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STUDIES ON THE INTRACEREBRAL METABOLISM OF ANTICONVULSANT DRUGS—I. PERFUSION OF PRIMIDONE THROUGH THE ISOLATED BRAIN OF THE RAT

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Abstract—Primidone and phenobarbital (each 85 nmoles/ml were separately perfused through the isolated brain of the rat. After 5 min of perfusion similar amounts of primidone and phenobarbital were taken up into the brain; for both drugs the concentration ratio between brain and perfusion medium was about 0.2. However, after 2 hr of perfusion the mean concentration ratio for primidone was about 0.55; for phenobarbital it was about 0.9 thus indicating a better uptake of phenobarbital. In two regions (hypophysis, mesencephalon) the concentration of phenobarbital was significantly higher than in perfusion medium. During 2 hr of perfusion of primidone, substantial quantities of phenobarbital and PEMA were formed amounting to 1400 pmoles for each metabolite. The highest concentration of the metabolites was found in septum, hypothalamus, hypophysis and mesencephalon. The *in situ* metabolism of primidone in the intact brain was demonstrated for the first time.

As a function of hepatic enzyme activity primidone is converted in animals and man into two major metabolites, namely phenylethylmalonamide (PEMA) and phenobarbital [1, 2].

The anticonvulsant properties of primidone and its metabolites are different from each other [2–4]. On the other hand the synergistic anticonvulsant effect of PEMA and phenobarbital was demonstrated by Gallagher and Baumel [5].

The activity of several drug metabolising enzymes in the intact brain has recently been shown after perfusion of oestradiol-17β through the isolated brain of the rat [6]. The question arose whether prodrugs like primidone are converted by the brain into their active metabolites. This may be of clinical importance if the prodrug passes the blood–brain-barrier more easily than the metabolites formed by the liver.

The present study was undertaken to investigate the metabolism of primidone in the intact brain. The isolated perfused rat brain seemed to be a suitable biological model. The perfusion method has been recently published in detail [7].

MATERIALS AND METHODS

Chemicals. All solvents and reagents used were of p.a. grade or of HPLC-purity (Merck, Darmstadt, F.R.G. and C. Roth, Karlsruhe, F.R.G.). Primidone (5-ethyl-5-phenylhexahydropyrimidine-4,6-dione) was kindly donated by ICI-Pharma, Plankstadt, F.R.G., and phenylethylmalonamide by Desitin, Hamburg, F.R.G. Phenobarbital (5-ethyl-5-phenyl-barbituric acid) and butabarbital {5-ethyl-5-(1-methylpropyl)-barbituric acid} were purchased from Serva, Heidelberg, F.R.G. Fluorocarbon FC 43 was from 3M Company, Neuss, F.R.G., and Pluronic F 68 from Erbslöh, Düsseldorf, F.R.G.

Perfusion technique. Male Sprague Dawley rats (200–210 g; S. Ivanovas, Kisslegg, F.R.G.) were anaesthetized by intraperitoneal injection of urethane (0.9 g/kg body weight) and atropine (0.01 mg/kg). The operation procedure was performed as described [7]. Both internal carotid arteries were cannulated; the pterygopalatine arteries which supply extracerebral structures were ligated. The jugular veins were opened and immediately after commencement of perfusion the neck was ligated to prevent leakage of perfusion fluid through the vertebral arteries. A fluorocarbon FC 43 emulsion with Pluronic F68 as macromolecular component was taken as perfusion medium. Perfusions were carried out as cyclic perfusions (2 hr) or as non-cyclic once-through perfusions (5 min).

During once-through perfusions the venous medium was collected in fractions. A pulsatory perfusion pressure was used (systolic pressure: 120 mmHg, diastolic pressure: 100 mmHg, pulse rate: 280/min).

The following parameters of the functioning state of the brains were monitored: EEG, mean vascular resistance (calculated from flow rate and mean perfusion pressure), oxygen consumption as well as the molar ratios of ATP to ADP and lactate to pyruvate in brain tissue. For further details see [7, 8].

A quantity of 13.6 μmole of primidone or phenobarbital were dissolved in 160 ml perfusion medium using ultrasonication. The concentration of the respective drug in perfusion medium was 85 nmoles/ml. After perfusion, the brains were quickly removed from the skull, cooled to 4° and prepared into 10 regions: cerebral cortex, cerebellum, corpus stria-

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tum, septum, thalamus, hippocampi, hypothalamus, hypophysis, mesencephalon and pons/medulla oblongata.

