Biochemical Pharmacology, Vol. 36, No. 13, pp. 2139–2144, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

EFFECT OF CHRONIC PRIMIDONE TREATMENT ON FOLATE-DEPENDENT ONE-CARBON METABOLISM IN THE RAT

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(*Received* 22 *July* 1986; *accepted* 4 *December* 1986)

Abstract—Rats were treated chronically with primidone (100 mg/kg/12 hr, p.o.) for up to 8 weeks. The effects of this treatment on one-carbon metabolism were determined in brain and liver. Serine hydroxymethyltransferase activity increased in both brain (44%) and liver (50%). Methylenetetrahydrofolate reductase activity increased in liver (26%) with a significant correlation to the length of treatment, but in brain it was unchanged. Methyltetrahydrofolate:homocysteine methyltransferase activity increased in brain (43%) with a significant correlation to length of treatment, but in liver no effect was observed. Methionine adenosyltransferase activity in brain was significantly lower than control at only one point after 8 weeks of chronic treatment. *S*-Adenosylmethionine concentration in liver increased gradually (23%) during treatment. *S*-Adenosylhomocysteine concentrations decreased in brain (33%) and increased in liver (23%) with chronic primidone treatment. These data support the hypothesis that chronic primidone treatment leads to folate depletion through interference with folate metabolism.

Chronic anticonvulsant therapy has been associated with folate deficiency. Phenytoin, in particular, as well as phenobarbital and primidone have been shown to induce folate deficiency in epileptic patients [1–3]. Carbamazepine [4–7] and valproate [7, 8] have also been implicated as interfering in folate function but the evidence is not as strong. This involvement of several anticonvulsants in folate depletion has led to speculation that the folate depletion may be required for anticonvulsant action [9]. This is supported by observations that folate derivatives are epileptogenic if allowed free access to the brain [10–12]. However, controlled studies of folate supplementation generally agree that in most cases repletion of plasma folate levels has no significant effect on seizure frequency [9]. There are exceptions [9] and, indeed, changes in the concentration or metabolism [9, 13–16] of anticonvulsants have been attributed to folate supplementation.

Folate deficiency has been known to cause megaloblastic anemia [1–3]. In less severe cases, folate deficiency has been associated with neurological [17–20], psychiatric [21–23] and intellectual [24] deficiencies as well. In fact, folate deficiency has been associated with a poor prognosis for the treatment of depression [25]. Indeed, it is possible that the folate depleting property of anticonvulsant therapy may be responsible for the high incidence of psychiatric morbidity in the epileptic population [26, 27].

While the mechanism(s) of anticonvulsant-induced folate depletion is/are unknown, several possibilities have been suggested. It is possible that chronic anticonvulsant therapy increases the requirement for folate by stimulating folate-dependent reactions or that anticonvulsant therapy somehow alters the dietary intake of folate. However, at present there is no evidence to support either of these hypotheses [28]. Other more likely possibilities exist, such as: (a) inhibition by anticonvulsants of absorption of folates from the intestine [28]; (b) induction by anticonvulsants of folate catabolic enzymes [29]; (c) anticonvulsant interference with folate metabolism causing an increase in the relative concentrations of the more labile derivatives of folate leading to degradation of folate and depletion of cellular pools [30]; and (d) displacement of folate from carrier proteins in serum leading to increased catabolism and excretion [31].

Another complicating factor with primidone is its conversion in the liver to phenobarbital. It has been suggested that phenobarbital may indeed be the active metabolite of primidone especially after chronic administration [32]. Because phenobarbital accumulates with chronic administration one might expect the effects of primidone on one-carbon metabolism to be similar or identical to those of phenobarbital.

Most research in this area has been done in humans. While the human is the most appropriate species in which to do medical research, there are significant disadvantages with regard to access to appropriate tissues, homogeneity of the experimental population, and treatment of control populations. We have developed a rat model for chronic primidone treatment which is minimally toxic and continuously protective.‖ We have used this model to examine the effect of chronic primidone treatment on folate-dependent one-carbon metabolism. Since

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‖ G. F. Carl and M. L. Smith, manuscript submitted for publication.

changes in the methyl side of one-carbon metabolism have been associated with the milder forms of folate deficiency, we have chosen to examine the enzymes involved in methyl synthesis and activation as well as the products of this system. We report here the results of that investigation.

