5-Methyl-5,6,7,8-tetrahydropteroyl Oligo-γ-L-glutamates: Synthesis and Kinetic Studies with Methionine Synthetase from Bovine Brain†

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ABSTRACT: The synthesis of 5-methyl-5,6,7,8-tetrahydropteroyl tri-, penta-, and heptaglutamate has been accomplished by reductive methylation of the tetrahydropteroyl oligoglutamate with formaldehyde, followed by purification on DEAE-Sephadex. The corresponding [5-^{14}C]methyltetrahydropteroyl oligoglutamates were prepared from $^{14}CH_2O$, and tested as substrates for methionine synthetase (EC 2.1.1.13) isolated from bovine brain. In all cases, the polyglutamate conjugates were better substrates (lower K_m, higher V_{max}) than the corresponding monoglutamate forms. In addition, the nonradioactive methyltetrahydropteroyl oligoglutamates inhibited the methylation of homocysteine by methyltetrahydrofolate. This indicates that the monoglutamate and polyglutamates compete for the same enzyme, and establishes a role for the ubiquitous methyltetrahydropteroyl oligoglutamates in mammalian methionine biosynthesis.

Methionine synthetase (EC 2.1.1.13) catalyzes the transmethylation reaction between the methyl donor, N^5-methyltetrahydrofolate, and homocysteine (Taylor and Weissbach, 1973). In fact, not only N^5-methyltetrahydrofolate but also the triglutamate can act as a methyl donor for the transmethylation reaction in bacteria and plants (Salem and Foster, 1972). The distinguishing feature between the enzymes which use the monoglutamate and those which use the higher glutamate derivatives is the fact that the former enzymes require vitamin B_{12} while the latter require phosphate and magnesium. In mammals, the enzyme has been isolated, partially purified, and studied extensively in terms of mechanism and kinetic properties (Burke et al., 1971). These studies show that the enzyme requires vitamin B_{12} and that it uses the monoglutamyl form as the methyl donor. Recent studies suggest that this transmethylation reaction is not limited to the methylation of homocysteine, but also may involve the biogenic amines (e.g., dopamine and serotonin) (Laduron et al., 1974). Studies from several laboratories have indicated that the predominant folate forms in mammalian tissue are 5-methyltetrahydropteroyl polyglutamates (Shin et al., 1972; Houlihan and Scott, 1972; Osborne-White and Smith, 1973; Brown et al., 1974). Since only small amounts of these conjugated folates have been available, the use of substituted folate polyglutamates as substrates for folate-dependent reactions has been rather limited (Blakley, 1969). The chemical synthesis of oxidized pteroyl polyglutamates (Godwin et al., 1972; Krumdieck and Baugh, 1969) has made it possible to obtain sufficient quantities of these conjugates to use them and their reduced derivatives as substrates for the many folate-dependent enzymes. We have previously described the synthesis of the 7,8-dihydro forms of pteroyl oligoglutamates and their use as substrates for the enzyme dihydrofolate reductase from several mammalian cell lines (Coward et al., 1974b). In the present paper we describe the synthesis of 5-methyl-5,6,7,8-tetrahydropteroyl polyglutamates and present kinetic studies of the interaction of these substrates and the enzyme methionine synthetase from bovine brain. A preliminary account of this work has been published previously (Coward et al., 1974a).