Extraction and purification of drugs. The different brain regions were weighed and separately homogenized in 10 vol of 0.3 moles/l perchloric acid. After addition of 2 ng butabarbital per mg tissue the homogenate was centrifuged at 2000 g_{av} for 10 min. The supernatant was neutralized at 4° with 3 moles/l KOH and extracted three times with the same vol of water-saturated ethylacetate. The organic phases were combined, evaporated to dryness under vacuum and the residue was dissolved in 5 ml 0.1 moles/l acetate buffer (pH 3.9). Fifteen microlitres of a suspension of activated carbon (preparation see below) were added and the mixture was shaken for 10 min. Subsequently it was centrifuged at 3000 g_{av} for 30 min. The supernatant was discarded and the pellet was centrifuged once more at 3000 g_{av} for 30 min. The supernatant was removed by a micropipette and discarded. The remaining carbon pellet, which was almost free of water, was twice suspended in ethylacetate/methanol (9:1, vol/vol).

The suspension was shaken for 10 min and subsequently centrifuged at 3000 g_{av} for 30 min. The organic supernatants were combined and evaporated to dryness under vacuum. The residue was dissolved in acetonitrile/water (27:73, vol/vol).

The perfusion medium was worked up in the following manner: 400 ng butabarbital were given to 200 µl perfusion medium. After the addition of 4 ml methanol, the emulsion disaggregated and two phases were formed. The fluorocarbon phase was discarded and the methanol/water-phase was evaporated to dryness under vacuum. The residue was dissolved in 4 ml water which was subsequently extracted three times with the same volume of ethylacetate. The organic phases were combined, evaporated to dryness under vacuum and the residue was dissolved in acetonitrile/water (27:73, vol/vol).

Suspension of activated carbon. Five hundred mg of activated carbon (Norit A, Serva, Heidelberg, F.R.G.) were suspended in 200 ml buffer pH 11.0 (21 g Na_2CO_3 and 0.42 g $NaHCO_3$ in 1000 ml water). The suspension was stirred for 30 min and subsequently put into a narrow vessel. After sedimentation, the supernatant was discarded. The remaining slurry (about 4 ml) contained about 120 mg/ml activated carbon.

High pressure liquid chromatography. A model SP 8700 chromatograph (Spectra Physics, Darmstadt, F.R.G.) was used. A model SP 8400 variable wavelength u.v.-detector (Spectra physics) was operated at 195 nm with 0.0025 A.U. for full scale. Injections were achieved with a high pressure sample injection valve (Rheodyne 7125 c/o Latek, Heidelberg, F.R.G.), fitted with a 20 µl sample loop. Chromatographic run was performed at 40° (column oven from Spectra Physics).

Before use, the mobile phase was degassed by ultrasonication; during chromatography the mobile phase was continuously gassed with helium. Chromatograms were recorded and computed by a SP 4100 integrator.

Primidone and phenobarbital were separated from each other on a RP 18 column (Nucleosil, 7 µm, 250 × 4.6 mm, Macherey & Nagel, Düren, F.R.G.), using acetonitrile/water (27:73, vol/vol) as mobile phase. Butabarbital was used as internal standard. After quantitation of primidone, the samples were chromatographed on a RP 2 column (Lichrosorb, 5 µm, 125 × 4.6 mm; Latek) using acetonitrile/water (12:88, vol/vol) as mobile phase. PEMA was quantitated in relation to primidone.

For further chromatographic characterization of the substances, the respective peaks in reversed phase chromatography were pooled and rechromatographed on silica gel (Lichrosorb Si 60, 10 µm, 250 × 4.6 mm; Chrompack, West-Berlin) with chloroform/methanol/14% (w/v) ammonium hydroxide (94.9/5.0/0.1, by vol) as mobile phase; detection was performed at 254 nm.

Equilibrium dialysis. Binding of primidone and phenobarbital in perfusion medium was determined by equilibrium dialysis. The dialysis was carried out at 28° using a Dianorm BFD (Diachemie, Zürich, Switzerland) with a Visking membrane (Serva). For further details, see [8].

RESULTS

Functional parameters of the isolated brains. The viability and functional state of the isolated brains were monitored by determination of different parameters including electrical activity of the cortex, flow rate, oxygen consumption, concentration of high energy phosphates as well as lactate in tissue. Additionally, some brains were investigated histologically.