MATERIALS AND METHODS

Animals. Male, Harlan–Sprague–Dawley rats (50–75 g) were maintained *ad lib.* on food and water with a 12 hr/12 hr light/dark cycle. All rats were fed every 12 hr by gastric gavage with either water or primidone suspended homogeneously in water (25 mg/ml) by continuous stirring. Each rat was weighed twice weekly, and the gavage volumes were adjusted appropriately to yield a dose of 100 mg primidone/kg every 12 hr. Each week six rats were transferred from the control group (water) to the treated group (primidone). Twelve hours after the last feeding the rats were decapitated. Final weights of the rats were 300–350 g.

Preparation of tissues. Brain and liver were quickly excised and frozen in liquid nitrogen and stored at −70° until completion of sacrifice. Then each tissue was weighed and homogenized in 4 vol. of 25 mM sodium ascorbate, pH 7.0. Three 1.0-ml aliquots of each liver homogenate were dispensed into separate tubes. These aliquots were diluted with 1.0 ml of the assay buffers for each of the following assays: 5-methyltetrahydrofolate:homocysteine methyltransferase; 5,10-methylenetetrahydrofolate reductase; and serine hydroxymethyltransferase. Three 1.0-ml aliquots of each brain homogenate were treated similarly, with a fourth being diluted with methionine adenosyltransferase assay buffer. All of these samples, including the remainder of the homogenates, were frozen for later analysis.

Enzymes assays. Serine hydroxymethyltransferase (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase; EC 2.1.2.1) (SHMT*) activity was assayed by the method of Taylor and Weissbach [33]. Methylenetetrahydrofolate reductase (5,10-methylenetetrahydrofolate:$FADH_2$ oxidoreductase; EC 1.1.99.15) (MTR) activity was measured by the method of Kutzbach and Stokstad [34]. Methyltetrahydrofolate transmethylase (5-methyltetrahydrofolate:L-homocysteine methyltransferase; EC 2.1.1.13) (MHMT) was assayed by the method of Clark *et al.* [35]. *S*-Adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase; EC 2.5.1.6) (MAT) was assayed by a modification of a combination of the double isotope method of Matthysse *et al.* [36] and the *S*-adenosylmethionine assay of Yu [37] as described previously [38].

Assay for AdoMet and AdoHcy. *S*-Adenosylmethionine (AdoMet) was assayed by the method of Yu [37], and *S*-adenosylhomocysteine (AdoHcy) was assayed according to the method of Schatz *et al.* [39].

* Abbreviations: SHMT, serine hydroxymethyltransferase; MAT, methionine adenosyltransferase; MHMT, methyltetrahydrofolate:homocysteine methyltransferase; MTR, methylenetetrahydrofolate reductase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; and 5-CH_3FH_4, 5-methyltetrahydrofolate.

RESULTS

SHMT catalyzes the transfer of the β-carbon of serine to tetrahydrofolate forming 5,10-methylenetetrahydrofolate, an important intermediate in one-carbon metabolism. SHMT activity increased with chronic primidone treatment in liver up to 6 weeks after initiation of treatment and then leveled off or possibly decreased toward the control activity (Fig. 1). Treatment would have to be extended beyond 8 weeks to determine if liver SHMT activity remains elevated or decreases with continued treatment. In brain, the SHMT activity increased in the first 2 weeks and remained elevated for the remainder of the treatment (Fig. 1).

The 5,10-methylenetetrahydrofolate formed as a product of the SHMT reaction can be used in the synthesis of thymidylate, oxidized to 5,10-methenyltetrahydrofolate or reduced to 5-methyltetrahydrofolate in a reaction catalyzed by methylenetetrahydrofolate reductase (MTR). Liver MTR activity increased as a function of the time of chronic treatment with primidone (Fig. 2), whereas brain MTR showed a tendency (albeit, not significant) toward higher activity with chronic primidone treatment.

The 5-methyltetrahydrofolate formed as a product of MTR activity donates its methyl group to homocysteine in a reaction that regenerates tetrahydrofolate and forms methionine. This reaction is catalyzed by MHMT which exhibited a higher specific activity in brain than in liver (Fig. 3). In addition, the MHMT activity in brain increased as a significant function of the time of chronic primidone treatment after some apparent delay (Fig. 3). In liver, on the other hand, chronic primidone treatment showed no effect on MHMT activity (Fig. 3).

