Inhibition of Thymidylate Synthetase by Some Analogs of Tetrahydrofolic Acid

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SUMMARY

A series of derivatives of tetrahydrofolate and dihydrofolate were investigated as substrates or inhibitors of thymidylate synthetase. N^{10}-Methyltetrahydrofolate is a competitive inhibitor of the enzyme, $K_I = 6.3 \times 10^{-5}$ M. All other analogs tested inhibited in either a noncompetitive or uncompetitive manner. The relationship between the structure and the activities of the analogs as substrates or inhibitors is discussed.

INTRODUCTION

The mechanism of inhibition of thymidylate synthetase is of particular interest because of the importance of this enzyme in the synthesis of DNA. Until now only few compounds have been investigated as potential inhibitors or thymidylate synthetase. Among these, 5′-fluorodeoxyuridylic acid (1, 2), tetrahydrohomofolic acid (3), tetrahydroaminopterin and some of its derivatives (4, 5) were found to inhibit thymidylate synthetase; however, little work has been done concerning the nature of the inhibition of this enzyme by tetrahydrofolate analogs.

In this study, a number of analogs of tetrahydro and dihydrofolic acid and tetrahydroaminopterin were investigated as substrates for and inhibitors of thymidylate synthetase. The type of inhibition exerted by each of these analogs has been examined in detail. On the basis of these experiments it has been possible to pinpoint the structural features of the tetrahydrofolic acid molecule that are necessary for its coenzyme activity. Furthermore, certain relationships between alterations in structure and the inhibitory properties of some analogs of tetrahydrofolate and tetrahydroaminopterin have been established.

This information is useful for the design of potential inhibitors of thymidylate synthetase. A preliminary report on these findings has been presented (6).

MATERIALS AND METHODS

Folic acid, aminopterin, and amethopterin were purchased from Sigma Chemical Company and purified by crystallization as magnesium salts from hot water. The salts were converted to the free acids by precipitation with 5 N HCl from hot aqueous solutions. 9-Methylfolic acid, 10-methylfolic acid, 9,10-dimethylfolic acid, 3′,5′-dichloroaminopterin, 3′,5′-dichloroamethopterin, 3′,5′-dichlorofolic acid, diopterin, folic acid diamide, and 5-formyltetrahydrofolic acid (folinic acid) were obtained from the Lederle Laboratories of the American Cyanamid Company; N^{10}-methylpteroic acid was obtained from the Calco division of the American Cyanamid Company. The first five compounds in this group were purified by chromatography on a cellulose column developed with ethanol–1 N NH_4OH (60:40 v/v). 3′,5′-Dichlorofolic acid, diopterin, folic acid diamide, and N^{10}-methylpteroic acid were used without purification. Pter-

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oylaspartic acid was a gift of Dr. A. W. Schrecker (National Cancer Institute, Bethesda, Maryland), 2,6-diamino-5-methyl-4-(3′,4′-dichlorophenyl)-pyrimidine of Dr. B. R. Baker (University of California, Santa Barbara, California) and *N*-(2-quinoxalylmethyl)-*p*-aminobenzoylglutamic acid of Dr. L. Goodman (Stanford Research Institute, Menlo Park, California). 7-Methylfolic acid (7), 4-aminopteroic acid (8), and 2,4-diaminopteridine (9) were synthesized in this laboratory. The two former compounds were purified by chromatography on cellulose as described above.

All compounds were examined for purity by means of descending paper chromatography using Whatman paper No. 3 and two solvents: propanol–1 N NH_4OH (60:40) and 0.5 M aqueous K_2HPO_4.