Materials and Methods

N^5-Methyltetrahydrofolate (Sigma) and N^5-[^{14}C]methyltetrahydrofolate (Amersham/Searle), 60 Ci/mol, were purchased as the barium salt and were converted into the sodium salt by dissolving 1 part methyltetrahydrofolate barium salt in 3 parts 0.1 *M* $NaHCO_3$, 5 parts 20 m*M* 2-mercaptoethanol, and 2 parts 0.5 *M* Na_2HPO_4. The precipitated barium phosphate was removed by centrifugation, and the clear supernatant solution was frozen (−68°) until used. N^5-Methyltetrahydropteroyl oligo-γ-glutamates were prepared by reductive methylation of the oxidized pteroyl polyglutamates in a manner similar to that described for the preparation of 5-methyltetrahydrofolate (Blair and Saunders, 1970). A typical preparation and purification of a N^5-[^{14}C]methyltetrahydropteroyl polyglutamate is as follows: pteroyl-γ-glutamyl-γ-glutamyl-γ-glutamate (50 μmol) (Coward et al., 1974b) in 4 ml of 66 m*M* Tris buffer (pH 7.8) under a nitrogen atmosphere, was treated with 80 mg (2.1 mmol) of $NaBH_4$ in 3 portions over a period of 25–30 min. After the mixture was stirred for 1.25 hr at ambient temperature, spectral analysis indicated complete conversion of the fully oxidized pteroyl triglutamate to the corresponding tetrahydro form (Blakley, 1969). Excess $NaBH_4$ was neutralized by 5 *N* acetic acid and the pH adjusted to 7.8 with 1 *N* NaOH. [^{14}C]Formaldehyde (300 μmol, 0.9 ml of a 1% aqueous solution, specific activity 1 Ci/mol) was added and after 5 min, 46 mg (1.2 mmol) of $NaBH_4$ was added in 4 portions over a period of 5 min. The reaction mixture was incubated at 45° under nitrogen for 90 min. At the end of this time the spectral analysis indicated complete conversion of the tetrahydropteroyl trigluta-

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mate to the 5-methyltetrahydro form (Blakley, 1969). The reaction mixture was cooled to ambient temperature and 20 μl of 2-mercaptoethanol was added and the pH adjusted to 7.0 with 5 *N* acetic acid. The resulting solution was applied to a DEAE-Sephadex A-25 column (0.9 × 30 cm) and the product was purified by elution with a NaCl gradient (0.2–0.7 *N*) in 5 m*M* Tris (pH 7.5) containing 10 m*M* 2-mercaptoethanol. Columns were monitored spectrophotometrically and elution of the radioactive products was monitored by counting aliquots of appropriate tubes in the scintillation counter. The 5-methyltetrahydropteroyl triglutamate was eluted in 0.46 *M* NaCl (the 5-methyltetrahydropteroyl penta- and heptaglutamates were eluted in 0.48 and 0.56 *M* NaCl, respectively). In all cases the peak of 5-methyltetrahydropteroyl polyglutamate as determined spectrophotometrically (λ_{max} 290 nm, Blakely, 1969) and as determined by radioactivity coincided in the elution profile. Spectral analysis of the products eluted from DEAE-Sephadex indicated a percent yield of approximately 30–35% for the conversion of the fully oxidized pteroyl oligoglutamate to the corresponding 5-methyltetrahydropteroyl oligoglutamates. In all cases small amounts of impurities were eluted from the column prior to the desired product and a substantial amount of radioactive material which absorbed in the 270–300-nm region was eluted at higher salt concentration. The identity of this latter material was not further investigated. The tubes containing the purified 5-methyltetrahydropteroyl triglutamate were pooled and lyophilized and the salts removed by chromatography on Sephadex G-15 using a 5 m*M* triethylammonium bicarbonate buffer containing 10 m*M* mercaptoethanol (pH 8.0). This procedure was effective in separating the desired 5-methyltetrahydropteroyl triglutamate from all salts as indicated by monitoring the column for radioactivity, uv absorption, and conductance. The peak tubes were pooled and lyophilized and the residue was redissolved in 5 ml of 5 m*M* Tris buffer (pH 7.5) containing 10 m*M* 2-mercaptoethanol. This final solution was divided into 0.5-ml aliquots and stored at −20° under nitrogen. The procedure described above is the result of investigating various reaction conditions which lead to formation of the 5-methyltetrahydropteroyl polyglutamates from the fully oxidized form. Use of lower ratios of formaldehyde/pterin and borohydride/pterin, or shorter time of reaction with $NaBH_4$ (Blair and Saunders, 1970), resulted in lower conversion to the desired methylated product.

