Biochemical Pharmacology, Vol. 33, No. 21, pp. 3457–3463, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

EFFECT OF CHRONIC PHENOBARBITAL TREATMENT ON FOLATES AND ONE-CARBON ENZYMES IN THE RAT*

G. FRANKLIN CARL† and DENNIS B. SMITH

Neurology Research Veterans Administration Medical Center, Augusta, GA 30910, and Department of Neurology, Medical College of Georgia, Augusta, GA 30912, U.S.A.

(*Received* 19 *October* 1983; *accepted* 10 *February* 1984)

Abstract—Chronic oral phenobarbital treatment (50 mg/kg every 12 hr for 8 weeks), which was nontoxic and continuously protective against seizures in rats, significantly decreased folate concentration in liver (29%) but not in brain or plasma. The apparent activity of 5,10-methylenetetrahydrofolate reductase (MTR) in liver decreased with initiation of treatment but then increased with a significant correlation to the length of treatment. Phenobarbital also stimulated the activity of this enzyme slightly *in vitro*. Methionine adenosyltransferase (MAT) activity was inhibited by high concentrations of phenobarbital *in vitro* but was not affected *in vivo*. No significant effects of phenobarbital on the activities of serine hydroxymethyltransferase (SHMT) or 5-methyltetrahydrofolate:homocysteine methyltransferase (MHMT) were observed either *in vivo* or *in vitro*.

It has been established that chronic anticonvulsant therapy can lead to folate deficiency. While phenytoin is most often associated with this problem [1], phenobarbital [1, 2], primidone [1], carbamazepine [2, 3], and valproate [3] have also been implicated. It has been known for 30 years that anticonvulsant-induced folate deficiency can lead to megaloblastic anemia [4, 5], but more recently folate deficiency has been associated with psychiatric [6–8], neurological [9–12], and intellectual [13] deficiencies as well. Indeed, a recent study indicates that low serum folate concentration is a predictor of poor prognosis for the pharmacological treatment of depression [14]. In light of the relationship between folate deficiency and psychoses, Reynolds [15] has suggested that the anticonvulsant-induced folate deficiency observed in many epileptic patients may be responsible for the high incidence of psychiatric morbidity observed in this population [16].

Most investigations in this area have been conducted with human subjects. But it is difficult to elucidate mechanisms of interaction in humans with limited tissue accessibility, lack of homogeneity in the population, and limited ability to control studies. We previously developed a rat model to parallel treatment of epileptics with phenytoin [17]. Using this model, we demonstrated that chronic phenytoin treatment caused an immediate depression of liver folate levels followed by an eventual fall in brain folate concentration [18].

The mechanism(s) by which anticonvulsants induce folate deficiency is unknown, but several possibilities have been suggested: (1) inhibition of the absorption of folate from the intestine [19], (2) increased catabolism of folates by induction of catabolic enzymes [20], (3) interference with folate metabolism causing accumulation of the more labile forms of folate which are more easily degraded [21], (4) displacement of folate from carrier proteins in serum [1], (5) inhibition of cellular folate uptake [1], (6) increased requirement for folate by stimulating folate-dependent reactions [19] and (7) alteration of dietary intake of folate [19]. There is no evidence for either of the latter two suggestions and there is evidence both for and against the former five [19, 20]. There is clearly little agreement on the mechanisms underlying the depletion of folate coenzymes by anticonvulsant treatment.

We report here a model in the rat for chronic, oral, nontoxic, continuously protective phenobarbital treatment and the effects of this treatment on tissue folate concentrations and on the activities of four enzymes of one-carbon metabolism.

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 by gastric gavage every 12 hr with either phenobarbital in propylene glycol or propylene glycol. The rats were weighed every week, doses were adjusted, and a group of four or five rats was transferred from the control group (propylene glycol) to the treatment group (phenobarbital in propylene glycol). Twelve hours after the last feeding, the rats were either evaluated for seizure threshold using the volatile convulsant hexafluorodiethylether (Armageddon Chemical, Durham, NC) [22] or decapitated.

Preparation of tissues. Blood was collected from the carotid artery into a beaker containing heparin and sodium ascorbate. Cells were collected by centrifugation, and the plasma was aspirated and frozen. Brain and liver were quickly excised and frozen in

* Supported by the Veterans Administration.