Preparation of the tetrahydro derivatives. The tetrahydro derivatives of the above compounds were prepared by catalytic hydrogenation (platinum dioxide) in a solution consisting of 9 volumes of glacial acetic acid and one volume of ethylene glycol (10, 11). Generally, 100 mg of a given folic acid analog were hydrogenated in 10 ml of the acetic acid glycol mixture, using 70 mg of platinum dioxide as a catalyst. After 2 moles of hydrogen per mole of the analog had been consumed, the mixture was filtered into 250 ml of anhydrous ether. The tetrahydro derivatives that precipitated were collected by filtration and washed with several portions of dry ether. This operation was carried out in a chamber filled with carbon dioxide. The product was then dried under reduced pressure; the compounds were stored in evacuated vials at —10°. 5-Methyltetrahydrofolic acid was prepared by reduction of N^5,N^{10}-methylenetetrahydrofolic acid with sodium borohydride (12). Folinic acid and 5-methyltetrahydrofolic acid were purified on DEAE-cellulose columns in the hydroxy form (2.2 × 15 cm), with a linear gradient using 500 ml of 1% mercaptoethanol in the mixing flask and 500 ml 1 M NH_4OH containing 1% mercaptoethanol in the reservoir. N^{10}-Formyltetrahydrofolic acid was prepared by the direct formylation of tetrahydrofolic acid (13); the product of this reaction, N^5,N^{10}-methyltetrahydrofolate, was suspended in 1% aqueous mercaptoethanol and adjusted to pH 9 with NH_4OH. This solution was clarified by filtration and chromatographed on a DEAE-cellulose column as described above.

Preparation of the dihydro derivatives. With the exception of 7-methyldihydrofolic acid, the dihydro derivatives of all folic acid analogs were prepared by reduction of the folic acid derivatives with sodium dithionite in the presence of sodium ascorbate (14). The reduced compounds were isolated by precipitation with 2 N HCl and the precipitates washed several times with 0.001 N HCl and lyophilized. The dry compounds were stored in sealed vials at —10°.

7-Methyldihydrofolic acid was prepared by the reduction of 7-methylfolic acid with zinc in an alkaline solution in the following way: a mixture of 60 mg of 7-methylfolic acid, 200 mg of zinc dust, 1 ml of water and 0.2 ml of 5 N NaOH was stirred for 18 hours at room temperature. The mixture was diluted with 10 ml of 0.25 M Na_2HPO_4 solution containing 1% of mercaptoethanol. After removal of the unreacted zinc by filtration, the solution was chilled in ice and adjusted to pH 4 with 1 N HCl. The precipitate was centrifuged, washed twice with 0.1 M acetic acid, twice with acetone, and once with ether; the yield of crude material was 18 mg. The product was suspended in 1% aqueous mercaptoethanol and dissolved by addition of a drop of 1 N NaOH. This solution was applied to a column of DEAE-cellulose-bicarbonate (2.2 × 15 cm, 8 g DEAE-cellulose). The elution was carried out with a linear gradient using 720 ml of 1% mercaptoethanol in the mixing flask and 720 ml of 2 M NH_4HCO_3 containing 1% mercaptoethanol in the reservoir; fractions of 8–10 ml were collected. The absorbancy of the eluate was monitored at 280 mμ. The main peak appeared in fractions 70–85. These fractions were combined and lyophilized until all bicarbonate was removed. The product obtained had a UV spectrum identical with that reported by Blakley for 7,8-dihydrofolate (15).

Enzyme preparation. Escherichia coli B

was grown in 10 liters of a medium containing 53 g of Bacto-peptone, 106 g of Bacto yeast extract, 180.2 g of KH_2PO_4, 231.2 g of K_2HPO_4, and 106 g of dextrose. The medium was divided into 1-liter portions. Aeration of the growing culture was maintained by vigorous shaking. Samples were withdrawn at 30-minute intervals and turbidity was determined in a Klett-Summerson colorimeter. After about 3 hours of growth (turbidity 280–300), the culture was chilled in ice and the cells were harvested by centrifugation. The cells obtained from 10 liters of medium were washed once with 1 liter of 0.05 M Tris HCl buffer, pH 7.4, containing 0.001 M EDTA and 0.01 M mercaptoethanol and stored in the frozen state. The total yield of cells was about 65 g (wet weight).

Ten grams of the cells were ground with 10 g of Alcoa A 303 alumina and 20 ml of buffer (described above) were added slowly during grinding. The mixture was centrifuged for 20 min at 25,000 *g*. The nucleic acids were precipitated from the viscous supernatant fraction with one-half volume of 5% streptomycin sulfate and removed by centrifugation. To 42 ml of the supernatant, 10.2 g of solid ammonium sulfate were added, and after 10 min the precipitate was separated by centrifugation and discarded. Another 12 g of ammonium sulfate was added to the supernatant material and after 10 min of stirring the precipitated fraction containing the enzyme was separated by centrifugation. The precipitate was dissolved in 15 ml of the Tris buffer (described above) and the solution passed through a Sephadex G-25 column (2 × 5 cm) equilibrated with the same buffer. The filtrate containing the active enzyme was stored at −10°. Under these conditions its activity was retained for several weeks.