The sulfur of methionine is converted to a high energy sulfonium ion by reaction with ATP forming AdoMet and catalyzed by MAT. The MAT activity in brain was not affected significantly by chronic primidone treatment until late in the 8 week regimen when a significant decrease in AdoMet formation was observed (Fig. 4). MAT activity in liver was not measured since the interpretation of the results would be complicated by the presence of several isozymes with widely differing K_m values [40]. In brain there is apparently only one form of the enzyme [41].

The product of the MAT activities in liver was measured (i.e. AdoMet), and concentrations increased significantly as a function of the duration of treatment with primidone (Fig. 5). AdoMet was not measured in brain because there was not enough homogenate left after aliquots were taken for the other assays.

AdoMet, as the universal methyl donor, is involved in many transmethylation reactions. AdoHcy is a product of these transmethylations, almost all of which are inhibited by accumulation of this product. In brain, the AdoHcy concentration decreased initially with primidone treatment but then leveled off and remained at the lower concentration through 8 weeks of treatment (Fig. 6). In liver, on the other hand, the AdoHcy concentrations increased with treatment at least through 4 or 5

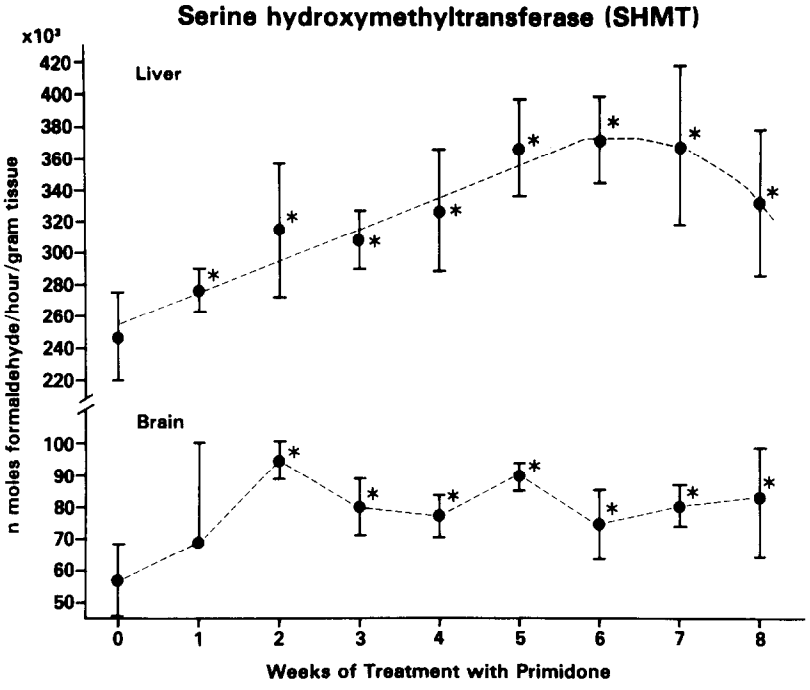

Fig. 1. Effect of chronic primidone treatment on the activity of serine hydroxymethyltransferase (SHMT) in liver and brain. Primidone was administered and enzyme activity was measured as described in Materials and Methods. Each point (1–8 weeks) represents the mean ± SD from six animals. Control points (0 week) represent the mean ± SD of thirteen to fifteen animals. An asterisk (*) indicates a significant difference from control at $P < 0.02$ (t-test). When liver activity was subjected to linear regression analysis for weeks 0–6, $\gamma = 0.971$, $P < 0.001$. Brain activity did not correlate with time of treatment.