The EEG, which exhibited a frequency of about 16 Hz and a mean amplitude of about 110 µV, remained unchanged during perfusion. Flow rate (2.5–2.7 ml/min/g wet wt) did not change during perfusion, indicating that the brains were free of edema. The ratios of the molar concentrations of ATP to ADP and lactate to pyruvate were 3.5 ± 0.4 and 14.1 ± 3.0, respectively ($\bar{x}$ ± S.D.; n = 6), thus indicating no lack of oxygen or acidosis in tissue. Oxygen consumption was 2.9 ± 0.3 µmoles/ml/g wet wt ($\bar{x}$ ± S.D.; n = 6). Microscopic examination of the brains (n = 2) revealed no signs of anoxia or edema in either grey or white matter even in those structures of the brain which were supplied by the basilar artery. A detailed study about the viability of the isolated brain of the rat after long-term perfusion is published elsewhere [7].

Distribution of phenobarbital and primidone in the brain. After perfusion, the brains were prepared into 10 regions, namely cerebral cortex, cerebellum, corpus striatum, septum, thalamus, hippocampi, hypothalamus, hypophysis, mesencephalon and pons/medulla oblongata. The wet weights of the respective regions are summarized in Table 1.

After 5 min of once-through perfusion, the concentrations of phenobarbital and primidone were similar in all brain regions. In hypophysis, mesencephalon and pons/medulla oblongata, the concentrations of phenobarbital were slightly higher, while the concentrations of primidone exceeded in hypothalamus, septum and cortex.

However, after 120 min of cyclic perfusion, the concentrations of phenobarbital in all regions

Table 1. Wet weights of 10 regions of rat brain after 2 hr of isolated perfusion. The values are mean values ± S.D. (n = 10)

Region	Wet wt (mg)
Cerebral cortex	621.2 ± 22.1
Cerebellum	266.0 ± 10.2
Corpus striatum	339.6 ± 15.4
Septum	27.0 ± 2.1
Thalamus	84.8 ± 5.4
Hippocampi	162.2 ± 6.9
Hypothalamus	36.6 ± 2.2
Hypophysis	8.3 ± 1.1
Mesencephalon	158.2 ± 3.4
Pons/medulla oblongata	170.6 ± 4.6

exceeded those of primidone. In hypophysis and mesencephalon the concentrations of phenobarbital were significantly higher than 85 pmoles/μl, indicating a high affinity of the binding sites for phenobarbital in tissue. The results are indicated in Fig. 1.

Binding of phenobarbital and primidone in perfusion medium. The binding of the anticonvulsant drugs in perfusion medium was tested by equilibrium dialysis. It was found to be 65 ± 3.2% for phenobarbital and 64 ± 3.9% ($\bar{x}$ ± S.D., n = 10), for primidone in a concentration range of 0.5 to 100 nmoles/ml.

Formation of phenobarbital and PEMA. Primidone (85 pmoles/μl) was perfused for 2 hr. After perfusion, two metabolites—namely phenobarbital and PEMA—were found in tissue and perfusion medium. The concentrations of the respective metabolites in the brain are indicated in Table 2. The highest concentrations of phenobarbital were found in hypophysis, cerebellum, septum and mesencephalon; the highest concentrations of PEMA

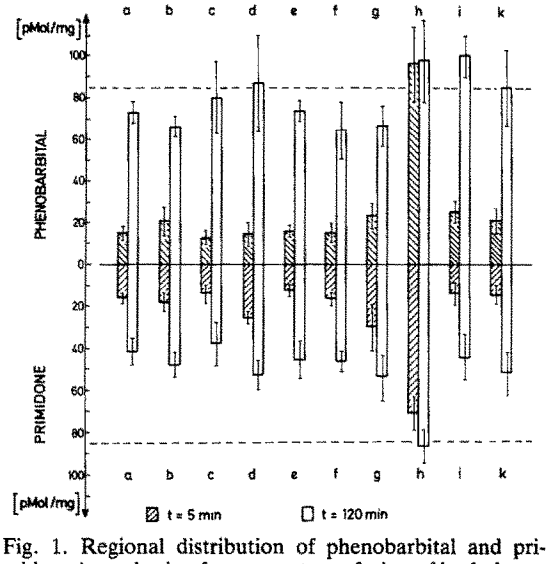

Fig. 1. Regional distribution of phenobarbital and primidone in rat brain after separate perfusion of both drugs, (each 85 pmoles/μl). Perfusion time: 5 min and 120 min. Mean values ± S.D. (n = 5) are given. a, cortex cerebri; b, cerebellum; c, corpus striatum; d, septum; e, thalamus; f, hippocampi; g, hypothalamus; h, hypophysis; i, mesencephalon; k, pons/medulla oblongata.