Enzyme assays. Thymidylate synthetase activity was assayed by the spectrophotometric method of Wahba and Friedkin (16), modified by increasing the concentration of deoxyuridylic acid. The incubation mixture contained 0.83 μmole of tetrahydrofolic acid, 48.3 μmoles of Tris-HCl buffer, pH 7.4, 13.5 μmoles of formaldehyde, 25 μmoles of $MgCl_2$, 50 μmoles of mercaptoethanol, 1 μmole of deoxyuridylic acid in total volume of 1.1 ml. The reaction was started by the addition of deoxyuridylate and was continued for 15 min at 20°. The change in optical density at 338 mμ was measured in a Beckman DU spectrophotometer; the incubation mixture from which deoxyuridylate was omitted served as the blank.

The analogs to be tested for their inhibitory effect were dissolved in 1 M mercaptoethanol and 0.05 ml of this solution was added to the incubation mixture.

To test for coenzyme activity the analogs were added in place of tetrahydrofolic acid at equimolecular amounts. The compounds were classified as inactive when there was no change in absorbancy at 338 mμ during the 1-hour period.

Determination of the inhibition constants. The inhibition constants for the various tetrahydrofolic acid analogs were calculated from the following equations (17):

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_I}\right)\frac{1}{A} + \frac{1}{V}$$

for competitive (1)

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_I}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{I}{K_I}\right)$$

for noncompetitive (2)

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_I}\right)$$

for uncompetitive inhibition (3)

where A = substrate concentration, I = inhibitor concentration, K = Michaelis constant, K_I = inhibition constant, V = maximal reaction velocity, and v = experimentally determined velocity.

RESULTS

Table 1 summarizes the substrate activity of several tetrahydrofolic acid analogs. Whereas tetrahydrofolic acid diamide, 7-methyltetrahydrofolic acid, tetrahydropteroylaspartic acid, and tetrahydrodiopterin showed some coenzyme activity, such activity was not found in the other derivatives tested.

TABLE 1
Substrate activity of tetrahydrofolic acid analogs

Substance	Percent of substrate activity
Tetrahydrofolic acid	100
N^{10}-Methyltetrahydropteroic acid	0
N^{5}-Methyltetrahydrofolic acid	0
N^{10}-Methyltetrahydrofolic acid	0
Tetrahydrofolic acid diamide	11.7
C^{7}-Methyltetrahydrofolic acid	1.4
Tetrahydrodiopterin	2.9
Tetrahydropteroylaspartic acid	1.6
Tetrahydroaminopterin	0
C^{9}-Methyltetrahydrofolic acid	0
3′,5′-Dichlorotetrahydrofolic acid	0.6

The substances were also tested as inhibitors of thymidylate synthetase. Tables 2 and 3 summarize the inhibition constants and the type of inhibition produced by the various tetrahydrofolic acid and tetrahydroaminopterin derivatives. Of the compounds studied, only N^{10}-methyltetrahydrofolic acid inhibited competitively with tetrahydrofolic acid and noncompetitively with deoxyuridylic acid (Fig. 1); the C^{9}-methyltetrahydrofolic acid was noncompetitive both with tetrahydrofolic acid and deoxyuridylic acid (Fig. 2).

All other tetrahydrofolic acid analogs, including tetrahydroaminopterin, were noncompetitive with tetrahydrofolic acid and uncompetitive with deoxyuridylic acid (Fig. 3). In contrast, all 7,8-dihydrofolic acid analogs tested, including dihydroaminopterin, were uncompetitive both with tetrahydrofolate and deoxyuridylate. The extent of the inhibitory activity of the various analogs is given by the inhibition constants. Of the monosubstituted derivatives of tetrahydrofolic acid, N^{10}-methyl, N^{5}-formyl, and C^{9}-methyl showed the strongest inhibitory effect. The inhibition was more pronounced when methyl groups were present simultaneously in positions N^{10} and C^{9}. It is of

TABLE 2
Inhibition constants and the type of inhibition of thymidylate synthetase by various tetrahydrofolic acid analogs

