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3-MERCAPTOPROPIONIC ACID: CONVULSANT PROPERTIES, EFFECTS ON ENZYMES OF THE γ-AMINOBUTYRATE SYSTEM IN MOUSE BRAIN AND ANTAGONISM BY CERTAIN ANTICONVULSANT DRUGS, AMINOOXYACETIC ACID AND GABACULINE

WOLFGANG LÖSCHER

Laboratory of Pharmacology and Toxicology, School of Veterinary Medicine, Free University, Koserstraße 20, D-1000 Berlin 33, GFR

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Abstract—Subcutaneous injection of 3-mercaptopropionic acid (MP) into mice caused severe convulsions which started after about 6 min and were paralleled by a large, reversible inhibition of glutamate decarboxylase (GAD) and activation of (GABA)-α-oxoglutarate aminotransferase (GABA-T) in the brain. The central (GABA) level, determined by a newly developed gas chromatographic method, was not altered after administration of the CD 97 of MP (60 mg/kg) but decreased after doubling or tripling the dose. Protection against the convulsions elicited by MP could be effected by pretreatment with phenobarbital, phenytoin, diazepam, carbamazepine, sodium valproate and trimethadione but not by ethosuximide and dimethadione. The ED 50's of the respective anticonvulsants against MP were similar to those determined against picrotoxin but, except in the case of trimethadione clearly less than those against strychnine-induced convulsions. All anticonvulsants effective against the convulsions of MP reversed the activation of GABA-T and tended to antagonize the inhibition of GAD caused by MP whereas dimethadione and ethosuximide were devoid of such action. The dissociative anaesthetic ketamine, which suppressed the MP-induced convulsions only at doses approaching the anaesthetic level, also failed to antagonize the alterations in the enzyme activities. Aminooxyacetic acid (AOAA) and gabaculine ((−)-5-amino-1, 3-cyclohexadiene carboxyclic acid) had a dose-dependent anticonvulsant effect against MP (ED 50 27 mg/kg s. c. and 135 mg/kg i. p., respectively) but at doses which were potentially (AOAA) or absolutely (gabaculine) lethal (LD 50 40 mg/kg and 62 mg/kg, respectively). Both drugs antagonized the inhibition of GAD caused by MP, reduced the activity of GABA-T to zero and raised the central level of GABA by more than 500 per cent.

The results suggest a role played by the GABA system in the convulsant action of MP and in the antiseizure activity of several clinically useful anticonvulsants as well as AOAA and gabaculine. With respect to the GABA system, MP seems to be a useful tool in studies of experimental epilepsy.

Since the role of γ-aminobutyric acid (GABA) as central inhibitory transmitter has become more and more established, there has been an increasing interest in a possible relationship between this amino acid and convulsive disorders (for review see [1]). A variety of convulsant agents have been shown to inhibit L-glutamate decarboxylase (GAD: EC 4.1.1.15), which catalyses α-decarboxylation of L-glutamate to form GABA and CO_2 and is believed to be the rate limiting enzyme that normally determines the steady-state levels of GABA in brain [2]. GAD activity is evidently sensitive to changes in levels of its cofactor pyridoxal phosphate and most of these convulsants cause inhibition of the enzyme by interference with the synthesis of pyridoxal phosphate or its coenzyme function. However, because of the wide range of other metabolic effects of such carbonyl trapping agents like isoniazid [3–4] or thiosemicarbazide (5), the significance of GAD inhibition in the causation of the seizures is not clear. More recently, a specific competitive inhibition of GAD with respect to glutamate had been shown for some mercapto acids with convulsant properties *in vitro* [6–7] and among these 3-mercaptopropionic acid proved to be most potent (see also [8]). *In vivo* studies demonstrated that convulsions caused by this agent were preceded by a decrease in both GABA levels and GAD activity in several brain areas [9–12]. In addition, the activity of γ-aminobutyrate–α-oxoglutarate aminotransferase (GABA-T; EC 2.6.1.19), the enzyme responsible for the degradation of GABA, was found to be activated by MP *in vivo* [9–10]. Because of these properties, MP proved to be a useful agent for studies on the neurophysiology and biochemistry of experimental epilepsy, however, data concerning the evaluation of protective agents against seizures induced by this convulsant are scanty. Phenobarbital, sodium valproate and aminooxyacetic acid (AOAA) had been shown to diminish or prevent convulsions elicited by MP [8, 12, 13], AOAA being known to increase the level of GABA in brain by inhibition of GABA-T [14–15]. Furthermore, the dissociative anaesthetic ketamine had been found to be a potent anticonvulsant with respect to MP [16] though ketamine itself inhibits GAD competitively *in vitro* [17].