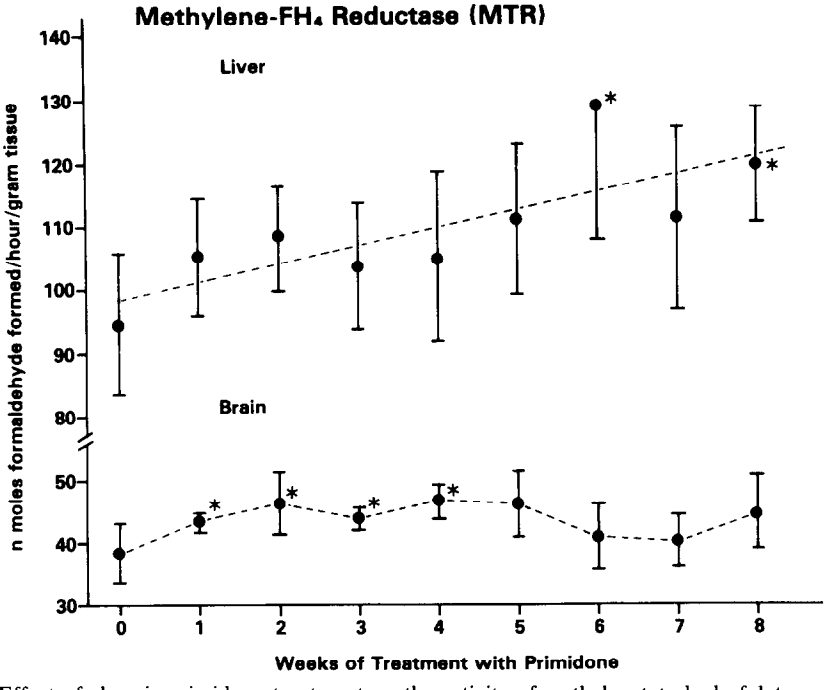

Fig. 2. Effect of chronic primidone treatment on the activity of methylenetetrahydrofolate reductase (MTR) in liver and brain. For details see the legend of Fig. 1. An asterisk (*) indicates a significant difference from control at $P < 0.01$ (t-test). Liver MTR activity correlated significantly with time of treatment by linear regression analysis, $\gamma = 0.757$, $P < 0.02$. Brain MTR activity did not correlate with time of treatment.

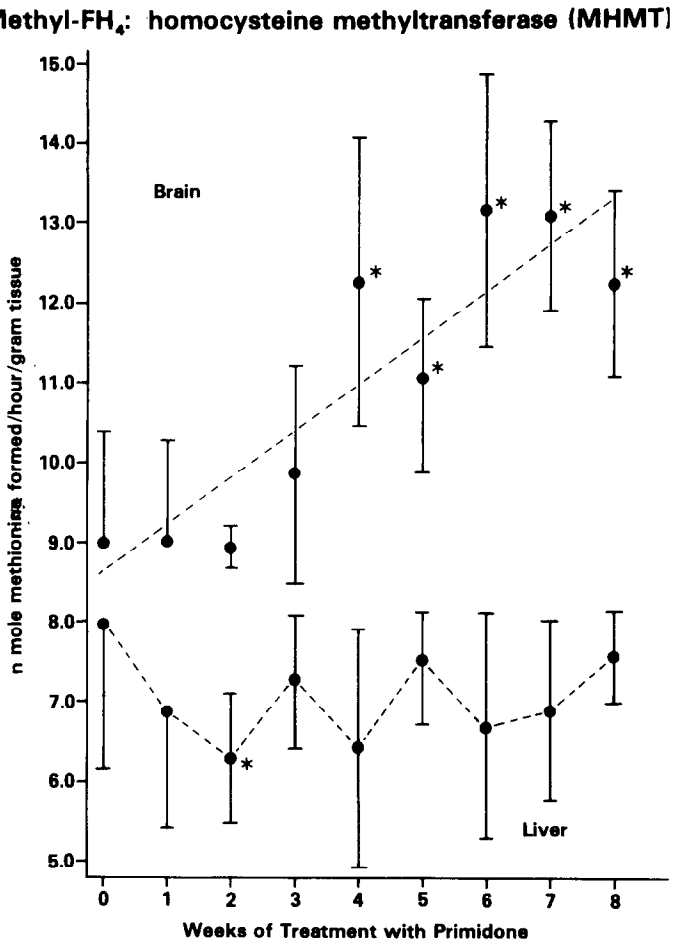

Fig. 3. Effect of chronic primidone treatment on the activity of methyltetrahydrofolate:homocysteine methyltransferase (MHMT) in brain and liver. An asterisk (*) indicates a significant difference from control at $P < 0.01$ (t-test). Brain MHMT activity correlated significantly with time of primidone treatment, $\gamma = 0.889$, $P < 0.001$ (linear regression analysis) but liver MHMT activity did not correlate with treatment time.

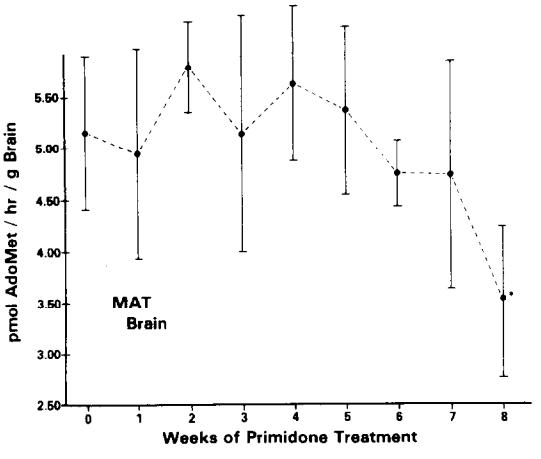

Fig. 4. Effect of chronic primidone treatment on the activity of ATP-methionine adenosyltransferase (MAT) in brain. For details, see legend of Fig. 1. An asterisk (*) indicates a significant difference from control at $P < 0.01$ (t-test).