Derivative	Type of inhibition		
	Versus tetrahydrofolate		Versus dUMP
		K_I (M)	
N^{10}-Methyltetrahydrofolic acid	Competitive	6.3×10^{-5}	Noncompetitive
C^{9}-Methyltetrahydrofolic acid	Noncompetitive	7.9×10^{-5}	Noncompetitive
$C^{9}N^{10}$-Dimethyltetrahydrofolic acid	Noncompetitive	2.2×10^{-5}	—
C^{7}-Methyltetrahydrofolic acid	Noncompetitive	5.6×10^{-4}	Uncompetitive
N^{5}-Formyltetrahydrofolic acid	Noncompetitive	2.5×10^{-4}	
N^{5}-Methyltetrahydrofolic acid	—	No inhibition at 5×10^{-4} M	—
N^{10}-Formyltetrahydrofolic acid	—	No inhibition at 5×10^{-4} M	—
N^{10}-Methyltetrahydropteroic acid	—	No inhibition at 5×10^{-4} M	—
3′,5′-Dichlorotetrahydrofolic acid	—	No inhibition at 5×10^{-4} M	—
Tetrahydrofolic acid diamide	Noncompetitive	7.5×10^{-4}	—
C^{7}-Methyldihydrofolic acid	Uncompetitive	3.8×10^{-3}	—
Tetrahydropteroylaspartic acid	—	No inhibition at 5×10^{-4}	—
Tetrahydrodiopterin	—	No inhibition at 5×10^{-4}	—
Quinoxalylmethylaminobenzoylglutamic acid	—	No inhibition at 5×10^{-4}	—

TABLE 3
Inhibition constants and the type of inhibition of thymidylate synthetase by various tetrahydroaminopterin derivatives

Substance	Type of inhibition		
	Versus tetrahydrofolate		Versus dUMP
		K_I (M)	
Tetrahydroaminopterin	Noncompetitive	1.12×10^{-5}	Uncompetitive
Dihydroaminopterin	Uncompetitive	1.5×10^{-5}	Uncompetitive
N^{10}-Methyltetrahydroaminopterin	Noncompetitive	1.97×10^{-6}	Uncompetitive
N^{10}-Methyldihydroaminopterin	Uncompetitive	1.9×10^{-5}	—
C^9-Methyltetrahydroaminopterin	Noncompetitive	2.7×10^{-4}	—
3′,5′-Dichlorotetrahydroaminopterin	Noncompetitive	3.02×10^{-5}	—
3′,5′-Dichloro-N^{10}-methyltetrahydroaminopterin	Noncompetitive	8.0×10^{-5}	—
N^{10}-Methyl-3′,5′-dichlorodihydroaminopterin	—	No inhibition at 5×10^{-4} M	—
3′,5′-Dichlorodihydroaminopterin	—	No inhibition at 5×10^{-4} M	—
4-Aminotetrahydropteroic acid	Noncompetitive	6.9×10^{-4} M	—
2,4-Diaminotetrahydropteridine	—	No inhibition at 5×10^{-4} M	—
2,6-Diamino-5-methyl-4-(3′,4′-dichlorophenyl)pyrimidine	—	No inhibition at 5×10^{-4} M	—

interest that whereas N^5-formyltetrahydrofolic acid was an inhibitor, N^5-methyltetrahydrofolic acid functioned neither as a substrate nor as an inhibitor. Similarly, N^{10}-formyltetrahydrofolic acid was inactive as a substrate and as an inhibitor. Substitution with a methyl at C^7 led to loss of substrate activity and the appearance of inhibitory activity (see 7-methyldihydro and 7-methyltetrahydrofolic acid, Tables 1 and 2).

Tetrahydroaminopterin, dihydroaminopterin, and some of their derivatives proved to be more effective inhibitors than the substituted tetrahydrofolic acid analogs.