Methionine synthetase was isolated from bovine brain and purified through the ammonium sulfate precipitation according to the procedure of Mangum et al. (1972). The ammonium sulfate pellets were stored at −20° until used and no loss of activity could be detected over a period of 3 months. Brains were obtained from calves which had not yet been weaned from stock-fed cows.[1] The enzyme was prepared for assay by thawing pellets and dissolving the resulting mix in 10 volumes of 0.05 *M* Tris buffer (pH 7.5). It should be noted that there was no difference in activity whether the enzyme was dissolved in 0.05 *M* Tris (pH 7.4) or 0.05 *M* sodium phosphate (pH 7.4). This is in contrast to the non-B_{12} enzyme described by Whitfield et al. (1970) where the enzyme is specific for phosphate buffer and has no activity in the presence of Tris buffer. The resulting enzyme solution was centrifuged at 37,500*g* for 20 min and the supernatant dialyzed for 18 hr against 2 × 100 volume changes of 0.05 *M* Tris buffer (pH 7.4) in order to remove ammonium sulfate and homocysteine used in the enzyme preparation. Following dialysis, the enzyme was centrifuged at 37,500*g* for 20 min and the supernatant used directly as an enzyme source. Protein concentration was determined by measuring the optical density at 280 nm assuming 1 mg = 1 OD unit; this method gave values which agreed well with results obtained using a micro biuret protein determination.

Methionine synthetase activity was determined according to the procedure of Kamely et al. (1973) except that 500 μM D,L-homocysteine (synthesized by Dr. W. Günther) was used. The purity of this material was routinely checked by use of Ellman's reagent (Ellman, 1959). For kinetic studies, the radioactive 5-methyltetrahydropteroyl polyglutamates (1200 cpm/nmol) were substituted for the monoglutamate, 5-methyltetrahydrofolate. For competition studies concentrations from 5 to 40 μM unlabeled 5-methyltetrahydropteroyl polyglutamate were added to assays containing 37.5–500 μM [5-^{14}C]methyltetrahydrofolate. The diminished rate of radioactive methionine formation observed in the presence of polyglutamyl alternate substrates (e.g., Figure 2) precluded the use of methyltetrahydrofolate concentrations of less than 37.5 μM in the competition experiments. All reactions were then incubated at 37° for 1 hr under nitrogen and the reactions terminated by addition of 0.8 ml of ice-cold water. The radioactive product, [^{14}C]methionine, was separated from the radioactive methyl donor by column chromatography on AG 1-X8 (Bio-Rad) in Pasteur pipets. The columns were washed with 1.0 ml of ice-cold water and the total effluent was collected in a scintillation vial, dissolved in 15 ml of Aquasol (New England Nuclear), and counted in a Packard TriCarb liquid scintillation counter. Methionine was identified as the radioactive product in a typical effluent, by paper chromatography as described by Mudd et al. (1970). Reaction velocities are expressed as nanomoles of methionine formed per hour per milligram of protein, and are the average of at least two separate determinations which differed by less than 10%. In order to be able to compare the results of the non-B_{12} methionine synthetase assay with those obtained in the assay described above, the concentrations of several reagents in the non-B_{12} reaction were different than those described by Whitfield et al. (1970) (see Results and Discussion).

In order to ensure that the activity observed with the 5-methyltetrahydropteroyl oligo-γ-glutamates was not due to their prior hydrolysis to 5-methyltetrahydrofolate, conjugase (glutamate carboxypeptidase, EC 3.4.12.10) activity was checked at pH 4.5 and pH 7.4, according to the procedure of Krumdieck and Baugh (1970). At pH 4.5, conjugase activity equal to approximately 3.5 nmol of 5-methyltetrahydropteroyl triglutamate hydrolyzed/(hr/mg of preparation) was present in both dialyzed and nondialyzed bovine brain preparations. However, when conjugase activity was measured at pH 7.4 (0.1 *M* sodium phosphate or 0.1 *M* Tris buffer), the pH of the methionine synthetase assay, no conjugase activity was detectable in either dialyzed or nondialyzed preparations.

Results

Component Requirements for the Methionine Synthetase Reaction. The use of 10^{-2} *M* homocysteine as an enzyme stabilizer during isolation of the bovine methionine synthe-

[1] Some differences in the kinetic properties of the enzyme isolated from calves which had not yet been weaned from pasture-fed cows have been noted. These diet-dependent differences are being investigated further.

