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EFFECTS OF PHENOBARBITONE AND PHENYTOIN ON FOLATE CATABOLISM IN THE RAT

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Abstract—The effects of phenobarbitone and phenytoin on the catabolism of oral [2-^{14}C] and [3′,5′,7,9-^{3}H] folic acid were investigated. Normal rats were found to excrete an excess of ^{3}H-labelled compounds into the urine and ^{14}C-labelled compounds into the faeces. Phenytoin abolished this urinary ^{3}H imbalance and also delayed and prolonged the overall excretion of radioactive material. Phenobarbitone appeared to increase the amounts of urinary scission products in the first 24 hr but over the 0–72 hr period both anticonvulsants decreased folate polyglutamate catabolism.

As the anticonvulsants used in these experiments decreased folate catabolism in the rat it is unlikely that the megaloblastic anaemia caused by chronic anticonvulsant therapy is due to induction of the enzymes responsible for folate breakdown *in vivo*.

Folate metabolism in man and the rat has been extensively studied and considerable catabolism has been shown to occur [1–3]. This catabolism is subject to alteration by several factors including malignancy [4]. Pheasant *et al.* [3] proposed a scheme to describe the physiological distribution of oral folic acid. Reduced folates are absorbed to form part of a folate monoglutamate pool. They are then either excreted in the bile to undergo enterohepatic circulation or are taken into the tissues where they are converted to polyglutamate derivatives. These latter compounds are eventually degraded to *p*-aminobenzoyl-L-glutamate (*p*-ABG) and various pteridines via scission of the C9-N10 bond [5] and excreted into the urine after acetylation to *p*-acetamidobenzoyl-L-glutamate (*p*-AcBG). Folates excreted into the bile may also undergo C9–N10 scission. The resulting pteridines are poorly reabsorbed from the gastro-intestinal tract and are excreted mainly in the faeces, although some may be further metabolised by the gut microflora [6]. The glutamate moiety is removed forming *p*-aminobenzoic acid (*p*-ABA) which is acetylated during reabsorption and then excreted in the urine as *p*-acetamidobenzoic acid (*p*-AcBA).

The actual mechanisms of scission are not known. Oxidative cleavage of the C9–N10 bond may be achieved by a chemical process or via catalysis by the microsomal mixed function oxidases.

Phenobarbitone and phenytoin are known to induce these enzymes in the rat [7], and are also widely used as anticonvulsant drugs to treat epilepsy [8]. Reynolds [9] noted that the drug-induced megaloblastic anaemia occurring in epileptics is usually associated with phenytoin therapy, either alone or in combination with phenobarbitone. In all cases this anaemia responds well to folic acid therapy. It was therefore decided to further investigate the effects of phenytoin and phenobarbitone on folate catabolism in the rat.

MATERIALS AND METHODS

Three groups of healthy male WAB/Not rats (180–280 g body weight) supplied by Dr M. Pymm (University of Nottingham) were used.

(A) Fifteen rats were dosed orally with a mixture of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight).

(B) Fourteen rats were dosed intraperitoneally with phenobarbitone sodium (80 mg/kg body weight) at 24 hr-intervals for 3 days. On the 4th day they received oral radiolabelled folic acid as per group A.

(C) Fifteen rats were dosed intraperitoneally with phenytoin (100 mg/kg body weight) for 10 days prior to oral dosing with radiolabelled folic acid as per group A. Treatment with phenytoin was then continued until the end of the experiment.

After the radioactive dose the animals were housed in metabolism cages (Jencons Metabowls, Jencons (Scientific) Ltd.) designed to allow the separate collection of urine and faeces. Samples were collected for periods of 0–24 hr, 24–48 hr for group B, and for a further 24 hr for groups A and C. Urine was collected into 0.05 M sodium phosphate buffer, pH 7.0, containing (2% w/v) sodium ascorbate and protected from light. At the end of each collection period 5 rats from each group were killed by cervical dislocation and the gut, liver and kidney removed to determine the distribution of retained radioactivity. All samples were stored at −15°C in the presence of sodium ascorbate until analysed.

Measurement of radioactivity. The radioactivity retained in the tissues and faeces was determined after freeze drying using a Beckman biological material oxidiser (10). Urine and column effluents were counted using a Beckman LS7500 liquid scintillation counter.

