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CHANGES IN HEPATIC FOLYLPOLYGLUTAMATE PATTERN IN PHENOBARBITONE-TREATED RATS

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Abstract—Folate deficiency is a common unpleasant secondary effect of anticonvulsant therapy. In order to contribute to the knowledge of biochemical mechanisms leading to this condition, the effects of two i.p. high doses of phenobarbitone administered to the rat (acute treatment) on the distribution of hepatic folate derivatives have been studied. A significant decrease of unsubstituted tetrahydro- and dihydropteroylpentaglutamates and 5,10-methylenetetrahydropentaglutamates was observed. The hypothesis that a lower availability of NADPH, which is utilized for hydroxylation reactions in phenobarbitone metabolism, may limit folate reduction is proposed.

Folylpolyglutamates are the preferred substrates for the enzymes of one-carbon metabolism and, as recently pointed out, also inhibitors of a number of folate-dependent enzymes for which they are not substrates [1]. The polyglutamate chain length depends on the species and on the tissues. Animal tissues may contain up to eight γ-glutamyl residues; penta- and hexaglutamates are the predominating forms in mammalian liver [2]. There is some evidence suggesting that one-carbon flux through various folate-dependent reactions may be regulated by varying the glutamate chain length [3]; in fact the enzymes that commit one-carbon units to the various pathways appear to show different specificities for chain length [4]. Therefore, it is possible that folate deficiency symptoms appear as a consequence of the impossibility of synthesizing folate derivatives with the correct number of glutamyl residues.

It has been known for many years that folate deficiency is a common finding in chronic anticonvulsant therapy, and can lead to megaloblastic anaemia [5]; in less severe cases, vitamin depletion has recently been associated with psychiatric, neurological and intellectual deficiencies as well [6, 7]. For this reason Reynolds [8] has ascribed the psychiatric morbidity often observed in epileptic anticonvulsant-treated population to the drug-induced folate depletion. Up to now the mechanism by which anticonvulsants can lead to the secondary vitamin deficiency is still unknown; several hypotheses have suggested a possible interference of the drugs on the folate intestinal absorption [9], on folate-dependent reactions [10] and on folate catabolism [11]; these possibilities have evidence both for and against them. More recently, studies of Carl and co-workers [12–14] on the effect of various chronic anticonvulsant treatments on folate-dependent enzymes in rat liver and brain are in favour of the hypothesis that the drugs lead to folate depletion by interfering with folate metabolism.

In previous works [15, 16] we have observed that, in the rat, "acute" i.p. administration of phenobarbitone causes a decrease in hepatic longer chain pteroylpolyglutamates. In the present study under the same experimental conditions, we have expanded our investigations to the examination of the changes of the individual folylpolyglutamate homologs by different extraction and separation procedures.

MATERIALS AND METHODS

Materials. Synthetic $PteGlu_n$† ($n = 1$–7) were obtained from Dr B. Schirks Laboratories (Jona, Switzerland). Bio-Gel P2 (200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA). Chemicals and solvents were obtained from the Sigma Chemical Co. (Poole, U.K.) and Merck (Darmstadt, F.R.G.).

Animals and tissue preparation. Male albino rats of Wistar strain, weighing 150–200 g were housed for a month in an air-conditioned, windowless room with light from 9.00 p.m. to 9.00 a.m. and maintained on a purified diet containing pteroylmonoglutamate (15 mg/kg of diet) supplied from 9.00 a.m. to 5.00 p.m. with water *ad lib*. [17]. After this period the animals weighing 250–300 g were divided into two groups: one group of 12 rats was i.p. injected with phenobarbitone (20 mg/mL in 0.9% NaCl) once daily for two days at a dose of 80 mg/kg body wt. Another group of 12 animals was injected with appropriate amounts of 0.9% NaCl solution, adjusted at the same pH as the phenobarbitone solution (control). All the rats were killed 24 hr after their last injection and livers were promptly removed and rinsed in degassed, argon-saturated 0.111 N HCl and blotted on filter paper. Livers weighing approximately 12 g were individually homogenized in 9 vol. (w/v) of the degassed, argon-saturated 0.111 N HCl.

Differential cleavage procedure. The homogenates were pooled two by two and subjected to the differential cleavage procedure as described by Eto and Krumdieck [18]. This procedure permits the

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† Abbreviations used: PteGlu, pteroylglutamate; pABGlu, p-aminobenzoylglutamate; AzoGlu, azo-*p*-aminobenzoylglutamate.