† Address correspondence to: G. F. Carl, Neurology Research (151), VA Medical Center, Downtown Division, Augusta, GA 30910.

liquid nitrogen and stored at −80° until completion of sacrifice. Then each tissue was weighed and homogenized in 4 vol. of 25 mM sodium ascorbate, pH 7.0. Four 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; serine hydroxymethyltransferase; and the folate microbiological assay. Four 1.0-ml aliquots of each brain homogenate were treated similarly with a fifth being diluted with methionine adenosyltransferase assay buffer. All of these samples, including the remainder of the homogenates, were frozen for later analysis.

Folate determination. Folates were determined in homogenates of brain and liver diluted 1:1 with 0.10 M sodium phosphate, pH 7.0, and in plasma by microbiological assay as previously described [17].

Phenobarbital determinations. Phenobarbital and 5-ethyl-5-tolylbarbituric acid (internal standard) were extracted from 1.0 ml of acidified (HCl) tissue homogenate into 8 ml dichloroethane. The aqueous layer was aspirated and discarded, and the organic layer was filtered through phase separating paper (Whatman 1PS). The phenobarbital was then back-extracted into 4 ml of 0.5 N NaOH. This solution was acidified and re-extracted with 6 ml dichloroethane. The aqueous layer was aspirated and the dichloroethane was transferred to a clean tube and evaporated. The residue was suspended in 2 ml of 0.5 N HCl:MeOH (4:1, v:v) and washed twice with 4 ml hexane. The methanolic HCl solution was then extracted twice with 4 ml chloroform. The chloroform was evaporated to dryness and the residue was dissolved in 100 μl Methelute (trimethyl anilinium hydroxide, 0.2 M in methanol, Pierce Chemical Co., Chicago, IL) and assayed by gas chromatography on a 6 ft × 2 mm, 5% OV 17 column, isothermally at 210°, with an injection port temperature of 300° and a detector temperature of 300°. Retention times were 9.3 min for phenobarbital and 13.5 min for 5-ethyl-5-tolylbarbituric acid.

Plasma phenobarbital was assayed using the enzyme-multiplied-immuno-technique (EMIT) method (Syva Co., Palo Alto, CA).