Column chromatography. DE52-cellulose chromatography and Sephadex G15 gel filtration

were performed as described by Barford *et al.* [11]

Extraction of retained folates. Livers were removed, quickly chilled and washed in ice-cold buffer. Retained folates were extracted into 4 volumes of boiling 0.05 M-sodium phosphate buffer, pH 7.0, containing sodium ascorbate (2% w/v). After boiling for 10 min the extract was cooled, homogenised, centrifuged to remove preciptated protein and the supernatant retained.

Chemicals. (2-^{14}C) Folic acid (specific activity 58.2 mCi/mmol) (96% radiochemically pure) and (3′,5′,7,9-^{3}H) folic acid (specific activity 500 mCi/mmol) (95% radiochemically pure) were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). The distribution of the tritium label was found to be 42.5% at the 3′ and 5′ positions on the benzene rine, 32% at position 9 and 25.5% at the 7 position by tritium nuclear magnetic resonance spectroscopy (Radiochemical Centre batch analysis sheet H/2392) (Fig. 1). Compounds for calibration purposes were

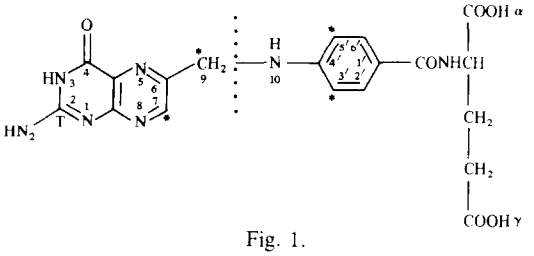

Fig. 1.

obtained as follows: folic acid was purchased from Koch Light (Colnbrook, Bucks); *p*-aminobenzoic acid, *p*-aminobenzoyl-L-glutamic acid, phenytoin sodium and phenobarbitone sodium from Sigma Chemical Company Limited (Kingston-upon-Thames) and *p*-acetamidobenzoic acid from Aldrich Chemical Company (Middlesex). 10-Formylfolate was prepared by the method of Blakley [12] and *p*-acetamidobenzoyl-L-glutamate by the method of Baker *et al.* [13]

RESULTS

The excretion of radioactivity into the urine and faeces of each group of rats is shown in Table 1. The normal animals excreted large amounts of radioactivity into the urine and faeces over the first 24 hr after which excretion rates were far lower. The excretion of the two isotopes was unbalanced in each sample with an excess of ^{3}H appearing in the urine and of ^{14}C in the faeces. However, if excretion is summed over 0–24 hr then the total isotope excretion figures are very close at 38.7% ^{14}C and 38.5% ^{3}H. The rats pretreated with phenobarbitone showed qualitatively the same pattern seen in normal rats. However, the 0–24 hr urinary excretion figures were greater than normal ($p = 0.01$), whereas the 0–24 hr faecal radioactivity was lower than normal ($p = 0.05$). Phenytoin pretreatment abolished the urinary ^{3}H excess. Significantly less ^{3}H appeared in both the 0–24 hr period ($p = 0.05$) and over the total 0–72 hr period ($p = 0.05$). The 48–72 hr urinary ^{3}H radioactivity, however, was significantly higher than normal. Greater amounts of ^{14}C also appeared in later urine samples. This suggests a delayed and prolonged excretion of radioactive material into the urine. Faecal radioactivity was greater in each time period for both isotopes ($p = 0.01$) and the imbalance between ^{14}C and ^{3}H levels was not as great as that found in normal rats.

Table 2 shows the tissue distribution of radioactivity together with overall recovery figures. No major differences are apparent except that the radioactive content of the liver appears lower in the animals pretreated with phenytoin ($p = 0.05$) and slightly higher in the phenobarbitone pretreated rats. This latter elevation is not statistically significant. Sequential chromatography of urine samples on DE52-cellulose and Sephadex G 15 columns allowed the identification of a number of metabolites listed in Table 3 (described in greater detail elsewhere [3]). Both groups of rats treated with anticonvulsants excreted more unchanged folic acid than normal rats. The two major urinary metabolites in all three groups were 5-methyltetrahydrofolate (5-MeTHF) and 10-formyltetrahydrofolate (10-CHOTHF). The