Table 2. Distribution of phenobarbital (PB) and phenylethylmalonamide (PEMA) in the brain after perfusion of primidone (85 pmoles/μl) for 2 hr. The values are mean values ± S.D. (n = 5) and given in pmoles/mg wet wt for brain regions and pmoles/ml for perfusion medium

	PB	PEMA
Cerebral cortex	0.4 ± 0.1	0.5 ± 0.2
Cerebellum	1.6 ± 0.6	0.6 ± 0.2
Corpus striatum	0.5 ± 0.2	0.7 ± 0.3
Septum	1.6 ± 0.9	2.9 ± 1.3
Thalamus	0.7 ± 0.2	0.8 ± 0.2
Hippocampi	0.3 ± 0.1	0.5 ± 0.2
Hypothalamus	1.0 ± 0.5	2.5 ± 0.9
Hypophysis	1.7 ± 0.8	6.6 ± 1.9
Mesencephalon	1.5 ± 0.8	0.6 ± 0.2
Pons/medulla oblongata	0.5 ± 0.2	0.5 ± 0.1
Perfusion medium	traces	0.7 ± 0.1

were measured in hypophysis, septum and hypothalamus. In perfusion medium, only small amounts of PEMA, (0.7 pmoles/ml), and traces of phenobarbital could be detected.

Altogether about 1400 pmoles phenobarbital as well as PEMA were found. Nearly 50% of the total amount of phenobarbital and 40% of the total amount of PEMA were contained in two regions. In cerebellum and mesencephalon 425.6 pmoles and 237.3 pmoles, respectively, of phenobarbital were detected, while in cerebral cortex and corpus striatum 310.6 pmoles and 239.7 pmoles, respectively, of PEMA were found.

DISCUSSION

In the present study, a technique for the perfusion of the isolated brain of the rat was applied that would maintain the functional state of the organ comparable to the *in vivo* state. Under these experimental conditions the intracerebral metabolism of primidone to phenobarbital and PEMA could be shown for the first time. This is of particular interest because primidone, an anticonvulsant agent, is metabolized at the site of action into two metabolites which exhibit antiepileptic activity on their part. It is well known that the anticonvulsant effect of primidone, phenobarbital and PEMA is a synergistic one [5, 9].

The question arises whether the intracerebral concentrations of phenobarbital and PEMA in patients treated with primidone are exclusively determined by the respective plasma levels or whether they are additionally influenced by the formation of phenobarbital and PEMA inside the brain. The relevance of the intracerebral formation may depend (a) on the amounts of phenobarbital and PEMA formed in the brain during chronic treatment; (b) on the binding of both metabolites to plasma proteins and tissue binding sites; (c) on the permeability of the blood–brain-barrier for phenobarbital and PEMA which may be different in the various brain regions.

The main tissue localisation of the enzymes responsible for the biotransformation of primidone in man is presumed to be the liver [1]. It is suggested that biotransformation of primidone in man may be influenced by interactions among primidone, pheno-

barbital and PEMA [9]. From the findings of perfusion studies with rat livers it was concluded that biotransformation of primidone may be simultaneously influenced by the processes of metabolite induction (phenobarbital) and metabolite inhibition (PEMA) [10].

It is well known that phenobarbital appears slowly in the brain after oral or intravenous application [11]. This is in accordance with the findings of the present study; when phenobarbital or primidone were perfused for 5 min, the brain levels of the respective drugs were similar. However, after 2 hr of perfusion, the concentrations of phenobarbital exceeded those of primidone. In the present study, a fluorocarbon emulsion was used as perfusion medium. As determined by equilibrium dialysis, the percentage of unbound drug was similar for both primidone and phenobarbital (about 35%). The higher uptake of phenobarbital into the brain in the present study may be attributed to the greater lipid solubility of the drug.

In the experiments described here the mean levels of primidone in the isolated perfused brain increased from about 17 nmoles/g (after 5 min) to about 47 nmoles/g (after 2 hr). The tissue levels of phenobarbital, formed from primidone during 2 hr, were 20–100 times lower (see Table 2). However, Baumel *et al.* [2] administered orally 31.25 mg/kg to rats and after 2 hr investigated plasma and brain; in the latter neither phenobarbital nor PEMA could be detected. Assuming a bioavailability for primidone of 90% and a V_D of 0.8 l/kg [12, 13] the above mentioned oral dose would correspond to a concentration of about 162 nmoles/ml in an isolated brain perfusion system. This is nearly twice the concentration of primidone used in the present study. From these data it may be speculated that after acute application of 31.25 mg/kg primidone or after a lower dose, levels of phenobarbital and PEMA in the brain may be attributed to the intracerebral metabolism of primidone.