Enzymes assays. Serine hydroxymethyltransferase

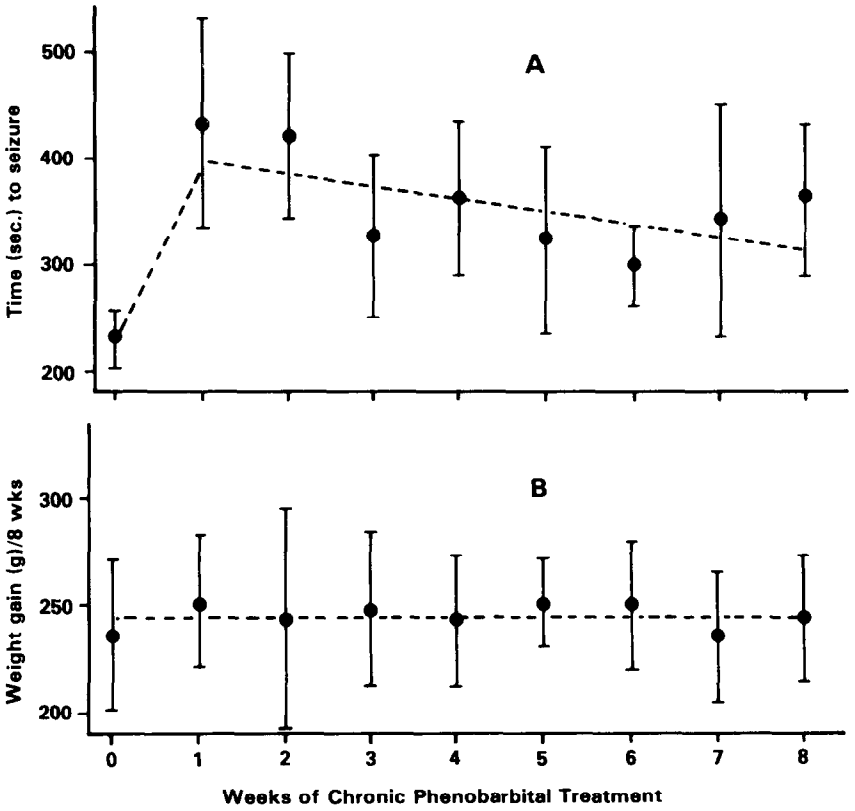

Fig. 1. Evaluation of chronic phenobarbital treatment in rats. (A) Degree of protection against seizures induced by hexafluorodiethylether as a function of length of treatment. (B) Weight gain as a function of length of treatment. Either phenobarbital (25 mg/ml in propylene glycol) or propylene glycol (2.0 ml/kg) was administered orally to each animal every 12 hr for 8 weeks. Groups of five animals were transferred from the control (propylene glycol) to the treatment (phenobarbital in propylene glycol) each week. Doses were adjusted twice per week, and the weight of each rat was recorded at the end of each week. Twelve hours after the final dose the animals were exposed to hexafluorodiethylether and the length of time (sec) required to produce a tonic-clonic seizure was recorded. Data are represented as mean ± S.D. of five or more observations.

(L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase; EC 2.1.2.1) (SHMT*) activity was assayed by the method of Taylor and Weissbach [23]. Methylenetetrahydrofolate reductase (5,10-methylenetetrahydrofolate:$FADH_2$ oxidreductase; EC 1.1.99.15) (MTR) activity was measured by the method of Kutzbach and Stokstad [24]. Methyltetrahydrofolate transmethylase (5-methyltetrahydrofolate:L-homocysteine methyltransferase; EC 2.1.1.13) (MHMT) was assayed by the method of Clark *et al.* [25]. *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.* [26] and the *S*-adenosylmethionine assay of Yu [27] as described previously [18].

* Abbreviations: SHMT, serine hydroxymethyltransferase; MAT, methionine adenosyltransferase; MHMT, methyltetrahydrofolate: homocysteine methyltransferase; and MTR, methylenetetrahydrofolate reductase.

RESULTS

Phenobarbital administered orally every 12 hr at 50 mg/kg provided continuous protection (Fig. 1A) against seizures induced 12 hr after the final dose by inhalation of hexafluorodiethylether. At the same time the phenobarbital exhibited no apparent toxicity as determined by the lack of effect of phenobarbital treatment on weight gain of the rats (Fig. 1B). However, the protection provided by this chronic treatment with phenobarbital decreased with length of treatment (Fig 1A) as might be expected considering the fact that phenobarbital induces enzymes for its own catabolism [28]. Indeed, the plasma, liver and brain concentrations of phenobarbital decreased in parallel with the decrease in protection, but plasma phenobarbital concentration was maintained at levels considered therapeutic for humans throughout the 8 weeks of treatment (Fig. 2).

The chronic oral administration of phenobarbital caused a significant decrease (29%) in folate concentration in the liver and a slight apparent (but not