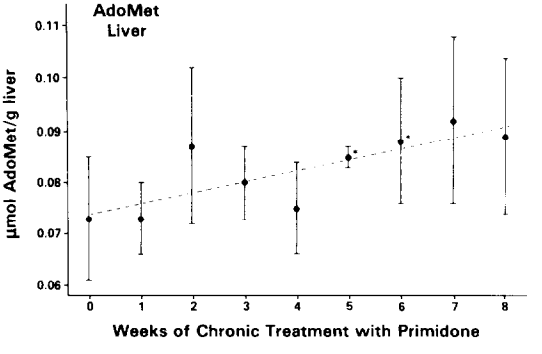

Fig. 5. Effect of chronic primidone treatment on the concentration of *S*-adenosylmethionine (AdoMet) in liver. AdoMet was assayed as described in Materials and Methods. For other details, see the legend of Fig. 1. An asterisk (*) indicates a significant difference from control at $P < 0.01$ (t-test). AdoMet concentration in liver correlated significantly with time of primidone treatment, $\gamma = 0.795$, $P < 0.01$ (linear regression analysis).

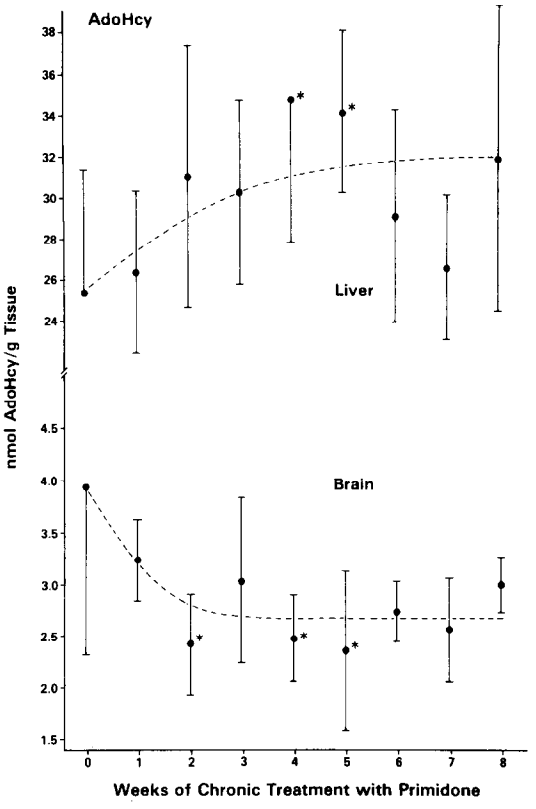

Fig. 6. Effect of chronic primidone treatment on the concentration of *S*-adenosylhomocysteine (AdoHcy) in liver and brain. AdoHcy was assayed as described in Materials and Methods. Each point (1–8 weeks) represents the mean ± SD of four to six animals. Control points (0 week) represent the mean ± SD of ten to twelve animals. An asterisk (*) indicates a significant difference from control at $P < 0.02$ (*t*-test).

weeks (Fig. 6). Whether this increased concentration is maintained by continued treatment is difficult to determine from our data.

DISCUSSION

The effects of chronic primidone treatment on one-carbon metabolism are obviously quite different in liver and brain, the only similarity being an apparent increase in the activity of the major one-carbon generating enzyme (SHMT) in both tissues (Fig. 1). Whether this indicates that primidone stimulates a generalized one-carbon turnover is unknown.

In liver there was an apparent increase in methyl synthesis as the activity of the MTR increased in this tissue with primidone treatment (Fig. 2). The MHMT activity in liver, however, was unaffected by primidone treatment (Fig. 3), indicating that the 5-methyltetrahydrofolate (5-CH_3FH_4) synthesized by the apparent increase in MTR activity may not be used by MHMT, the only enzyme that uses 5-CH_3FH_4 as substrate, in the liver but may be made for export. Since folate concentrations are higher in liver than in other tissues [42] and since liver folate concentrations are the first to be affected by folate-depleting drugs [38], it is reasonable to conclude, as previously suggested [42], that liver serves as a storage organ for folate. Moreover, it might also be suggested that liver stores folate in the form of 5-CH_3FH_4. This form constitutes a major portion of total liver folate, 35–60% [42], and it is the major circulating form. Therefore, stored folate might be accessed more easily if it is already in the one-carbon form needed for circulation.

