa that had tightly bound 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) (20). In fact, purification was dependent upon following the bound radioactivity over several different columns. Only when passed over an anion exchange column did the folate ligand dissociate (5). Tightly binding inhibitors are effective at concentrations similar to that of the enzyme (21). In the studies described here, GNMT was present at a concentration of about 0.09 \( \mu M \) (for the tetrameric enzyme), and 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) was present at concentrations of 0.1–0.3 \( \mu M \). Under these conditions, a significant amount of enzyme and inhibitor should be present in an essentially irreversible complex, and the assumptions upon which the Michaelis-Menten and the Hill equations are based do not apply (22). It is therefore surprising that the positive cooperativity of GNMT is increased with increasing amounts of inhibitor. One would expect the reaction rate to be decreased when significant amounts of enzyme are tied up in an essentially irreversible complex but not that the cooperativity of the enzyme would increase. Fig. 8A indicates that at a concentration of 0.1 \( \mu M \) about 5% of 5-CH\(_3\)-H\(_4\)PteGlu\(_5\) was bound, and at 0.3 \( \mu M \) about 30% was bound at pH 7.4 (i.e. 95 and 70%, respectively, were unbound). It may be that the decreased amounts of enzyme and inhibitor are not sufficient to significantly alter the fit of the data to the Hill equation. Fig. 8A also shows that it is possible to completely inhibit the enzyme, but this requires far greater concentrations of CH\(_3\)-H\(_4\)PteGlu\(_5\) (about 8 \( \mu M \)).

Ogawa and Fujikawa (4) noted that liver GNMT exhibited positive cooperativity with respect to AdoMet when measured at pH 7.4. It was also stated that positive cooperativity was noted at pH 8.8. Interaction of AdoMet with the liver enzyme also showed positive cooperativity when measured by the quenching of the intrinsic fluorescence at pH 7.4 (23) or by the binding of radioactive AdoMet at pH 7.2 (24). Limited proteolysis of rat liver GNMT removed an 8-residue N-terminal peptide, which changed the kinetics from sigmoid to hyperbolic (24). The crystal structure of the recombinant rat liver GNMT has recently been published (10). Crystals were grown at pH 6.5. The tetrameric structure shows the N terminus of each subunit deeply associated with the other three subunits. We
have not observed positive cooperativity of the rat liver enzyme at pH 9.0 in the absence of 5-CH₃-H₄PteGlu₅. This suggests that the tetrameric enzyme is in a looser configuration at pH 9.0 than it is at pH 7.0 and that the presence of the inhibitor causes a change in conformation or association, which permits interaction among the subunits. We have also obtained evidence that binding of 5-CH₃-H₄PteGlu₅ results in a change in the intrinsic fluorescence of GNMT. Konishi and Fujioka (24) showed that 4 mol of AdoMet were bound per tetramer, suggesting the existence of four active sites. This has been confirmed by the crystal structure, which revealed that AdoMet remained bound to each monomer of the enzyme upon crystallization.

Inhibition of the liver enzyme by 5-CH₃-H₄PteGlu₅ occurred at both pH 9.0 and 7.0. Binding and inhibition by 5-CH₃-H₄PteGlu₅ closely paralleled each other at both pH values (Fig. 8). Complete inhibition of activity was observed at pH 7.0 when only 1 mol of ligand was bound per mol of tetrameric enzyme. At pH 9.0, a maximum of 1.6 mol of ligand were bound per mol of tetrameric enzyme. Fu et al. have suggested that 5-CH₃-H₄PteGlu₅ binds to the AdoMet binding site (10); however, this appears to be extremely unlikely. Aside from the limited similarity between the pterin ring and the purine ring, the two compounds are quite different in structure. AdoMet has a net positive charge, and the folate polyglutamate has multiple negative charges. AdoMet binds to four sites per tetramer and shows positive cooperativity; 5-CH₃-H₄PteGlu₅ binds to one site per tetramer and shows negative cooperativity. It is therefore possible that 5-CH₃-H₄PteGlu₅ may then restrict access of AdoMet to its binding sites. These suggestions should be clarified by locating 5-CH₃-H₄PteGlu₅ within the crystal structure of the molecule.

The concentration of 5-CH₃-H₄PteGlu₅ in rat liver has been estimated to be about 9.1 μM (25). From the same reference, the concentration of AdoMet can be estimated to be about 0.4 mM. The apparent Kₘ for AdoMet is about 0.39 mM (liver enzyme; Table II). Complete inhibition occurs at 10 μM 5-CH₃-H₄PteGlu₅ (Fig. 8A), and 50% inhibition occurs at about 0.27 μM (Fig. 6). Therefore, the concentrations of these compounds in the liver are in the range where changes could modulate the activity of the enzyme.

The complete inhibition of a tetrameric enzyme containing four active sites by a single mole of inhibitor is highly unusual. It would be of benefit, however, for the regulation of GNMT under physiologic conditions, because the enzyme activity can be completely turned on or off through a relatively narrow concentration range of 5-CH₃-H₄PteGlu₅. If there were four sites for binding and inhibition, then complete inhibition would require 4 times as much 5-CH₃-H₄PteGlu₅ much more than is actually present (25).

The binding of 5-CH₃-H₄PteGlu₅ to GNMT is similar in many aspects to the binding of folate polyglutamates to deoxyhemoglobin. Benesch and co-workers (26, 27) showed that folate polyglutamates are bound to deoxyhemoglobin tetramers at a site to which 2,3-diphosphoglycerate is also bound. Only a single molecule of the folate polyglutamate was bound to the deoxyhemoglobin tetramer. Both 5-CH₃-H₄PteGlu₅ and PteGlu₇, as well as the polyglutamate form of methotrexate were bound with dissociation constants ranging from 0.025 to 0.14 mM at a site that involves the central cavity of the deoxyhemoglobin tetramer. In oxymyoglobin, the central cavity is too small to accommodate the folate polyglutamate (28). It is tempting to think that 5-CH₃-H₄PteGlu₅ may bind to the GNMT tetramer in a similar manner. The availability of the crystal structure of the enzyme makes such studies now possible.

Acknowledgments—We acknowledge Byron Glenn and W. Decha-Umphai for excellent technical assistance. In addition, we thank Drs. D. W. Horne and R. J. Cook for useful discussion on methodology. We also are grateful to Dr. R. L. Kishiuk for discussion on the interaction of folate polyglutamate with GNMT.

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E.-J. Yeo, W. T. Briggs, and C. Wagner, unpublished results.