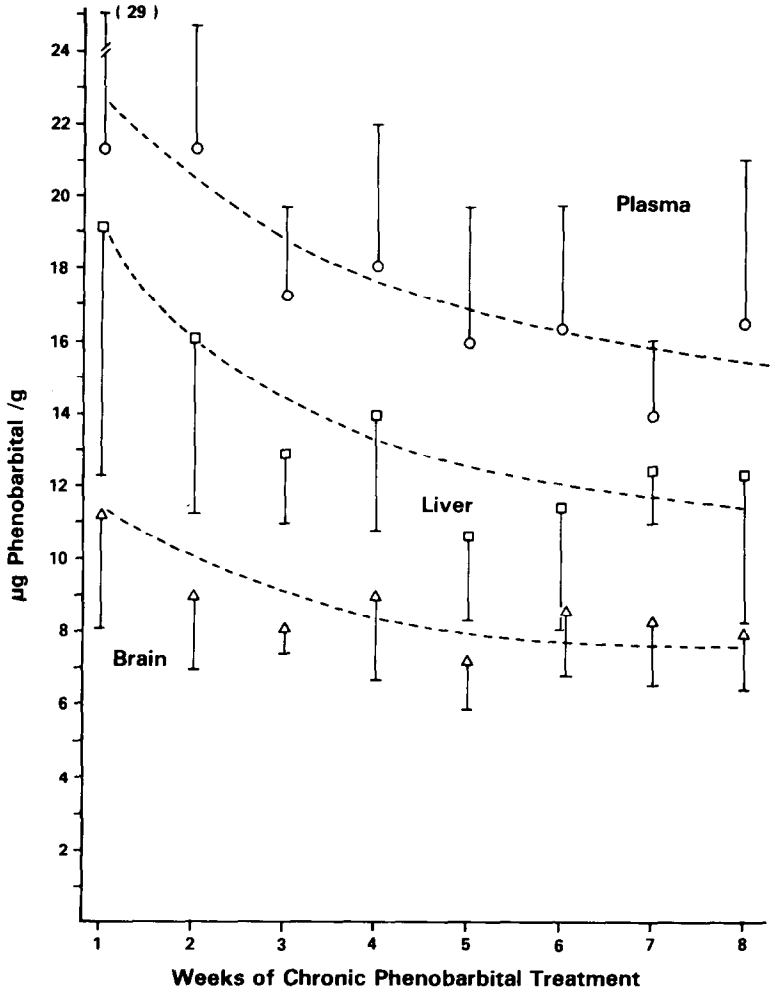

Fig. 2. Change in plasma and tissue phenobarbital concentrations with length of chronic phenobarbital treatment. Rats were treated as described in Fig. 1 and decapitated 12 hr after the final phenobarbital dose. Tissue phenobarbital was measured by gas chromatography as described in Methods, and plasma phenobarbital was assayed by EMIT. Data are represented as mean ± S.D. of eight or more observations.

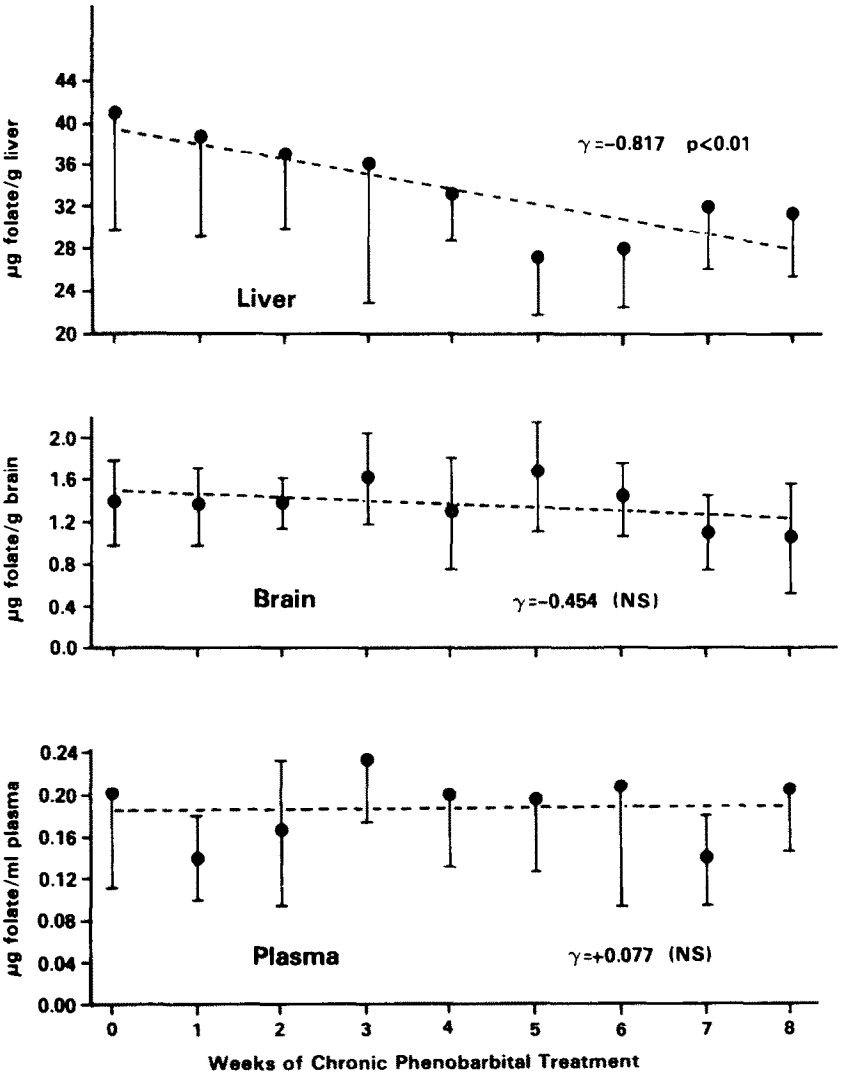

Fig. 3. Change in folate concentration in liver, brain and plasma with length of chronic phenobarbital treatment. Rats were treated as described in Fig. 1 and decapitated 12 hr after the final treatment. Folates were determined by microbiological assay using *Lactobacillus casei* (ATCC 7469) as previously described [17]. Data are represented as mean ± S.D. of eight or more observations. Key: γ = correlation coefficient; and NS = not significant to 95% confidence.