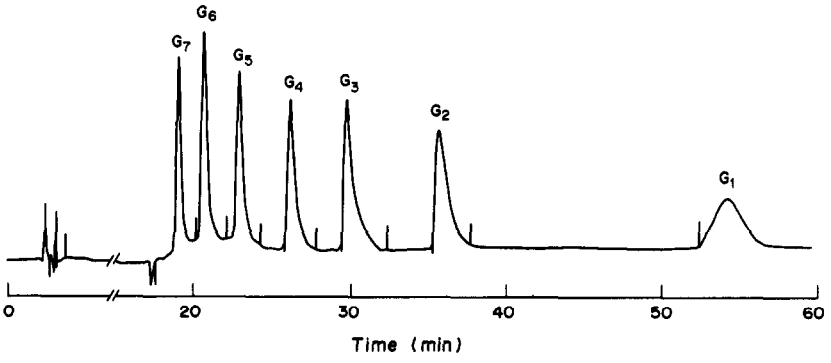

Fig. 1. Separation of synthetic $AzoGlu_n$ by reversed-phase HPLC on Spherisorb S5 ODS-1 (0.46 × 25 cm). A.U. = 2×10^{-2}; $\lambda = 556$ nm. The details of the HPLC conditions are given under Materials and Methods. The symbols G_1, G_2, G_3, etc. indicate the number of glutamyl residues.

quantitation of three different pools of 1-C substituted and unsubstituted folates:
pool 1 is made up of 5,10-methylenetetrahydrofolates, unsubstituted tetrahydro- and dihydrofolates;
pool 2 is made up of 5,10-methyltetrahydrofolates;
pool 3 is made up of 5,10-methenyl-, 10-formyl-, 5-formyl- and 5-formiminotetrahydrofolates.

Selective cleavage of C9–N10 bond of the folates of pool 1 (route I), pools 1+2 (route II), pools 1+2+3 (route III) was performed and the resulting $pABGlu_n$ were subjected to the Bratton-Marshall reaction by which they were converted to the corresponding $AzoGlu_n$, purified on Bio-Gel P2 columns (1.5 × 14 cm) and concentrated by lyophilization [19].

Synthetic pteroylglutamates were subjected to the reductive cleavage as described by Brody *et al.* [20] which converted them into the corresponding $pABGlu_n$. $AzoGlu_n$ derivatives were prepared from the latter compounds by the Bratton-Marshall procedure, purified on Bio-Gel P2 columns (0.7 × 4 cm) and concentrated by lyophilization.

Analytical determination. The lyophilized $AzoGlu_n$, both from standard and hepatic folates, were dissolved in suitable volumes of mobile phase and analysed by reversed-phase HPLC. AzoGlu separations were performed on a 0.46 × 25 cm column packed with 5 μm Spherisorb S5 ODS-1 with a guard column containing the same material (Phase Separation Ltd), according to Eto and Krumdieck [19] with the only modification of solvent B: 1-propanol 14%: acetic acid 7.5%: water 78.5%.

The HPLC system was a Waters Data Module M730, Model 721 programmable system controller, equipped with Lambda Max Model 481 LC variable wavelength spectrophotometer. The effluent was monitored at 556 nm.

Calculations and statistics. Glutamate chain length of $AzoGlu_n$ derivatives of the hepatic folates was determined by comparing the retention times corresponding to each peak with those of the authentic synthetic markers whose elution pattern is shown in Fig. 1. The amount of each polyglutamate of routes I, II and III was evaluated by automatic integration of the area under each peak and external standard quantitation. The absolute amounts of each polyglutamate of pools 1, 2 and 3 were calculated as follows:
pool 1 = route I;
pool 2 = route II − route I;
pool 3 = route III − route II.

Statistical comparisons between groups were carried out using the Student's *t*-test. A level of significance of $P < 0.05$ was chosen.

RESULTS

Figure 2 shows typical elution patterns of the $AzoGlu_n$ derivatives of the hepatic folates of control and phenobarbitone-treated rats (route III); detected derivatives are hexa-, penta- and tetraglutamates in both groups of animals; heptaglutamates are found in traces. Similar elution profiles are obtained from route I and II in control and treated animals.