Table I: Component Requirements of Assay System.[a]

Assay System	nmol of Methionine formed/(hr mg)	% Activity
Complete B_{12}	4.7	100
– Homocysteine	0.3	6.4
– 2-ME	0.08	1.7
– SAM	0.16	3.4
– B_{12}	3.1	66.0
– Enzyme	0	0
Complete B_{12}[b]	5.3	100
– 2-ME, SAM, and B_{12}	0	0
Non-B_{12}[c]	0	0

[a] Bovine brain methionine synthetase was prepared according to the procedure described under Materials and Methods and dialyzed overnight against 2 × 100 volume changes of 0.05 *M* Tris-HCl buffer (pH 7.5) containing no homocysteine. The enzyme was assayed in the standard system at 37° for 1 hr as described under Materials and Methods. Except as noted, the complete B_{12} assay mixture contained in a final volume of 0.2 ml the following components: sodium phosphate buffer, pH 7.4 (20 μmol); 2-mercaptoethanol (2-ME) (24 μmol); *S*-adenosylmethionine (SAM) (50 nmol); cyano-B_{12} (10 nmol); D,L-homocysteine (100 nmol); N^5-[^{14}C]methyltetrahydrofolate (100 nmol); 50 μl of enzyme; and water to volume. Appropriate blanks were run for each set of conditions. [b] Substrate was N^5-[^{14}C]methyltetrahydropteroyl triglutamate (50 nmol). [c] Enzyme isolated as in (a). The complete assay mixture contained in a final volume of 0.2 ml: sodium phosphate buffer, pH 7.4 (20 μmol), D,L-homocysteine (100 nmol); N^5-[^{14}C]methyltetrahydropteroyl triglutamate (50 nmol); $Mg(OAc)_2$ (20 nmol); 1,4-dithiothreitol (2 μmol); 50 μl of enzyme; and water to volume. Appropriate blanks were run.

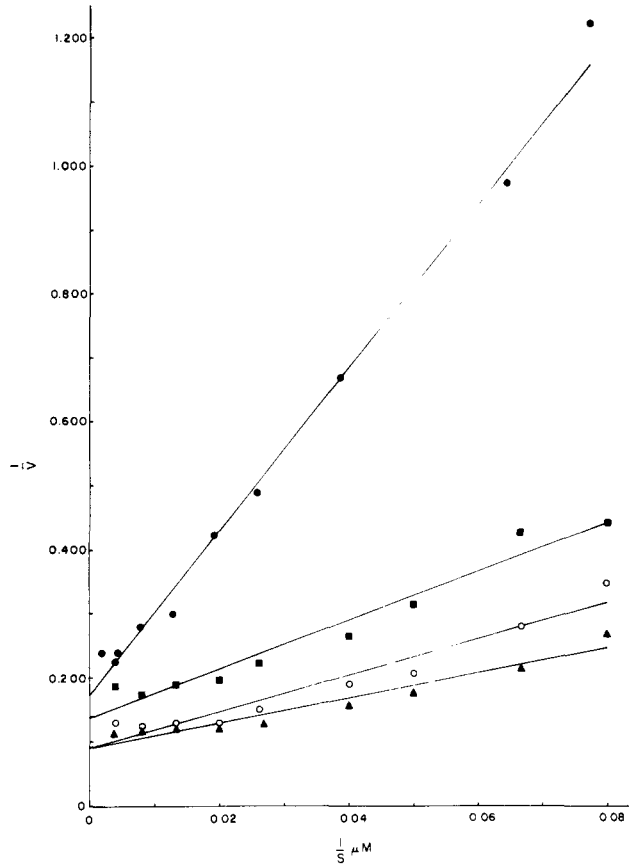

FIGURE 1: Kinetic data for the methylation of homocysteine by N^5-[^{14}C]methyltetrahydropteroyl mono- (●), tri- (■), penta- (○), and heptaglutamate (▲).

Table II: Kinetic Constants for Bovine Brain Methionine Synthetase.