statistically significant) decrease in the brain but had no effect on plasma folate throughout the 8 weeks of treatment (Fig. 3).

Chronic treatment with phenobarbital exhibited no significant effects *in vivo* on any of the four enzymes examined in the brain although MHMT showed a tendency toward increased activity with treatment and SHMT showed a tendency toward decreased activity (Fig. 4). In the liver, on the other hand, MTR activity showed a highly significant positive correlation with length of treatment, especially evident when the control is dropped as the zero time point (Fig. 4E). Indeed, the significant ($P < 0.02$) drop in MTR activity after 1 week of treatment indicates an initial inhibitory (repression) effect, followed by the increase in activity with continued treatment. Phenobarbital in high concentrations seemed to have a slight but significant stimulating effect on brain MTR activity *in vitro* (Table 1). However, it is very unlikely that such a direct effect is responsible for the *in vivo* effect since only a small effect is observed *in vitro* using a phenobarbital concentration approximately twenty times that generated *in vivo*.

Liver SHMT activity showed a significant negative correlation with length of phenobarbital treatment (Fig. 4G). While the same trend was exhibited by the brain, both *in vivo* (Fig. 4F) and *in vitro* (Table 1), neither of these effects was significant.

Phenobarbital (1.0 mM) exhibited a slight but significant inhibition of brain MAT activity *in vitro* (Table 1). However, no effect of phenobarbital treatment was observed for this enzyme in brain *in vivo* (Fig. 4A). Liver MAT activity was not assayed because of the existence of several isozymes with different K_m values for methionine in this tissue [29].

