Serine Transhydroxymethylase. Affinity of Tetrahydrofolate Compounds for the Enzyme and Enzyme–Glycine Complex*

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ABSTRACT: Serine transhydroxymethylase has been shown previously to combine with glycine and tetrahydrofolate to form a complex absorbing at 495 mμ [Schirch, L., and Mason, M. (1963), *J. Biol. Chem. 238*, 1032]. This paper shows that N^5-methyltetrahydrofolate and N^5-formyltetrahydrofolate form complexes with serine transhydroxymethylase and glycine which exhibit absorption maxima at 506 mμ. The absorbance of these complexes near 500 mμ has been used as a way of determining the affinity of the tetrahydrofolate derivatives and glycine for the enzyme. The data show that N^5-methyltetrahydrofolate, N^5-formyltetrahydrofolate, and tetrahydrofolate have a five- to tenfold greater affinity for the enzyme–glycine complex than for the enyzme.

The studies also show that glycine has a greater affinity for the complexes of the enzyme with the above-named tetrahydrofolate compounds than for the enzyme. N^5-Methyl- and N^5-formyltetrahydrofolates were shown to be competitive inhibitors of tetrahydrofolate in the enzymatic conversion of serine to glycine.

Serine transhydroxymethylase catalyzes the interconversion of serine and glycine as shown in eq 1.

$$\text{L-serine} + FH_4{}^1 \rightleftharpoons \text{glycine} + N^{5,10}\text{-methylene-}FH_4 \quad (1)$$

Schirch and Mason (1963) have previously shown that the enzyme combines with glycine and tetrahydrofolate to form an enzyme–substrate complex absorbing maximally at 495 mμ. This colored complex provides a convenient method for measuring the affinity of substrates and inhibitors for the enzyme (Schirch and Jenkins, 1964a). This paper describes the affinity of tetrahydrofolate and several of its metabolic derivatives for the enzyme and enzyme–glycine complex. The results indicate that substitution on the 5-nitrogen of tetrahydrofolate by either a methyl or a formyl group does not interfere with its binding to the enzyme. The possible physiological significance of the results are discussed.

Experimental Section

Methods and Materials. Tetrahydrofolate was purchased from Nutritional Biochemicals Corp. and purified as previously described (Schirch and Jenkins, 1964b). The concentrations of tetrahydrofolate reported in the following experiments were determined by measuring enzymatically the concentrations of $N^{5,10}$-methylenetetrahydrofolate formed after the addition of an excess of formaldehyde (Huennekens *et al.*, 1963). Only 1-L-tetrahydrofolate is measured by this method.

N^5-Methyltetrahydrofolate was prepared and purified according to the method of Mangum (1963). The purified compound exhibited an A_{max} at 290 mμ and an A_{min} at 245 mμ. N^5-Formyl- and N^{10}-formyltetrahydrofolates were gifts from G. R. Greenberg. The concentrations of these tetrahydrofolate compounds were determined spectrophotometrically and, therefore, represent the concentrations of *dl* mixtures. Serine transhydroxymethylase was purified as previously described (Schirch and Jenkins, 1964a). All preparations used had an $A_{280}:A_{430} = 10$ ratio, which is characteristic of enzyme preparations that are 90–100% pure (Schirch and Mason, 1963). Glycine was purchased from Nutritional Biochemicals Corp.

The enzymatic activity of serine transhydroxymethylase was measured at 30° as previously described (Schirch and Mason, 1962). All spectrophotometric studies were performed with a Beckman DB spectrophotometer connected to a Sargent SRL recorder. A scale expander was used with several experiments.

Results

We have previously shown that solutions of serine transhydroxymethylase, glycine, and tetrahydrofolate exhibit a large absorption maximum at 495 mμ (Schirch and Mason, 1963). Several other derivatives of tetrahydrofolate have been tried in this system to gain some insight into the structural requirements for binding of the tetrahydrofolate molecule. When N^5-formyl- and N^5-methyltetrahydrofolates are substituted for tetrahydrofolate an absorption maximum at 506 mμ

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[1] The following abbreviations are used: FH_4, tetrahydrofolate; E, serine transhydroxymethylase; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides.