Ligand[a]	V_{max}[b]	K_m (μM)	K_{ii} (μM)[c]
$CH_3H_4PteGlu_1$	5.8	73.1	
$CH_3H_4PteGlu_3$	7.2	24.4	26.8
$CH_3H_4PteGlu_5$	10.1	27.7	26.0
$CH_3H_4PteGlu_7$	11.1	22.3	22.1

[a] CH_3H_4Pte, 5-methyl-5,6,7,8-tetrahydropteroyl. [b] nmoles of methionine formed/(hr mg of protein). [c] Obtained in competition experiments as described in the text.

tase prevented a demonstration of the requirement for homocysteine. It was determined that homocysteine could be removed from the enzyme prior to assay without seriously affecting the enzyme stability; preparations dialyzed against buffer containing no homocysteine had 92% of the activity of the preparations dialyzed against 10^{-3} *M* homocysteine. Consequently, the following experiments were performed using bovine brain 5-methyltetrahydrofolate–homocysteine transmethylase which had been dialyzed for 18 hr at 4° against 2 × 100 volume changes of 0.05 *M* Tris-HCl buffer (pH 7.5) containing no homocysteine.

The component requirements of the assay are shown in Table I. Omission of cyano-B_{12} produced only a 34% loss of activity, which is in agreement with data reported by Taylor and Weissbach (1967). These authors suggested that endogenous B_{12} is tightly bound, and exogenous B_{12} does not act as a cofactor, but rather as a minor part of the mercaptoethanol reducing system. In the complete assay system, the reaction was linear over a protein range of 0.05–0.25 mg, and for as long as 90 min. A K_m determination for DL-homocysteine using the standard system and enzyme dialyzed against 2 × 100 volume changes of 0.05 *M* Tris-HCl buffer (pH 7.5) yielded a value of 53.3 μ*M*.

The possibility existed that a non-B_{12} methionine synthetase, such as found in bacteria and plants (Salem and Foster, 1972), was present in the partially purified bovine brain preparation and was responsible for the activity seen with the 5-methyltetrahydropteroyl oligo-γ-glutamates. In order to test for the presence of non-B_{12} methionine synthetase activity under the conditions used to obtain the above results, the assay of Whitfield et al. (1970) was modified slightly (Table I). Under these conditions, no [^{14}C]methionine formation could be detected. As a further check that the bovine brain transmethylase satisfied the criteria established for a B_{12}-requiring enzyme, 250 μ*M* [5-^{14}C]methyltetrahydropteroyl triglutamate was assayed in the modified B_{12} system (Kamely et al., 1973). As expected for a B_{12} enzyme, there was no detectable methionine formation from methyltetrahydropteroyl triglutamate in the absence of *S*-adenosylmethionine and the 2-mercaptoethanol, cyano-B_{12} reducing system (Table I).

Evaluation of Kinetic Data. Using enzyme preparations dialyzed for 18 hr against 2 × 100 volume changes of 0.05 *M* Tris-HCl buffer (pH 7.5), K_m values were obtained for N^5-[^{14}C]methyltetrahydropteroyl mono-, tri-, penta-, and heptaglutamates as substrates for bovine brain methionine synthetase (Table II). The corresponding Lineweaver–Burk plots are shown in Figure 1. All plots were drawn from data subjected to a least-squares analysis on an IBM 1130 computer. It can be seen that the K_m values for the 5-methyltetrahydropteroyl oligoglutamates are two- to threefold lower

than the corresponding value for the monoglutamate, and the V_{max} values are twice as high.

Inhibition studies were carried out using N^5-[^{14}C]methyltetrahydrofolate as substrate and unlabeled 5-methyltetrahydropteroyl tri-, penta-, and heptaglutamates as inhibitors. Lineweaver–Burk plots, such as shown in Figure 2, clearly establish that the tri-, penta-, and heptaglutamate forms are noncompetitive inhibitors of homocysteine methylation by the monoglutamate, 5-methyltetrahydrofolate. Replots of these data revealed that the inhibition is I-linear, S-parabolic (Cleland, 1970), leading to K_{ii} values of 26.8, 26.0, and 22.1 μM for the tri-, penta-, and heptaglutamates, respectively. These values are in good agreement with the K_m values obtained using the 5-methyltetrahydropteroyl oligoglutamates as substrates. This is reasonable, since the observed inhibition presumably results from the fact that the higher conjugates can act as alternate substrates in the transmethylation reaction.