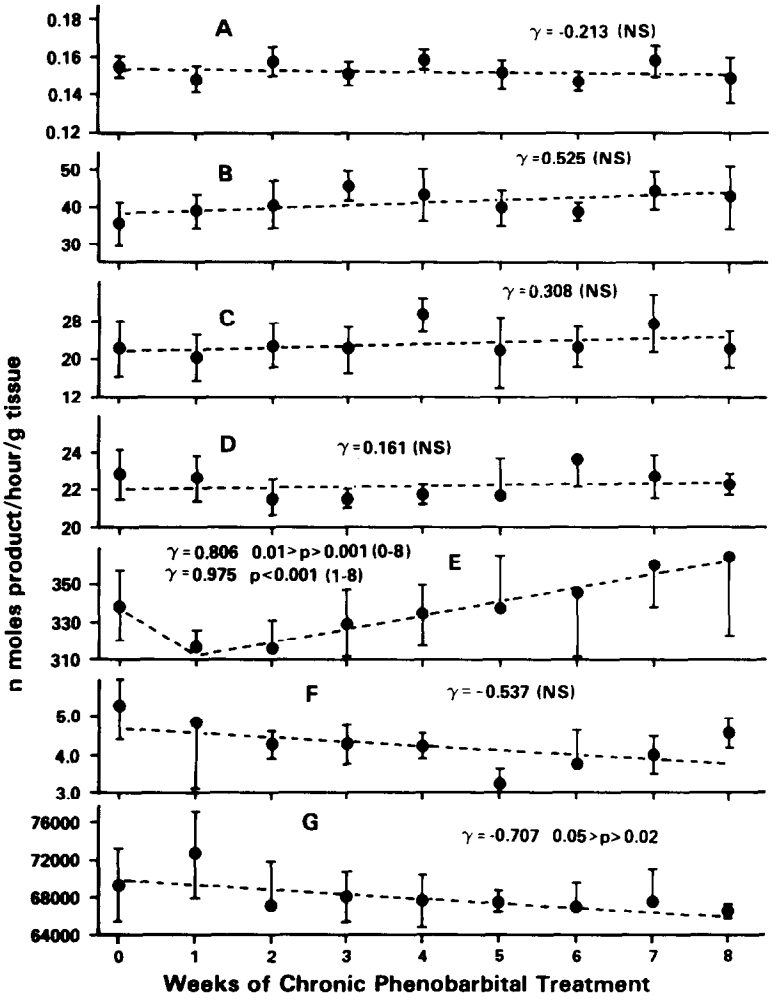

Fig. 4. Change in the activities of four one-carbon enzymes with length of chronic phenobarbital treatment. Rats were treated, tissues were prepared, and enzymes were assayed as described in Methods. (A) methionine:ATP adenosyltransferase activity in brain; (B) 5-methyltetrahydrofolate:homocysteine methyltransferase (MHMT) activity in brain; (C) MHMT activity in liver; (D) 5,10-methylenetetrahydrofolate reductase (MTR) activity in brain; (E) MTR activity in liver; (F) serine hydroxymethyltransferase (SHMT) activity in brain; (G) SHMT activity in liver. Data are represented as mean ± S.D. of five or more observations. Key: γ = correlation coefficient; and NS = not significant to 95% confidence.

Table 1. Effect of 1.0 mM phenobarbital on the activities of four one-carbon enzymes *in vitro**

	Enzyme activity (nmoles/hr/g brain)		
	Control	Phenobarbital	P
SHMT	7.19 ± 0.95 (4)	6.28 ± 0.59 (4)	> 0.1
MTR	16.8 ± 0.5 (4)	18.0 ± 0.6 (4)	< 0.02
MHMT	45.4 ± 1.0 (3)	46.1 ± 1.9 (3)	> 0.1
MAT	0.157 ± 0.008 (5)	0.140 ± 0.009 (5)	< 0.02

* The enzymes serine hydroxymethyltransferase (SHMT), 5,10-methylenetetrahydrofolate reductase (MTR), 5-methyltetrahydrofolate:homocysteine methyltransferase (MHMT) and methionine:ATP adenosyltransferase (MAT) from rat brain homogenates were assayed in the absence and presence of 1.0 mM phenobarbital as described in Methods. The number of independent assays is given in parentheses.

This would make interpretation of data, such as that generated here, impossible.

DISCUSSION

Even though the chronic oral treatment of rats with phenobarbital at 50 mg/kg every 12 hr maintained the plasma concentration at levels considered to be in the low therapeutic range for humans and resulted in no apparent toxic effects in the animals, we found a significant reduction of the folate concentration in the livers of treated animals. This depletion, very probably of folates stored in the liver, was correlated significantly with the length of treatment with phenobarbital. However, the depletion of liver folates caused by phenobarbital is not nearly as extensive as the depletion caused by phenytoin under similar circumstances [18]. In addition, phenobarbital treatment showed a trend toward causing folate depletion in brain but this trend did not reach statisti-

cal significance. In contrast, phenytoin has been shown to have significant effects on brain folate concentration after 8 weeks [18]. It is possible that higher doses or the extension of chronic treatment with phenobarbital might cause a significant reduction of folate concentration in this organ.

Smith and Racusen [30] found that chronic treatment with phenobarbital caused a significant reduction in plasma folate concentration in rats, an observation we were not able to confirm. There are several differences between our study and that of Smith and Racusen [30] which could account for the different results. They administered an aqueous solution of phenobarbital at 32 mg/kg intraperitoneally once per day for 10 weeks attaining plasma levels of approximately 30 μg/ml 4–5 hr after injection. Our mode of administration, 50 mg/kg in propylene glycol orally every 12 hr, maintained phenobarbital concentrations in a range (15–25 μg/ml 12 hr post-administration) considered to be low therapeutic in humans. Unfortunately, these investigators did not measure tissue folates in their animals. It would have been interesting to see if liver and brain folate concentrations fell prior to the observed decrease in plasma folate, since phenytoin [18] and phenobarbital apparently cause a decrease in liver folate first.