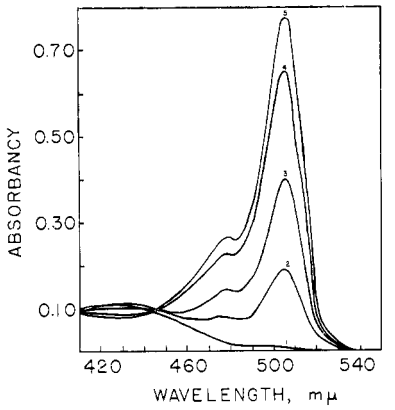

FIGURE 1: Effect of 5-methyltetrahydrofolate on the spectrum of a solution of 0.01 M glycine and serine transhydroxymethylase (1.2 mg/ml) in 0.05 M potassium phosphate–0.002 M mercaptoethanol, pH 7.3. Curve 1, spectrum of glycine and enzyme; curves 2–5, spectrum of glycine–enzyme solution after the addition of tetrahydrofolate; curve 2, 4.2×10^{-6} M; curve 3, 1.3×10^{-5} M; curve 4, 3.4×10^{-5} M; curve 5, 7.6×10^{-5} M.

with a shoulder at 468 mμ is observed. Figure 1 shows the effect of increasing concentrations of N^5-methyltetrahydrofolate on the spectrum. Essentially identical results were obtained when N^5-formyltetrahydrofolate was used instead of N^5-methyltetrahydrofolate.

The large molar absorptivity constant of the compound absorbing near 500 mμ gives a rapid and accurate method for determining the affinity of these tetrahydrofolate compounds for the enzyme. Florini and Vestling (1957) have described a method for determining the affinity constants for reactions involving two substrates. Using their graphical method we have determined the equilibrium constants for the following reactions.

$$E^1 + FH_4 \overset{K_2}{\rightleftharpoons} E{\cdot}FH_4 \quad (2)$$

$$E + Gly^1 \overset{K_3}{\rightleftharpoons} E{\cdot}Gly \quad (3)$$

$$E{\cdot}FH_4 + Gly \overset{K_4}{\rightleftharpoons} E{\cdot}FH_4{\cdot}Gly \quad (4)$$

$$E{\cdot}Gly + FH_4 \overset{K_5}{\rightleftharpoons} E{\cdot}FH_4{\cdot}Gly \quad (5)$$

$$E + N^5\text{-methyl-}FH_4 \overset{K_6}{\rightleftharpoons} E{\cdot}N^5\text{-methyl-}FH_4 \quad (6)$$

$$E{\cdot}Gly + N^5\text{-methyl-}FH_4 \overset{K_7}{\rightleftharpoons} E{\cdot}N^5\text{-methyl-}FH_4{\cdot}Gly \quad (7)$$

$$E{\cdot}N^5\text{-methyl-}FH_4 + Gly \overset{K_8}{\rightleftharpoons} E{\cdot}N^5\text{-methyl-}FH_4{\cdot}Gly \quad (8)$$

$$E + N^5\text{-formyl-}FH_4 \overset{K_9}{\rightleftharpoons} E{\cdot}N^5\text{-formyl-}FH_4 \quad (9)$$