It has been shown in this animal model that therapeutic doses of phenytoin [38], phenobarbital [43] and primidone [44] deplete liver folates. Whether this depletion is in response to demands for folate elsewhere (which are met by exporting folate from the liver) is unknown, but it is interesting to note that treatment of the rat with phenobarbital [45] or primidone [44] depletes polyglutamates of folates relatively rapidly. This may be an indication that folates are exported from the liver in response to treatment with these anticonvulsants. The mechanism of this effect is unknown. However, if liver is actively exporting 5-CH_3FH_4, then the increase of the activities of SHMT (Fig. 1) and MTR (Fig. 2) might be explained as a response to an increased need for the synthesis of 5-CH_3FH_4. This hypothesis is supported by the lack of effect of primidone treatment on the activity of MHMT (Fig. 3), the only enzyme that uses 5-CH_3FH_4 as a substrate. Phenobarbital, like primidone, also causes an increase in MTR activity with no concomitant effect on MHMT activity [45].

While the effect of primidone treatment on MAT activity in the liver could not be evaluated in the crude tissue preparations used in this study, the concentrations of the product of this enzyme, *S*-adenosylmethionine (AdoMet), were measured and found to increase with treatment time (Fig. 5). Whether this increase is due to increased synthesis or decreased use is unknown, but if the lack of effect of the treatment on MHMT activity (Fig. 3) indicates that methionine regeneration is not increased, and if the treatment does not cause an increase in methionine concentrations in the liver, then it is possible that decreased use of AdoMet could be responsible for the increased AdoMet concentrations. There are two pathways that use AdoMet, polyamine biosynthesis and transmethylation. The product of the latter reaction, *S*-adenosylhomocysteine (AdoHcy), which is a potent inhibitor of most transmethylations, also increases in liver with primidone treatment (Fig. 6), indicating that transmethylations may be decreased and that accumulation of AdoMet may be caused by decreased use. From the data presented here, it can be seen that one-carbon metabolism is affected significantly in liver by chronic primidone treatment, but the mechanism of this effect remains unclear. However, it is apparent that primidone does have an effect on folate-dependent metabolism, and it is likely that some of the effects that primidone exerts on tissue and plasma folate concentrations are mediated via these metabolic effects.

In brain, the metabolic consequences of primidone treatment on one-carbon metabolism are quite different from those in liver. Although the SHMT activity in brain increased slightly (Fig. 1), the MTR activity was unaffected (Fig. 2) by chronic primidone treatment. While it is possible that methyl synthesis did not increase, the MHMT activity did increase

(Fig. 3), indicating greater use of the methyl group in the synthesis of methionine. Whether or not this effect of primidone is related to the high specific activity of MHMT in nerve endings in brain [46] is unknown. Increased methionine regeneration in brain, however, does not necessarily translate into increased transmethylation capacity. It has been shown that substantial increases in methionine concentration in brain do not result in large increases of AdoMet concentrations probably because of the saturability of the MAT activity in brain [41]. If the MAT in brain is saturated at normal methionine concentrations, then a decrease in MAT activity [as observed here late in the treatment (Fig. 4)] would most likely result in decreased AdoMet synthesis. This conclusion is supported by the observation that AdoHcy concentration decreased in brain with primidone treatment (Fig. 6). However, this decrease occurred early in the treatment, and the AdoHcy concentration remained at the lower level.

The data presented here support the hypothesis that the anticonvulsant primidone affects folate concentrations in tissue and plasma by interfering with folate-dependent metabolic processes. Although the primary site of this interference has not yet been shown, the data presented here, combined with earlier observations [44], indicate an interaction of primidone with the synthesis of folylpolyglutamates.

Acknowledgements—The authors appreciate the excellent technical assistance of Ms. Carolyn DeLoach and Mr. Jerome Patterson, the artistic and technical expertise of the staff of the Medical Media Service of the Augusta Veterans Administration Medical Center, and the quality secretarial help of Ms. Pauline Thompson. These studies were supported by the Medical Research Service of the Veterans Administration (G. F. C.) and by the Epilepsy Foundation of America (R. A. S.).

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