Table 1 shows pteroylpolyglutamate distribution in pools 1, 2 and 3 in liver of control and treated rats. Pentaglutamates are the major component of every pool followed by hexa- and tetraglutamates in both groups of animals. The total amount of hepatic folates compares well with our previous results using microbiological assay [15]. In control animals different percentages of penta- and hexaglutamates found in all pools by us in comparison with Eto and Krumdieck [19] are probably due to dietary contribution and/or to rat strain employed.

Phenobarbitone treatment causes a significant decrease ($P < 0.001$) in pentaglutamate content of pool 1, which groups unsubstituted tetra- and dihydrofolates and 5,10-methylenetetrahydrofolates. Moreover, a slight but not statistically significant decrease appears in 5-methyltetrahydropentaglutamates (pool 2). It must be remarked that greater biological variability has been found in pool 2 and 3 derivatives and that their differential assays are subjected to a double error of determination.

Table 2 shows total pteroylpolyglutamate content of each pool, that is hexa- +penta- +tetraglutamates, expressed both as absolute values and as percentages of the sum of the three pools.

Total folate derivative amount (pool 1+

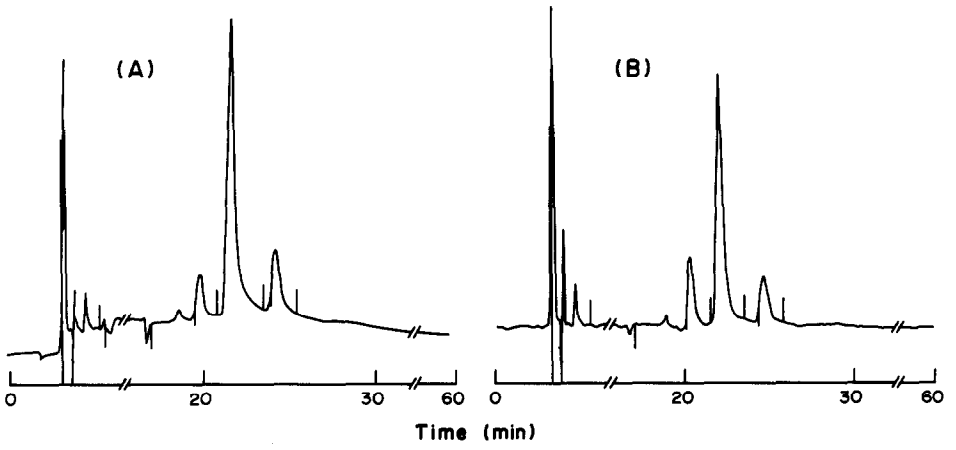

Fig. 2. Separation of AzoGlu$_n$ derived from rat liver folates (route III) by reversed-phase HPLC. (A) control rats. (B) phenobarbitone-treated rats.

Table 1. Distribution of liver folates of control and phenobarbitone-treated rats

	Polyglutamate chain length	Control (nmol/g liver)	Treated (nmol/g liver)
Pool 1	G_4	1.62 ± 0.17	0.73 ± 0.40
(methylene+	G_5	8.41 ± 0.21	4.85 ± 0.44†
unsubstituted)	G_6	2.04 ± 0.19	2.12 ± 0.17
Pool 2	G_4	0.68 ± 0.33	0.47 ± 0.19
(methyl)	G_5	3.21 ± 0.67	2.28 ± 0.68
	G_6	1.75 ± 0.42	2.08 ± 0.46
Pool 3	G_4	1.47 ± 0.42	0.68 ± 0.20
(methenyl+formyl+	G_5	3.97 ± 0.87	4.41 ± 0.68
formimino)	G_6	1.40 ± 0.38	1.83 ± 0.61
Total	G_4	3.77 ± 0.31	1.88 ± 0.26
	G_5	15.59 ± 0.58	11.54 ± 0.60*
	G_6	5.19 ± 0.33	6.03 ± 0.41

The symbols G_4, G_5 and G_6 indicate the number of glutamyl residues. Each value represents the mean ± SE of results obtained from 12 animals pooled two by two. Each measurement was performed in triplicate. Total represents the sum of folates carrying the same number of glutamyl residues.

* Significantly different from control, $P < 0.01$, by Student's *t*-test.

† Significantly different from control, $P < 0.001$, by Student's *t*-test.

pool 2 + pool 3) appears 20% lower in treated animals, which is due almost entirely to pool 1 pentaglutamate decrease.