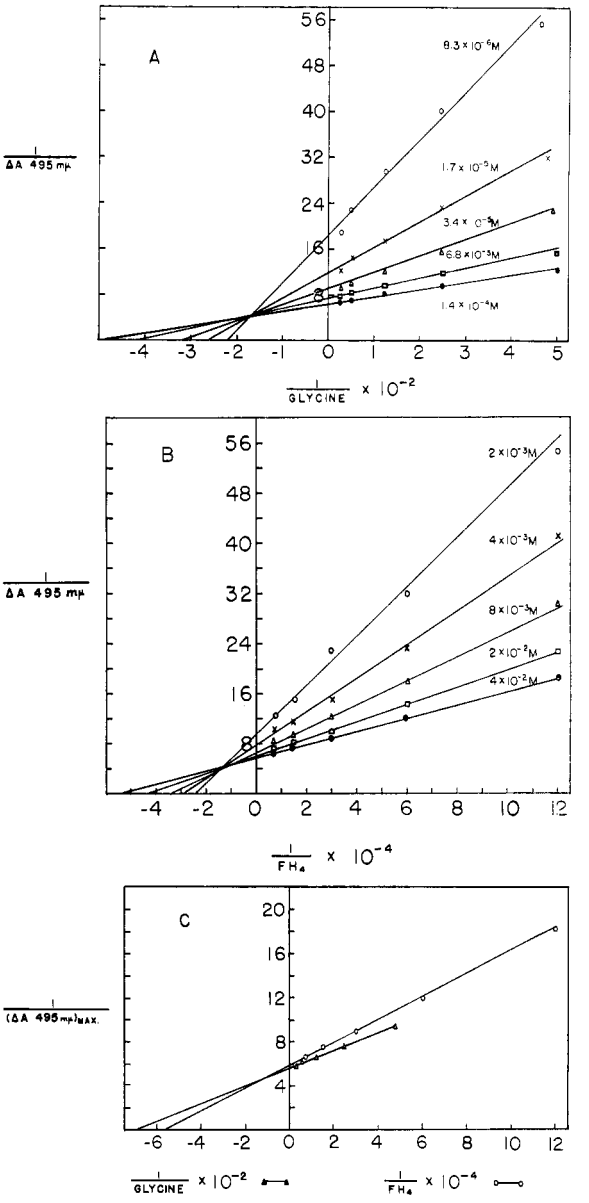

FIGURE 2: Effect of the concentration of glycine and tetrahydrofolate on the absorbancy at 495 mμ of a solution of serine transhydroxymethylase (0.06 mg/ml) in 0.025 M potassium phosphate–0.002 M mercaptoethanol, pH 7.3. (A) The numbers on the lines are the concentrations of glycine employed in that particular experiment. (B) The numbers on the lines are the concentrations of tetrahydrofolate employed in that particular experiment. (C) The intercepts of graphs A and B are plotted against the reciprocals of the concentrations of glycine and tetrahydrofolate according to the method of Florini and Vestling (1957).

$$E{\cdot}Gly + N^5\text{-formyl-}FH_4 \overset{K_{10}}{\rightleftharpoons} E{\cdot}Gly{\cdot}N^5\text{-formyl-}FH_4 \quad (10)$$

$$E{\cdot}N^5\text{-formyl-}FH_4 + Gly \overset{K_{11}}{\rightleftharpoons} E{\cdot}Gly{\cdot}N^5\text{-formyl-}FH_4 \quad (11)$$