Figure 4 shows the relationship between enzyme activity and enzyme concentration both in the presence and in the absence of

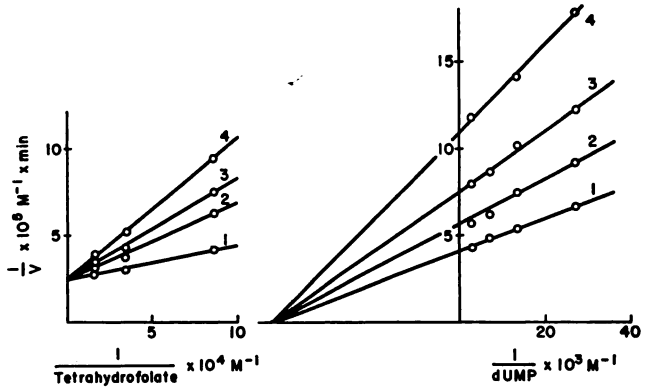

FIG. 1. *Inhibition of thymidylate synthetase by N^{10}-methyltetrahydrofolic acid*

In both cases the following inhibitor concentrations were used: *1*, no inhibitor; *2*, 1.5×10^{-4} M; *3*, 3.0×10^{-4} M; *4*, 6.0×10^{-4} M.

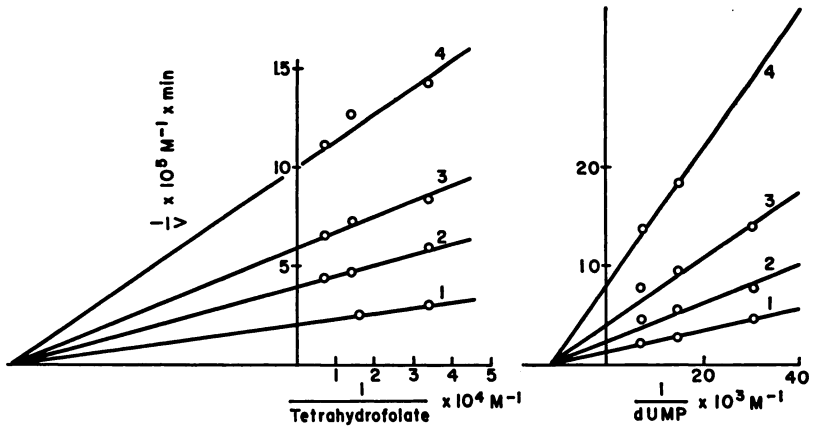

FIG. 2. *Inhibition of thymidylate synthetase by 9-methyltetrahydrofolic acid*

In both cases the following inhibitor concentrations were used: *1*, no inhibitor; *2*, 1.5×10^{-4} M; *3*, 3.0×10^{-4} M; *4*, 6.0×10^{-4} M.

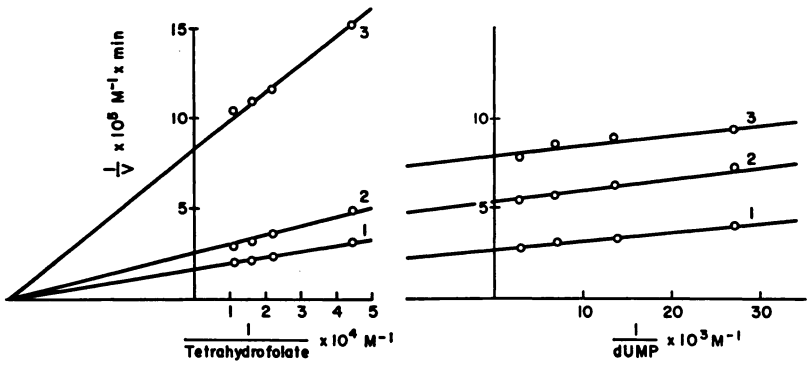

FIG. 3. *Inhibition of thymidylate synthetase by tetrahydroamethopterin*

In both cases the following inhibitor concentrations were used: *1*, no inhibitor; *2*, 7.3×10^{-6} M; *3*, 3.65×10^{-4} M.

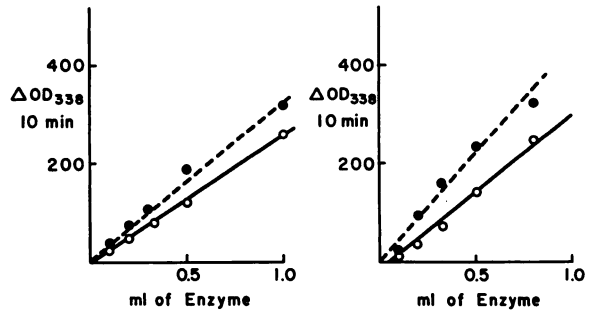

FIG. 4. *Effect of enzyme concentration on the reaction rate in the absence* (●) *and presence* (○) *of inhibitors*

Dihydroamethopterin (3×10^{-6} M) on the left and tetrahydroamethopterin (7.8×10^{-6} M) on the right.

dihydro- and tetrahydroaminopterin. The linear dependence in all cases shows that the inhibition exerted by the 4-amino analogs tested is not stoichiometric. This is in contrast to the stoichiometric inhibition of folate reductase by aminopterin and amethopterin (18).