The initial decrease in MTR activity in the liver followed by a gradual increase in activity with continued phenobarbital treatment indicates that we are observing two different effects. It is interesting that neither of these effects occurred in the brain. Since there is no evidence to indicate that the liver and brain enzymes are different, their difference in response to chronic phenobarbital treatment indicates that differences that exist between the brain and the liver in response to phenobarbital treatment cause different effects on the activity of the enzymes. It is possible that direct effects by phenobarbital on folate transport or metabolism translate into effects on the enzyme. It is also possible that direct effects on the enzymes could translate into the observed changes in folate concentration. On the other hand, both of these effects might be indirect results of other effects of phenobarbital. For example, phenobarbital has been shown to increase fluidity in rat liver microsomal membranes by increasing the methylation of phosphatidylethanolamine to phosphatidyl-*N*-methyl-ethanolamine [31], to decrease the concentration of *S*-adenosylmethionine, the universal methyl donor, in mouse brain [32], to increase the concentration of *S*-adenosylhomocysteine, the product of methylation reactions, in mouse brain [32], and to decrease the *in vivo* activity of mouse brain protein carboxymethyltransferase after chronic treatment [32]. These observations indicate that phenobarbital has a significant effect on methylation processes. Since the folates are involved as carriers and since MTR is the enzyme that synthesizes methyl groups *de novo*, it is not surprising that these factors may be affected by chronic phenobarbital treatment. Indeed, *S*- adenosylmethionine is a potent inhibitor of MTR activity and *S*-adenosylhomocysteine partially reverses this inhibition [24]. It has also been shown that the brain and liver are quite different in their abilities to generate *S*-adenosylmethionine [33]. The liver is capable of producing large increases of *S*-adenosylmethionine in response to increased methionine levels but the brain is not [33]. This is due to the presence of a high K_m, high V_{max} isozyme of methionine adenosyltransferase in the liver while the brain exhibits only a low K_m, low V_{max} activity which is saturated at normal concentrations of methionine [33]. It can be seen then that the differences in the synthesis of *S*-adenosylmethionine between brain and liver combined with the effects of *S*-adenosylmethionine and *S*-adenosylhomocysteine on MTR activity and the effects of phenobarbital on *S*-adenosylmethionine and *S*-adenosylhomocysteine concentrations could cause the observed effects on MTR activity in rat liver. It is also possible that the observed depletion of liver folate might be a result of these effects.

To explain the negative correlation between length of phenobarbital treatment and the activity of serine hydroxymethyltransferase (SHMT) in liver (with the same trend, although not statistically significant, in brain), it is tempting to speculate that the increased levels of *S*-adenosylhomocysteine, the product of methylation reactions, caused the observed increase in MTR activity which then lead to increased levels of 5-methyltetrahydrofolate, which, in turn, inhibited the activity of SHMT. SHMT is indeed inhibited by 5-methyltetrahydrofolate, but the inhibition is competitive with the substrate tetrahydrofolate [34] which, in our assay, is added to a concentration (5×10^{-4}M) that would overwhelm any possible contribution by endogenous 5-methyltetrahydrofolate to the mileau of the assay reaction. Another observation, that the activity of SHMT in mouse brain declines with age in the young animal [35], may mislead one to believe that we are seeing this effect independent of the phenobarbital treatment. However, all of our animals were the same age at the time of sacrifice. Therefore, it is possible that younger animals (the longer the treatment, the younger the animal when treatment was begun) are more sensitive to phenobarbital treatment, especially with regard to SHMT activity, but an independent effect is not likely.

In comparing the effect of phenobarbital on folate metabolism to that of phenytoin, it is obvious that phenytoin is more potent in depleting folate than phenobarbital, but it is also obvious that phenobarbital has a significant effect on folate-dependent one-carbon metabolism. In this respect, the data presented here tend to support the hypothesis that phenobarbital interferes (probably indirectly) with folate metabolism to change the steady state leading to the decline in liver folate concentration.

Acknowledgements—The authors appreciate the excellent technical assistance of Mr. Jack Hazel and Mr. James Houston, and the invaluable aid of Medical Media and Mrs. Nan Simpson in the preparation of this manuscript. This study was supported by the Medical Research Service of the Veterans Administration.

REFERENCES

1. E. H. Reynolds, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and R. P. Schmidt), p. 247. Raven Press, New York (1972).