Table 1. Radioactivity recovered in the urine and faeces after the administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Samples	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine						
0–24 hr	23.4 (2.3)	31.8 (3.2)	30.3 (2.6)	37.5 (1.6)	19.4 (2.7)	20.5 (3.0)
24–48 hr	1.6 (0.3)	8.1 (2.3)	1.9 (0.1)	2.9 (0.2)	4.7 (1.3)	5.4 (1.6)
48–72 hr	0.3 (0.1)	1.0 (0.3)	n.d.	n.d.	3.2 (1.3)	4.7 (1.8)
Faeces						
0–24 hr	15.3 (4.3)	6.7 (2.1)	6.9 (2.3)	4.6 (1.5)	22.4 (2.5)	13.6 (1.6)
24–48 hr	1.3 (0.4)	0.8 (0.2)	1.8 (0.9)	1.7 (0.9)	5.5 (0.78)	4.6 (0.6)
48–72 hr	0.14 (0.04)	0.6 (0.2)	n.d.	n.d.	4.8 (0.95)	4.1 (1.0)
Total 0–48 hr	41.6	47.4	40.9	46.7	52.0	44.1
Total 0–72 hr	42.0	49.0	n.d.	n.d.	60.0	52.9

Results are expressed as a percentage of the dose (± S.E.M.) n.d. = Not determined.

Table 2. Recovery of radioactivity from urine and faeces up to 48 hr and in the tissues at 48 hr after administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid to rats (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Sample	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine	25.0	39.9	32.2	40.4	24.1	25.9
Faeces	16.6	7.5	8.7	6.3	27.9	18.2
Liver	19.1 (1.8)	21.6 (2.3)	25.6 (1.5)	23.6 (1.4)	11.8 (1.4)	11.9 (1.9)
Kidney	1.6 (0.2)	2.0 (0.5)	2.1 (0.1)	2.1 (0.1)	1.6 (0.3)	1.1 (0.2)
Small intestine + colon	2.3 (0.3)	2.5 (0.2)	2.8 (0.1)	2.8 (0.1)	2.1 (0.3)	2.1 (0.2)
Total	64.6	73.5	71.4	75.2	67.5	58.2

Results are expressed as a percentage of the dose (± S.E.M. where appropriate).

phenobarbitone pretreated rats excreted the highest levels of these and the phenytoin treated the lowest. 5,10-Methylenetetrahydrofolate (5,10-CH_2THF) was present in similar amounts in the urine of normal and phenobarbitone treated rats but was undetectable in the urine of the phenytoin treated animals. However, two unidentified species were present in the 24–72 hr urine of these rats. The peaks eluted separately from DE52-cellulose at a molarity of 0.8–0.85 M and from Sephadex G15 at fraction number 33 as a single peak. Increased amounts of C9–N10 scission products (^{14}C-only and ^{3}H-only labelled compounds; ^{3}H at C9 is lost in scission process [4] and ^{3}H at C7 is lost in subsequent hydroxylation) were found in the phenobarbitone pretreated rats whereas the opposite occurred in the phenytoin treated animals. Sephadex G15 gel filtration of liver homogenates from each group showed the major radioactive species present to be folate polyglutamate.

DISCUSSION

Theories put forward to explain anticonvulsant-induced anaemia include an impairment of absorption of folates from the gastrointestinal tract [14,15]; the induction of enzymes which require folate coenzymes [16] and the induction of enzymes responsible for the breakdown of the folate molecule via C9–N10 scission [17].

In this paper we have compared the metabolism of an oral dose of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid in normal rats and in rats pretreated with two compounds known to induce hepatic mixed function oxidases. If degradation of the folate molecule through oxidative cleavage of the C9–N10 bond is catalysed by these microsomal enzymes then we would expect to see a greater proportion of the urinary metabolites present as scission products (Fig. 1). However, the phenytoin and phenobarbitone treated rats excreted more unchanged folic acid than the normal group and similar amounts of 5-MeTHF and 10-CHOTHF.