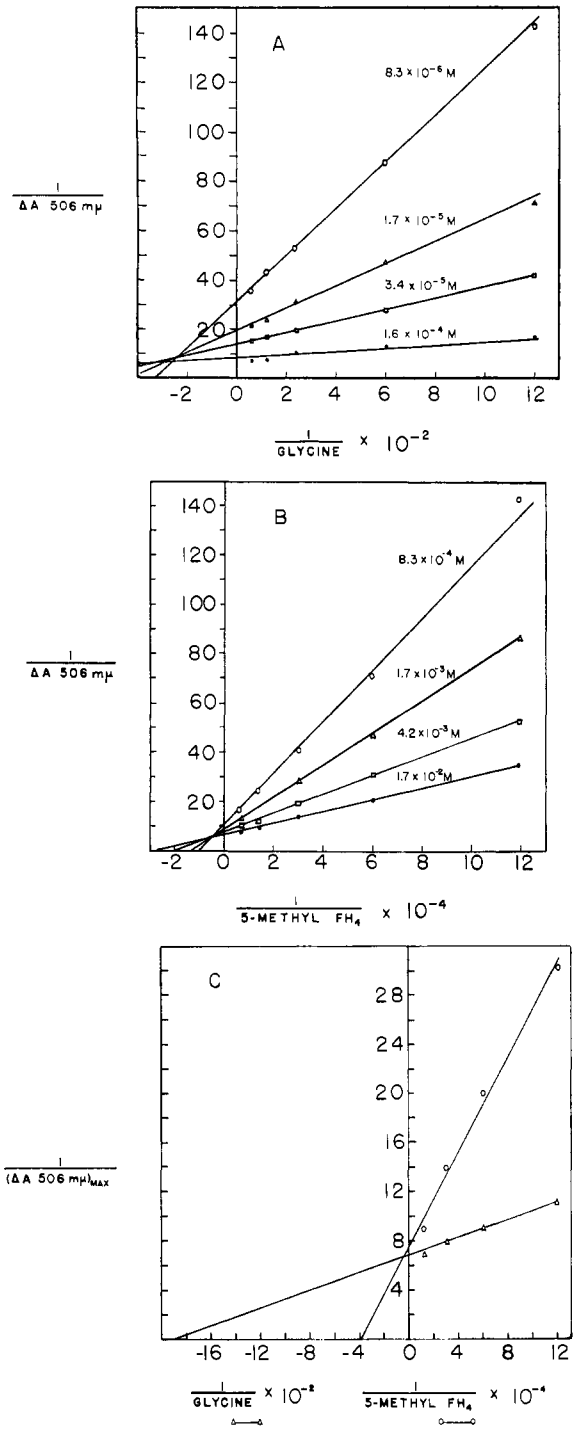

FIGURE 3: Effect of the concentration of glycine and N^5-methyltetrahydrofolate on the absorbancy at 506 mμ of a solution of serine transhydroxymethylase (0.4 mg/ml) in 0.025 M potassium phosphate–0.002 M mercaptoethanol, pH 7.3. (A) The numbers on the lines are the concentrations of glycine employed in that particular experiment. (B) The numbers on the lines are the concentrations of N^5-methyltetrahydrofolate employed in that experiment. (C) The intercepts of graphs A and B are plotted against the reciprocals of the concentrations of glycine and tetrahydrofolate according to the method of Florini and Vestling (1957).

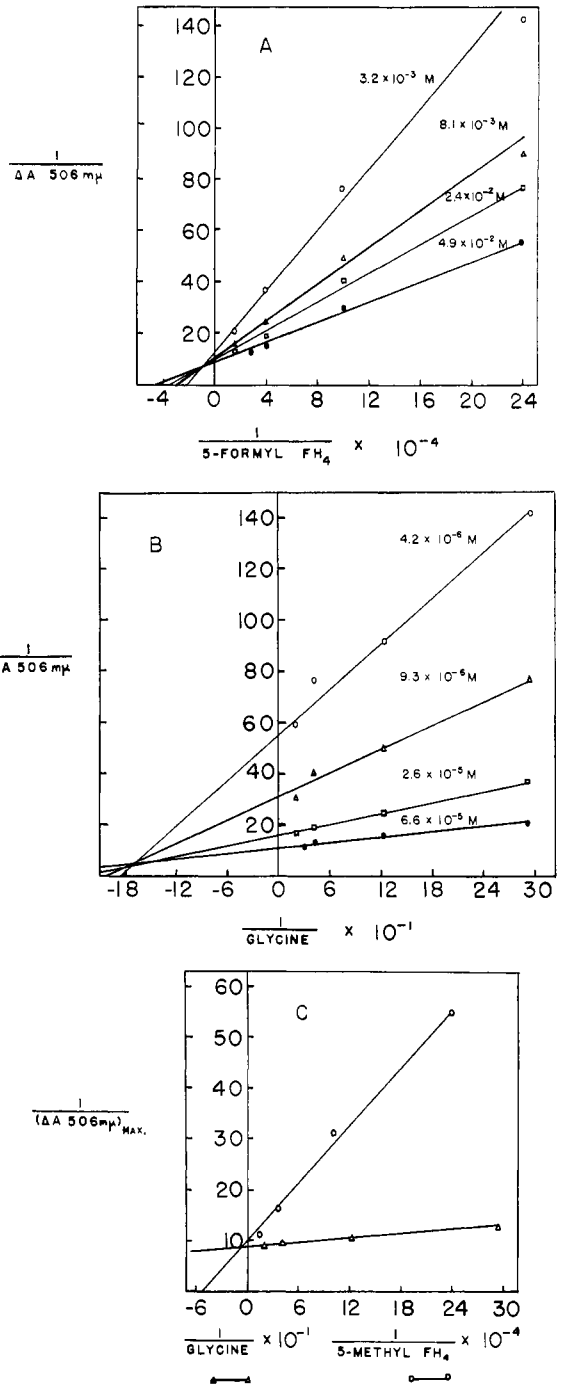

FIGURE 4: Effect of the concentration of glycine and N^5-formyltetrahydrofolate on the absorbancy at 506 mμ of a solution of serine transhydroxymethylase (0.4 mg/ml) in 0.025 M potassium phosphate–0.002 M mercaptoethanol, pH 7.3. (A) The numbers on the lines are the concentrations of glycine employed in that particular experiment. (B) The numbers on the lines are the concentrations of N^5-formyltetrahydrofolate employed in that particular experiment. (C) The intercepts of graphs A and B are plotted against the reciprocals of the concentrations of glycine and N^5-formyltetrahydrofolate according to the method of Florini and Vestling (1957).