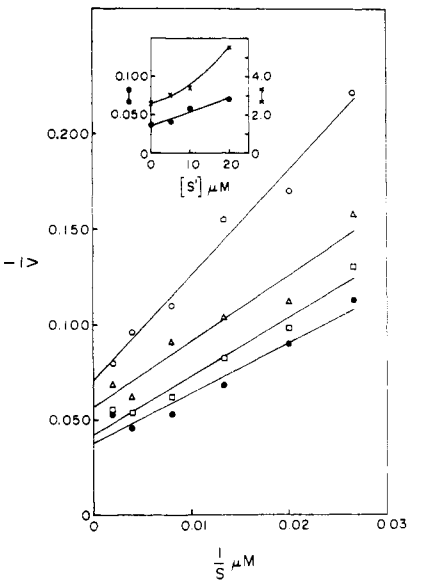

FIGURE 2: Kinetic data for the inhibition of N^5-[^{14}C]methyltetrahydrofolate-dependent methylation of homocysteine by N^5-methyltetrahydropteroyl heptaglutamate at concentrations of 0 μM (●), 5 μM (□), 10 μM (△), and 20 μM (○) heptaglutamate (S'). Inset: replot of slope (x) and intercept (●) vs. concentration of heptaglutamate.

Discussion

The primary finding of this work, namely that 5-methyltetrahydropteroyl oligoglutamates are substrates for the enzyme methionine synthetase, lends further support to the idea that the conjugates of folic acid can act as substrates as well as, or better than, the unconjugated folate, and therefore are not simply storage forms. Blakley (1957) previously reported that tetrahydropteroyl triglutamate appeared to be a better cofactor than the monoglutamate for rabbit muscle serine transhydroxymethylase, while Loughlin et al. (1964) reported that a crude 5-methyltetrahydropteroyl triglutamate was nearly equal to the monoglutamate as a substrate for hog kidney methionine synthetase. Both studies were limited by the scarcity of purified polyglutamate forms, and the possibility that conjugase activity was present in the enzyme preparation. Recently we found that the oligoglutamates of folate and dihydrofolate were as good or better substrates than corresponding monoglutamates for purified mammalian dihydrofolate reductase (Coward et al., 1974b). In addition, Kisliuk et al. (1974) have recently reported that several oligoglutamate conjugates of 5,10-methylenetetrahydrofolate are more effective than the monoglutamate as substrates for the enzyme thymidylate synthetase from *Lactobacillus casei*.

In the present work, it has been found that methionine synthetase partially purified from bovine brain is quite stable to dialysis in the absence of homocysteine, thus allowing a more accurate assessment of the K_m value for homocysteine (53 μM) than previously found by Mangum et al. (1972). This enzyme system fits the criteria for a B_{12}-requiring enzyme as demonstrated in the experiments dealing with the component requirements of the assay system, especially the reducing system components (Table I). That exogenous B_{12} did not substantially affect activity is not unusual in the light of results of other investigators using this reducing system (Taylor and Weissbach, 1967). Evidence that a non-B_{12} enzyme was not active under the present assay conditions was provided by using a modified assay of Whitfield et al. (1970). This does not mean that a non-B_{12} enzyme may not be present in mammalian tissue, but rather that under the present isolation and assay conditions, it was not responsible for the results found when 5-methyltetrahydropteroyl oligo-γ-glutamates were tested as enzyme substrates.

The K_m values for the 5-methyltetrahydropteroyl tri-, penta-, and heptaglutamates were between 25 and 30 μM, as compared with a K_m of 75 μM found for the monoglutamate. In addition, the V_{max} values obtained with the oligoglutamates were twice the V_{max} values obtained with the monoglutamate. However, a pattern which indicated a preference for one of the oligoglutamates as a more effective substrate was not observed. All oligomers above the monoglutamate were approximately equal in substrate activity. The inhibition studies (Figure 2) revealed that the oligoglutamates produced a noncompetitive inhibition vs. 5-methyltetrahydrofolate, with K_i values approximating the K_m's. The noncompetitive type of inhibition is significant in that it eliminates the possibility of separate forms of bovine brain methionine synthetase active specifically with each of the 5-methyltetrahydropteroyl oligo-γ-glutamates. It supports either the concept of one enzyme form which utilizes both the monoglutamate and the polyglutamate derivatives, but has a preference for the polyglutamate derivatives; or, the less likely possibility of multiple forms of the enzyme, each of which utilizes the monoglutamate derivative, but shows specificity for a particular polyglutamate.