The phenobarbitone treated group does appear to excrete more ^{3}H-only labelled fragments, 11.5% of the dose as opposed to the normal value of 7.6% but his increase was restricted to the first 24 hr and returned to normal on the second day. In contrast the phenytoin treated group excrete less (5.0%). These figures may only reflect changes in the short term turnover of folates within the rat. A better estimate of the extent of tissue folate catabolism may be obtained using the formula

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as } p\text{-AcBG 24–48 hr}}{\%\ \text{Radioactivity retained at 24 hr}} \times 100\%.$$

The distribution of the ^{3}H label throughout the folate molecule is such that C9–N10 cleavage leaves 42.5% of the label in the glutamyl containing fragment. Appropriate correction must therefore be made for this. This allows an estimation to be made of the breakdown product of tissue folate polyglutamates as a percentage of the total body burden of polyglutamate. There are a number of ways of assessing the

Table 3. Metabolites present in the urine 0–48 hr following the administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Metabolites	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic acid	1.5	1.8	3.4	3.8	5.4	3.7
5MeTHF	7.6	9.8	10.5	11.1	6.2	4.2
10CHOTHF	6.7	10.1	8.0	8.8	5.1	5.4
5,10-CH_2THF	3.3	3.8	2.5	2.6	0	0
Novel Folates	0	0	0	0	1.6	1.6
^{14}C-only fragments	4.3	0	6.7	0	3.2	0
^{3}H-only fragments	0	9.52	0	11.5	0	5.0
$^{3}H_2O$	0	2.2	0	0.6	0	0.8

The results are expressed as the percentage of the dose given.

Table 4. Excretion of the catabolites of folate polyglutamates

Group	% ^{3}H of the dose retained in the body after 24 hr	% ^{3}H dose excreted as *p*-AcBG at 24–48 hr	Extent of breakdown (%)
(a) Calculations based on total radioactivity retained in the body at 24 hr i.e. Dose administered − (Total excreted in urine and faeces over 0–24 hr)			
Normal	61.5	3.3	5.4
Phenobarbitone	57.9	2.58	4.5
Phenytoin	65.9	1.7	2.6
(b) Calculations based on radioactivity found in the liver, kidney and gut at 24 hr			
Normal	25.9	3.3	12.7
Phenobarbitone	27.0	2.58	9.6
Phenytoin	24.0	1.7	7.1
(c) Calculations based on radioactivity in the liver at 24 hr			
Normal	18.6	3.3	17.7
Phenobarbitone	21.4	2.58	12.1
Phenytoin	20.3	1.7	8.4

The results are given as the percentage of tissue radioactivity excreted as pAcBG where

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as } p\text{-AcBG 24–48 hr}}{\%\ \text{Radioactivity retained at 24 hr}} \times 100\%.$$

polyglutamate burden, as shown in Table 4. If the assumption is made that there is no loss of labelled folate polyglutamate from the body over 24 hr except through the urine and faeces and that the remainder of the dose is totally converted to polyglutamates then the extent of breakdown is shown in Table 4(a). As this assumption exaggerates the total body folate the catabolism may also be calculated using the radioactivity retained in the gut, liver and kidney at 24 hr (Table 4b) or the liver only at 24 hr (Table 4c).

Table 4 shows that no matter how the calculation is done the rats pretreated with the two anticonvulsants show a lower breakdown of polyglutamates than the normal animals. Phenytoin produces a greater decrease in breakdown than phenobarbitone and this trend is found in all three types of calculation.

Krumdieck *et al.* [18] and Kelly *et al.* [17] also investigated the effects of anticonvulsant therapy on folate metabolism. In a long term study [18] it was found that in man phenytoin accelerated the urinary loss of newly absorbed folate but did not increase the rate of catabolism. Although these results agree with those presented here, Kelly *et al.* [17] found that in the mouse phenytoin increased scission but phenobarbitone had no effect. This apparent discrepancy may be an example of species variation in the handling of folates or may be due to differing experimental procedures.

In conclusion, the induction of the hepatic microsomal enzyme system does not lead to an increase in folate catabolism. Both phenobarbitone and phenytoin did influence folate handling by the rat but as their actions were different the changes seem to be the result of some intrinsic property of the drugs themselves as opposed to an effect on hepatic mixed function oxidases. The apparent delay in absorption and increased loss of folates in the urine of phenytoin treated animals may offer a partial explanation for the folate deficiency sometimes associated with phenytoin therapy.

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