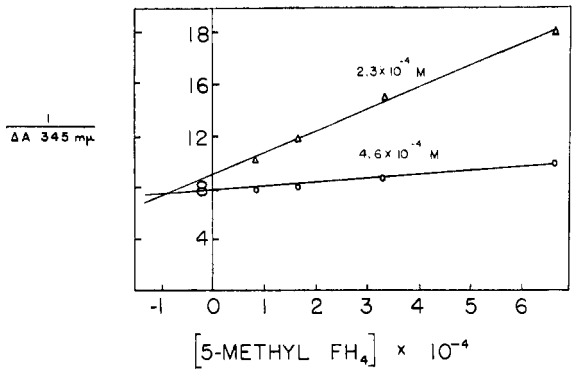

FIGURE 5: Inhibition of serine transhydroxymethylase by N^5-methyltetrahydrofolate. To 0.3 ml of 0.01 M potassium phosphate–0.002 M mercaptoethanol, pH 7.3, were added enzyme, tetrahydrofolate, and N^5-methyltetrahydrofolate. The reaction was started by the addition of 0.02 ml of 0.2 M L-serine. The numbers on the lines are the concentrations of tetrahydrofolate used in that experiment. The extent of the reaction was determined by measuring the amount of $N^{5,10}$-methylenetetrahydrofolate formed from serine and tetrahydrofolate. The $N^{5,10}$-methylenetetrahydrofolate was oxidized to $N^{5,10}$-methenyltetrahydrofolate by chicken liver $N^{5,10}$-methylenetetrahydrofolate dehydrogenase and TPN^+. The amount of TPNH formed was determined by measuring the increase in absorbancy at 345 mμ. The data were plotted according to the method of Dixon (1953).

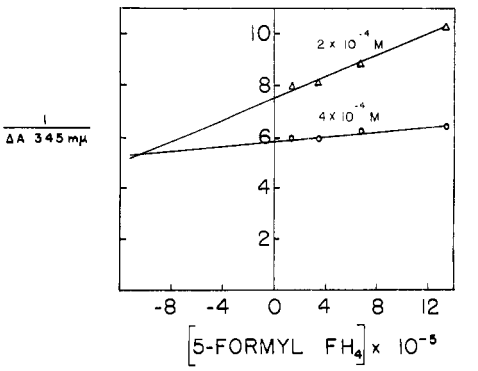

FIGURE 6: Inhibition of serine transhydroxymethylase by N^5-formyltetrahydrofolate. The reaction conditions were the same as described in Figure 5. The numbers on the lines are the concentrations of tetrahydrofolate used in that experiment.

The following experimental procedure was used to determine the first four equilibrium constants. To a cuvet containing serine transhydroxymethylase (0.6 mg/ml) and glycine were added microliter aliquots of a concentrated tetrahydrofolate solution. After each addition of tetrahydrofolate the absorbance of the solution at 495 mμ was measured. The reciprocal of the tetrahydrofolate concentration was then plotted against the reciprocal of the absorbancy at 495 mμ. The experiment was repeated several times using different levels of glycine. The reciprocal plots are given in Figure 2a. From the point of intersection of the lines one can obtain the value of K_2 as 7.7×10^{-5} M. A similar set of data is obtained by noting the changes in absorbancy at 495 mμ when increasing amounts of glycine are added to a solution containing enzyme and tetrahydrofolate. Figure 2b shows reciprocal plots at several tetrahydrofolate concentrations. The value obtained from this graph for K_3 is 5.9×10^{-3} M. The affinity of glycine for the enzyme–tetrahydrofolate complex (K_4) can be obtained from the data recorded in Figure 2a. A plot of the vertical intercepts against the reciprocal of the glycine concentrations gives a straight line which cuts the base line at $-1/K_4$ (Figure 2c). The value obtained for K_4 is 1.4×10^{-3} M. Figure 2c also shows the replotting of the data in Figure 2b to obtain a value of 1.7×10^{-5} M for K_5. The three graphs in Figure 2 show that tetrahydrofolate has a greater affinity for the enzyme–glycine complex than for the free enzyme and that glycine has a greater affinity for the enzyme–tetrahydrofolate complex than for the free enzyme.

A similar set of experiments was performed with solutions in which N^5-methyltetrahydrofolate was used instead of tetrahydrofolate. The absorbancy determinations were made at 506 mμ. Figure 3 shows the data obtained in these experiments. A value of 4×10^{-3} M for K_3 was calculated from Figure 3a. Figure 3b gives a value of 2×10^{-4} M for K_6. Figure 3c is a replotting of the data in Figure 3a,b. From this graph values of 2.2×10^{-5} and 5.2×10^{-4} M were obtained for K_7 and K_8, respectively.