2. F. Mauguiere, in *Folic Acid in Neurology, Psychiatry and Internal Medicine* (Eds. M. I. Botez and E. H. Reynolds), p. 317. Raven Press, New York (1979).
3. D. B. Smith and G. F. Carl, *Neurology* **32**, 965 (1982).
4. E. Mannheimer, F. Pakesch, E. E. Reimer and H. Vetter, *Medsche. Klin.* **47**, 1397 (1952).
5. J. Badenoch, *Proc. R. Soc. Med.* **47**, 426 (1954).
6. R. W. Strachan and J. G. Henderson, *Q. Jl. Med.* **36**, 189 (1967).
7. J. Sapira, S. Tullis and R. Mullaly, *Sth. med. J., Nashville* **68**, 776 (1975).
8. S. D. Shorvon, M. W. P. Carney, I. Chanarin and E. H. Reynolds, *Br. med. J.* **281**, 1 (1980).
9. J. H. Pincus, E. H. Reynolds and G. H. Glazer, *J. Am. med. Ass.* **221**, 496 (1972).
10. E. Melamed, A. Reches and C. Hershko, *J. neurol. Sci.* **25**, 93 (1975).
11. M. Manzoor and J. Runcie, *Br. med. J.* **1**, 1176 (1976).
12. M. I. Botez, M. Cadotte, R. Beaulieu, L. P. Pichette and C. Pison, *Can. med. Ass. J.* **115**, 217 (1976).
13. M. R. Trimble, J. A. Corbett and D. Donaldson, *J. Neurol. Neurosurg. Psychiat.* **43**, 1030 (1980).
14. A. Coppen and M. T. Abou-Saleh, *Br. J. Psychiat.* **141**, 87 (1982).
15. E. H. Reynolds, in *Advances in Epileptology: XIIth Epilepsy International Symposium* (Eds. M. Dam, L. Gram and J. K. Penry), p. 621. Raven Press, New York (1981).
16. J. Kogeorgos, P. Fonagy and D. F. Scott, *Br. J. Psychiat.* **140**, 236 (1982).
17. G. F. Carl and D. B. Smith, *Epilepsia* **24**, 494 (1983).
18. G. F. Carl and D. B. Smith, *J. Nutr.* **113**, 2368 (1983).
19. I. H. Rosenberg, J. Selhub and G. J. Dhar, in *Folic Acid in Neurology, Psychiatry, and Internal Medicine* (Eds. M. I. Botez and E. H. Reynolds), p. 95. Raven Press, New York (1979).
20. I. Chanarin, in *Folic Acid in Neurology, Psychiatry, and Internal Medicine* (Eds. M. I. Botez and E. H. Reynolds), p. 75. Raven Press, New York (1979).
21. D. Kelly, D. Weir, B. Reed and J. Scott, *J. clin. Invest.* **64**, 1089 (1979).
22. D. B. Smith and G. F. Carl, *Neurology* **32**, 965 (1982).
23. R. T. Taylor and H. Weissbach, *Analyt. Biochem.* **13**, 80 (1965).
24. C. Kutzbach and E. L. R. Stokstad, *Biochim. biophys. Acta* **139**, 217 (1967).
25. B. R. Clark, H. Ashe, R. M. Halpern and R. A. Smith, *Analyt. Biochem.* **61**, 243 (1974).
26. S. Matthysse, R. J. Baldessarini and M. Vogt, *Analyt. Biochem.* **48**, 410 (1972).
27. P. H. Yu, *Analyt. Biochem.* **86**, 498 (1978).
28. T. C. Butler, in *Antiepileptic Drugs: Quantitative Analysis and Interpretation* (Eds. C. E. Pippenger, J. K. Penry and H. Kutt), p. 265. Raven Press, New York (1978).
29. M. C. Liau, G. W. Lin and R. B. Hurlbert, *Cancer Res.* **37**, 427 (1977).
30. D. B. Smith and L. C. Racusen, *Archs. Neurol., Chicago* **28**, 18 (1973).
31. B. N. Rama Sastry, C. N. Statham, R. G. Meeks and J. Axelrod, *Pharmacology* **23**, 211 (1981).
32. R. A. Schatz, T. E. Wilens, S. B. Tatter and O. Z. Sellinger, in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), p. 675. Macmillan, New York (1982).
33. G. F. Carl, F. C. Benesh and J. L. Hudson, *Biol. Psychiat.* **13**, 661 (1978).
34. L. Schirch and M. Ropp, *Biochemistry* **6**, 253 (1967).
35. W. F. Bridgers, *J. Neurochem.* **15**, 1325 (1968).