DISCUSSION

The relationship between the structure of tetrahydrofolic acid analogs and their activity as substrates for and inhibitors of thymidylate synthetase, has been investigated. All but one of the inhibitors studied proved to be noncompetitive or uncompetitive with respect to both tetrahydrofolic acid and deoxyuridylic acid. Therefore, despite the structural similarities between the inhibitors and the substrate, it is impossible, on the basis of the study of the inhibitors, to reach any conclusion concerning the mode of binding of the substrate to the enzyme.

On the other hand, it has been observed that certain structural changes that lead to a decrease of the substrate activity of the tetrahydrofolic acid analogs also result in a decrease in the inhibitory activity of the tetrahydroaminopterin analogs. Thus, substitution of the 3′,5′-positions of tetrahydrofolic acid with chlorine atoms abolishes the substrate activity without producing inhibitory activity. This indicates that such modification reduces the attachment of the molecule to the enzyme. Similarly, substitution in the 3′,5′-position of tetrahydroaminopterin, tetrahydroamethopterin, and dihydroaminopterin leads in each case to a partial or complete loss of the inhibitory activity.

Since the amidation of the glutamate carboxyl groups markedly decreases the substrate activity of tetrahydrofolic acid, it appears that the free carboxyls are also essential for binding. Elimination of the glutamic acid moiety from tetrahydroaminopterin results in a considerable decrease of inhibitory activity (see 4-aminotetrahydropteric acid, Table 3). Thus, it appears that certain binding sites of the enzyme might be common for substrates as well as inhibitors.

The following areas of the tetrahydrofolic acid molecule seem to be essential for substrate activity.

1. Unsubstituted C^7-N^8 area. Compounds such as 7-methyldihydro- and 7-methyltetrahydrofolic acid had only minimal substrate activity (Table 1) and were very weak inhibitors of the enzyme (Table 2). That the C^7-N^8-dihydrobond is important for the attachment of the substrate and inhibitors to the enzyme is suggested by the fact that the nonreduced folate analogs do not interfere with enzyme activity.

2. The area of attachment of the one-carbon unit. Thus, substitution of N^5, C^9 and N^{10} atoms with either methyl or formyl groups leads to the loss of coenzyme activity. Such a loss it to be expected when either N^5 or N^{10} are substituted, because these atoms are involved in the transfer of the one-carbon moiety. The loss of activity resulting from the substitution of C^9 might be due to steric hindrance.

3. The intact *p*-aminobenzoate moiety. It is difficult to establish whether the N^{10} or the aromatic ring of *p*-aminobenzoic acid interacts with the enzyme. The effect of substitution of the aromatic ring with a halogen in positions 3′ and 5′ may be explained either by the steric hindrance caused by this modification or by the drop of nucleophilicity of N^{10}. Additional experimental evidence is necessary to distinguish between these two possibilities.

4. The glutamic acid moiety. The presence of the carboxyl groups as well as the distance between them seem to be essential for proper binding. Tetrahydropteroylaspartic acid shows only negligible substrate and no inhibitory activity. Extension of the distance between the glutamate carboxyls seems to have less effect than the shortening of the aliphatic chain of the amino acid. Friedkin observed that tetrahydropteroyl aminoadipate and tetrahydropteroyl aminopimelate were active as substrates and the analogous homotetrahydropteroyl compounds were inhibitors of the thymidylate synthetase (19). On the other hand, there was only slight substrate activity in several tetrahydropteroyl derivatives in which glutamate was substituted

by another monocarboxylic amino acid and appreciable loss of inhibitory activity in the case of analogous homotetrahydropteroyl derivatives (19). Since tetrahydrodiopterin has neither cofactor nor inhibitory effect, it is also possible that the proper distance between the free γ-carboxyl of glutamic acid and *p*-aminobenzoyl moiety is essential for binding.

Another area that remains to be considered is the pyrimidine moiety of the substrate. Whether this portion of the molecule is involved in the binding to the enzyme cannot be decided at present.

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