Figure 4 is the experimental data obtained when N^5-formyltetrahydrofolate replaces tetrahydrofolate. The absorbancy determinations were made at 506 mμ. The values of 6×10^{-3}, 1.3×10^{-4}, 1.9×10^{-5}, and 1.6×10^{-3} M were calculated for K_3, K_9, K_{10}, and K_{11}, respectively. Of particular interest is the difference of K_6 and K_7 for N^5-methyltetrahydrofolate and K_9 and K_{10} for N^5-formyltetrahydrofolate. These constants show that the affinities of N^5-methyl- and N^5-formyltetrahydrofolates for the enzyme–glycine complex are about an order of magnitude greater than their affinity for the enzyme. The values of the ten equilibrium constants are given in Table I.

The binding of N^5-methyl- and N^5-formyltetrahydrofolates to the enzyme suggests that they are inhibitors of the enzymatic reaction. Measuring the enzyme activity in the direction of serine cleavage, Figure 5 shows that N^5-methyltetrahydrofolate is a competitive inhibitor of tetrahydrofolate with a K_i of 9.2×10^{-5} M. N^5-Formyltetrahydrofolate is also a competitive inhibitor with a K_i of 1.3×10^{-4} M (Figure 6). The inhibitor constants are considerably higher than the values of K_7 and K_{10} obtained by the spectrophotometric method. It should be pointed out that K_7 and K_{10} are a measure of the affinity of N^5-methyl- and N^5-formyltetrahydrofolate for the enzyme–glycine complex

TABLE I: Summary of Equilibrium Constants.

Tetrahydrofolate Derivative Used to Obtain Equilibrium Constants	Reaction	$K \times 10^5$
Tetrahydrofolate	2	7.7
	3	590
	4	140
	5	1.7
N^5-Methyltetrahydrofolate	3	400
	6	20
	7	2.2
	8	52
N^5-Formyltetrahydrofolate	3	600
	9	13
	10	1.9
	11	160

while the inhibitor constants are a measure of their affinity for the enzyme–serine complex. The spectrophotometric data would predict that N^5-formyl- and N^5-methyltetrahydrofolate would be better inhibitors if glycine was present in the reaction mixture. This was confirmed by an experiment in which glycine was added to one-half the concentration of serine in the enzymatic assay. Under these conditions N^5-methyltetrahydrofolate exhibited a K_i of 5.8×10^{-5} M as compared to the 9.2×10^{-5} M obtained in the absence of glycine.

p-Aminobenzoylglutamate and N^{10}-formyltetrahydrofolate were also used in this study. These compounds did not form a colored complex with solutions of serine transhydroxymethylase and glycine. Also, they did not inhibit the enzymatic reaction. These data suggest that they do not bind to the active site.

Discussion

The interaction of N^5-methyl- and N^5-formyltetrahydrofolates with serine transhydroxymethylase may be important physiologically. This is suggested by the low levels at which the two compounds bind to the enzyme and the observation that a major proportion of the tetrahydrofolate in the cell is in the form of N^5-methyl and N^5-formyl derivatives (Schertel *et al.*, 1965; Noronha and Silverman, 1962).

The data show that both N^5-formyl- and N^5-methyltetrahydrofolates have a much higher affinity for the enzyme–glycine complex than they do for either the enzyme or enzyme–serine complex. This indicates that the amount of inhibition will be very sensitive to the concentration of glycine. This regulation of the degree of inhibition by the level of glycine might serve in the cell to prevent a large influx of glycine from depleting the one-carbon pool.

The data also show that glycine has a greater affinity for the enzyme–tetrahydrofolate complex than for the enzyme. This is also true for the enzyme–N^5-methyltetrahydrofolate and the enzyme–N^5-formyltetrahydrofolate complexes. There is about a fourfold increase in the affinity of glycine for the enzyme when it is combined with either tetrahydrofolate or N^5-formyltetrahydrofolate. The affinity of glycine for the enzyme–N^5-methyltetrahydrofolate complex is eightfold as great as its affinity for the enzyme. These data suggest that increasing levels of these tetrahydrofolate compounds in the cell would become increasingly more effective as inhibitors of the enzymatic interconversion of serine and glycine.

Blakley (1957) has shown that the folic antagonist tetrahydro-4-amino-4-deoxypteroylglutamate does not inhibit the enzymatic conversion of glycine to serine. Only relatively high concentrations of the antagonist dihydro-2-deaminopteroylglutamate were found to inhibit the reaction. These studies show that the substituents on the pteridine ring are important in the binding of tetrahydrofolate to serine transhydroxymethylase.

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