The existence of bacterial and plant methionine synthetases which do not require vitamin B_{12} but which require the 5-methyltetrahydropteroyl triglutamate as the methyl donor is well documented. The requirement for additional glutamates has been explained as necessary to bind the substrate to the enzyme. No information has previously been available to indicate a role for the oligoglutamate conjugates in mammalian methionine synthetases. This study indicates that polyglutamate forms of 5-methyltetrahydrofolate, which have been found in quantity in all mammalian tissues examined, and previously regarded as "storage forms" can act as substrates for this enzyme. In addition, there are data available which indicate that several pteroyl oligoglutamates are potent inhibitors of bacterial thymidylate synthetase (Friedkin et al., 1971; Kisliuk et al., 1974). The accumulated data thus support the idea that the polyglutamate forms may function both as the natural substrates and as potent regulators of intracellular folate-dependent reactions.

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References

Blair, L. A., and Saunders, K.J. (1970), *Anal. Biochem. 34,* 376.

Blakley, R. L. (1957), *Biochem. J. 65,* 345.

Blakley, R. L. (1969), The Biochemistry of Folic Acid and Related Pteridines, New York, N.Y., Wiley.

Brown, J. P., Davidson, G. E., and Scott, J. M. (1974), *Biochim. Biophys. Acta 343,* 78.

Burke, G. T., Mangum, J. H., and Brodie, J. D. (1971), *Biochemistry 10,* 3079.

Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., DeAngelis, L. M., and Bertino, J. R., (1974a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1507.

Coward, J. K., Parameswaran, K. N., Cashmore, A. R., and Bertino, J. R. (1974b), *Biochemistry 13,* 3899.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Friedkin, M., Crawford, E. J., and Plante, L. T. (1971), *Ann. N.Y. Acad. Sci. 186,* 209.

Godwin, H. A., Rosenberg, I. H., Ferenz, C. R., Jacobs, P. M., and Meienhofer, J. (1972), *J. Biol. Chem. 247,* 2266.

Houlihan, C. M., and Scott, J. M. (1972), *Biochem. Biophys. Res. Commun. 48,* 1675.

Kamely, D., Littlefield, J. W., and Erbe, R. W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2585.

Kisliuk, R. L., Gaumont, G., and Baugh, C. M. (1974), *J. Biol. Chem. 249,* 4100.

Krumdieck, C. L., and Baugh, C. M. (1969), *Biochemistry 8,* 1568.

Krumdieck, C. L., and Baugh, C. M. (1970), *Anal. Biochem. 35,* 123.

Laduron, P. M., Gommeren, W. R., and Leysen, J. E. (1974), *Biochem. Pharmacol. 23,* 1599, and references therein.

Loughlin, R. E., Elford, H. L., and Buchanan, J. M. (1964), *J. Biol. Chem. 239,* 2888.

Mangum, J. H., Stewart, B. W., and North, J. A. (1972), *Arch. Biochem. Biophys. 148,* 63.

Mudd, S. H., Levy, H. L., and Morrow, G., III (1970), *Biochem. Med. 4,* 193.

Osborne-White, W. S., and Smith, R. M. (1973), *Biochem. J. 136,* 265.

Salem, A. R., and Foster, M. A. (1972), *Biochem. J. 127,* 845.

Shin, Y. S., Williams, M. A., and Stokstad, E. L. R. (1972), *Biochem. Biophys. Res. Commun. 47,* 35.

Taylor, R. T., and Weissbach, H. (1967), *J. Biol. Chem. 242,* 1502.

Taylor, R. T., and Weissbach, H. (1973), *Enzymes, 3rd Ed. 9,* 121.

Whitfield, C. D., Steers, E. J., Jr., and Weissbach, H. (1970), *J. Biol. Chem. 245,* 390.