However, the picture may be different during chronic administration of primidone. Brain concentrations of primidone and phenobarbital, respectively, were measured in epileptic patients on the occasion of neurosurgical operations [14]. The patients had received treatment with primidone for at least one week beforehand. While the ratios of the concentrations in CSF and plasma were 0.81 for primidone and 0.43 for phenobarbital, thus representing the percentage of unbound drug in plasma, the tissue concentrations of the respective drugs were similar to those measured in plasma. Brain levels of phenobarbital were also measured in patients receiving solely this drug. No obvious difference was seen in the brain/plasma phenobarbital ratio when phenobarbital was derived from primidone rather than phenobarbital itself. From these findings it may be concluded that during chronic treatment with primidone the brain levels of phenobarbital are predominantly determined by the metabolism of primidone in the liver. However, the apparent half life of primidone in man is 7.7 hr [1]; this implies that a b.i.d. administration leads to substantial fluctuation of the plasma level. Furthermore, it has not been investigated to what extent the site of formation of phenobarbital influences its anticonvulsant effects.

After 2 hr of perfusion of primidone, the highest tissue concentrations were achieved in hypophysis, septum and hypothalamus. Correspondingly, substantial concentrations of the metabolites of primidone were found in these regions. The highest concentrations of phenobarbital were measured in hypophysis, cerebellum and septum, while PEMA predominated in hypophysis, septum and hypothalamus. The formation of the two metabolites could possibly be determined by the regional uptake of primidone. Additionally, it may be attributed to local enzyme activity; after perfusion of oestradiol-17β through the isolated brain of the rat, hypophysis, hypothalamus and septum contained the highest concentrations of the metabolites of the oestrogenic steroid [6].

In conclusion, the observation of a cerebral metabolism of drugs acting on CNS may be a new aspect in neuropharmacology. However, considering the substantial hepatic metabolism of most drugs, the relevance of a metabolic conversion at the site of action has to be established.

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REFERENCES

1. D. D. Schottelius, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and C. E. Pippenger), pp. 415–420. Raven Press, New York (1982).
2. I. P. Baumel, B. B. Gallagher, J. Dimicco and H. Goico, *J. Pharmac. exp. Ther.* **186**, 305 (1973).
3. D. D. Schottelius and R. W. Fincham, *Neurology* **28**, 409 (1978).
4. J. Oxley, S. Hebdige, J. Laidlaw, J. Wadsworth and A. Richens, in *Antiepileptic Therapy: Advances in Drug Monitoring* (Eds. S. I. Johannessen, P. L. Morselli, C. E. Pippenger, C. E. Richens, A. Schmidt and H. Meinardi), pp. 237–245. Raven Press, New York (1980).
5. B. B. Gallagher and I. P. Baumel, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and R. P. Schmidt), pp. 367–371. Raven Press, New York (1972).
6. M. Höller, K. Dengler and H. Breuer, *J. Steroid Biochem* **20**, 785 (1984).
7. M. Höller, H. Breuer and K. Fleischhauer, *J. Pharm. Methods* **9**, 19 (1983).
8. M. Höller, W. Grochtmann, M. Napp and H. Breuer, *Biochem. J.* **166**, 363 (1977).
9. R. W. Fincham, D. D. Schottelius, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and C. E. Pippenger), pp. 421–427. Raven Press, New York (1982).
10. J. Alvin, E. Goh and M. T. Bush, *J. Pharmac. exp. Ther.* **194**, 117 (1975).
11. E. W. Maynert, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and C. E. Pippenger), pp. 309–317. Raven Press, New York (1982).
12. R. Saller, Th. Berger, E.-M. Ulmer and D. Hellenbrecht, in *Praktische Pharmakologie*. F. K. Schattauer Verlag, Stuttgart (1983).
13. D. D. Schottelius, in *Antiepileptic Drugs* (Eds. D. M. Woodbury, J. K. Penry and C. E. Pippenger), pp. 405–414. Raven Press, New York (1982).
14. G. W. Hougton, A. Richens, P. A. Toseland, S. Davidson and M. A. Falconer, *Eur. J. clin. Pharmac.* **9**, 73 (1975).