Due to the observed changes, the per cent distribution of various derivatives grouped per pool is quite different in treated rats as compared with controls.

DISCUSSION

Acute phenobarbitone treatment significantly lowers liver folates as we have reported in previous works [15, 16], where we ascribed the decrease to "longer chain" derivatives. In this work the different extraction and the very sensitive HPLC assay procedure give us more detailed results. It appears now that phenobarbitone treatment causes a decrease of primarily pentaglutamates among longer chain derivatives; in particular the decrease involves reduced unsubstituted and methylene substituted derivatives; methyl substituted derivatives show a tendency, albeit not significant, to decrease. On the other hand, hexaglutamates are at the same level in livers of both groups. It is tempting to speculate that the latter derivatives have not been touched by the drug effect as they represent "old" folate [21]; we have in fact performed a very short time treatment, which has effected only the pentaglutamates, the predominant forms in rat liver, synthesized at a relatively rapid rate [22]. As a consequence it should be remarked that the proportion of hexa- and pentaglutamates has changed.

Table 2. Reduced, one-carbon substituted liver folylpolyglutamates of control and phenobarbitone-treated rats

	Control		Treated	
	nmol/g liver*	Percentage of total	nmol/g liver*	Percentage of total
Pool 1 (methylene+ unsubstituted)	12.07 ± 0.57	49.16	7.70 ± 1.01	39.59
Pool 2 (methyl)	5.64 ± 1.42	22.97	4.83 ± 1.33	24.83
Pool 3 (methenyl+formyl+ formimino)	6.84 ± 1.67	27.86	6.92 ± 1.49	35.58
Total (pools 1+2+3)	24.55 ± 1.22	100	19.45 ± 1.28	100

* Values represent the sum of various length folylpolyglutamates of each pool.

Carl *et al.* [23] performed chronic treatment of rats with primidone, which is rapidly converted to phenobarbitone and showed a 50% decrease of total pteroylpentaglutamates in liver within the first week of treatment. In spite of the different experimental conditions the results appear comparable; it is quite likely that we are observing the initial effect of phenobarbitone on pool 1 and 2 pentaglutamates.

It is our opinion that drug interference on polyglutamate metabolism could be at the level of reduction processes rather than polyglutamilation reaction. Phenobarbitone by inducing the cytochrome P450 system increases NADPH utilization for hydroxylation reactions and could diminish its availability for enzymes such as methylenetetrahydrofolate reductase (EC 1.1.1.68) and dihydrofolate reductase (EC 1.5.1.3); these enzymes, together with serine hydroxymethyltransferase (EC 2.1.2.1), are largely responsible for pool 1 and 2 derivative levels. The hypothesis that phenobarbitone could in some way enhance the conjugase activity is in our opinion unlikely because conjugase being unspecific would hydrolyse all polyglutamate forms and not only pentaglutamates.

The hepatic folate picture we have found is probably strongly dependent on the doses and on the time of treatment; Carl and Smith [13] have shown a progressive decrease in hepatic folate concentration significantly correlated with the length of phenobarbitone treatment, and an interesting biphasic behaviour of hepatic methylenetetrahydrofolate reductase. The activity of this enzyme, in fact, has a drop in the very initial phase of treatment and then increases with significant correlation to the length of treatment. In conclusion, acute phenobarbitone treatment seems to interfere with folate metabolism, particularly with the synthesis of pool 1 pentaglutamate derivatives and, as a consequence, with folate-dependent metabolic processes, thus leading to secondary vitamin deficiency.

Studies are being carried out on pteroylpolyglutamate distribution in various length phenobarbitone treatments.

REFERENCES

1. Matthews RG, Ghose C, Green JM, Matthews KD and Dunlap RB, Folylpolyglutamates as substrates and inhibitors of folate-dependent enzymes. *Adv Enzyme Regul* **26**: 157–171, 1986.
2. Scrimgeour KG, Biosynthesis of polyglutamates of folates. *Biochem Cell Biol* **64**: 668–674, 1986.
3. Foo SK and Shane B, Regulation of folylpoly-γ-glutamate synthesis in mammalian cells. *J Biol Chem* **257**: 13587–13592, 1982.
4. Ross J, Green J, Baugh CM, MacKenzie RE and Matthews RG, Studies on the polyglutamate specificity of methylene-tetrahydrofolate dehydrogenase from pig liver. *Biochemistry* **23**: 1796–1801, 1984.
5. Badenoch J, The use of labeled vitamin B12 and gastric biopsy in the investigation of anemia. *Proc R Soc Med* **47**: 426–427, 1954.
6. Shorvon SD, Carney MWP, Chanarin I and Reynolds EH, The neuropsychiatry of megaloblastic anaemia. *Br Med J* **281**: 1036–1038, 1980.
7. Trimble MR, Corbett JA and Donaldson D, Folic acid and mental symptoms in children with epilepsy. *J Neurol Neurosurg Psychiatry* **43**: 1030–1034, 1980.
8. Reynolds EH, Anticonvulsant drugs, folate metabolism and mental symptoms. In: *Advances in Epileptology. XIIth Epilepsy International Symposium, Copenhagen, Denmark, 1980* (Eds. Dam M, Gram L and Penry JK), pp. 621–625. Raven Press, New York, 1981.
9. Rosenberg IH, Selhub J and Dhar GJ, Absorption and malabsorption of folates. In: *Folic Acid in Neurology, Psychiatry and Internal Medicine* (Eds. Botez MI and Reynolds EH), pp. 95–111. Raven Press, New York, 1979.
10. Kelly D, Weir D, Reed B and Scott J, Effect of anticonvulsant drugs on the rate of folate catabolism in mice. *J Clin Invest* **64**: 1089–1091, 1979.
11. Chanarin I, Effects of anticonvulsant drugs. In: *Folic Acid in Neurology, Psychiatry and Internal Medicine* (Eds. Botez MI and Reynolds EH), pp. 75–80. Raven Press, New York, 1979.
12. Carl GF and Smith DB, The effect of chronic phenytoin treatment on tissue folate concentrations and on the activities of the methyl synthetic enzymes in the rat. *J Nutr* **113**: 2368–2374, 1983.
13. Carl GF and Smith DB, Effect of chronic phenobarbital treatment on folates and one-carbon enzymes in the rat. *Biochem Pharmacol* **33**: 3457–3463, 1984.
14. Carl GF, Gill MW and Schatz RA, Effect of chronic primidone treatment on folate-dependent one-carbon metabolism in the rat. *Biochem Pharmacol* **36**: 2139–2144, 1987.
15. Formiggini G, Bovina C, Marchi-Marchetti M and Marchetti M, Pteroylpolyglutamates in liver of phenobarbitone-treated rats. *Int J Vit Nutr Res* **53**: 390–393, 1983.

16. Formiggini G, Bovina C, Marchi-Marchetti M and Marchetti M, Effect of phenobarbitone on folic acid metabolism in the rat. *Pharmacol Res Commun* **16**: 467–478, 1984.
17. Potter VE, Baril EF, Watanabe M and Whittle ED, Systematic oscillations in metabolic functions in liver from rats adapted to controlled feeding schedules. *Fed Proc* **27**: 1238–1245, 1968.
18. Eto I and Krumdieck CL, Determination of three different pools of reduced one-carbon-substituted folates. II. Quantitation and chain-length determination of the pteroylpolyglutamates of rat liver. *Anal Biochem* **115**: 138–146, 1981.
19. Eto I and Krumdieck CL, Determination of three different pools of reduced one-carbon substituted folates. III. Reversed-phase high-performance liquid chromatography of the azo dye derivatives of *p*-aminobenzoylpoly-γ-glutamates and its application to the study of unlabeled endogenous pteroylpolyglutamates of rat liver. *Anal Biochem* **120**: 323–329, 1982.
20. Brody T, Shane B and Stokstad ELR, Separation and identification of pteroylpolyglutamates by polyacrylamide gel chromatography. *Anal Biochem* **92**: 501–509, 1979.
21. Leslie GI and Baugh CM, The uptake of pteroyl ^{14}C glutamic acid into rat liver and its incorporation into the natural poly-γ-glutamates of that organ. *Biochemistry* **13**: 4957–4961, 1974.
22. Shane B, High performance liquid chromatography of folates: identification of poly-γ-glutamate chain length of labeled and unlabeled folates. *Am J Clin Nutr* **35**: 599–608, 1982.
23. Carl GF, Eto I and Krumdieck CL, Chronic treatment of rats with primidone causes depletion of pteroylpentaglutamates in liver. *J Nutr* **117**: 970–975, 1987.