In this study, the ability of anticonvulsant drugs to antagonize the convulsions and related alterations in the activity of GAD and GABA-T induced by MP was evaluated. In addition, we investigated the effects of ketamine, AOAA and gabaculine ((−)-5-amino-1,3-cyclohexadiene carboxyclic acid), a naturally occurring neurotoxin isolated from Streptomyces toyocaensis, shown to be a powerful irreversible inhibitor of

GABA-T [18]. The anticonvulsant potency of the respective drugs against MP was compared to that against other convulsant drugs and maximal electroshock seizures.

MATERIALS AND METHODS

Materials

3-Mercaptopropionic acid, phenobarbital and 1,1,-1,3,3,3-hexafluoroisopropanol were obtained from Merck (Darmstadt). Phenytoin was kindly supplied by Leo Pharmaceutical Prod. (Ballerup, Denmark), ethosuximide, sodium valproate and α-methyl-α-propylsuccinimide by Desitin-Werk Carl Klinke GmbH (Hamburg). Carbamazepine and *d,l*-gabaculine-hydrochlorid were generous gifts from Ciba-Geigy AG (Basle, Switzerland). Trimethadione and dimethadione were kindly provided by Deutsche Abbot GmbH (Ingelheim), diazepam by Hoffmann-La Roche (Basle, Switzerland). Aminooxyacetic acid hemihydrochloride, pentafluoropropionic anhydride and δ-aminovaleric acid were obtained from Ferak (Berlin), pentetrazole from Knoll AG (Ludwigshaven), picrotoxin from Fluka AG (Ulm) and strychnine nitrate from Chemische Fabrik Tempelhof (Berlin). Ketamine hydrochloride was used as the commercial 5% solution (Ketanest®) and obtained from Parke-Davis Co. (München).

Animals

Male mice of the NMRI-strain (Bomholtgard A/S, DK-8680 Ry, Denmark) weighing 24–30 g were used. They were kept in groups of 10 in Makrolon® cages at an ambient temperature of 24–26° and fed on Altromin® standard food (Altrogge, Lage, Germany).

Convulsant CD50s of 3-mercaptopropionic acid (MP), picrotoxin, strychnine nitrate and pentetrazole were determined using groups of ten mice per dose. Mice were injected subcutaneously with solutions of the convulsants freshly prepared in water in a vol. of 10 ml/kg. Criterion of the convulsant effect was fully developed clonic seizures with loss of the righting reflexes appearing within 30 min after administration.

Evaluation of protective agents

1. Anticonvulsant drugs. Anticonvulsant potency of several anticonvulsant drugs was determined against the CD 97 of MP (60 mg/kg) and, if it had not previously been determined in the same strain of mice, against the CD 97 of picrotoxin (4 mg/kg), strychnine (1.2 mg/kg) and pentetrazole (100 mg/kg). Dose–effect curves were constructed in the usual way using groups of ten mice per dose. The anticonvulsant drugs were dissolved in water and administered orally in a vol. of 10 ml/kg, phenobarbital and phenytoin (brought into solution by adequate amounts of dilute NaOH) 2 hr, dimethadione 1.5 hr, diazepam (brought into solution by adequate amounts of dilute HCl) and carbamazepine (given as suspension in 5% gum acacia) 1 hr, ethosuximide, trimethadione and sodium valproate 45 min before the injection of the convulsants. The time intervals were chosen on the basis of previous studies [19–20] except in the case of dimethadione, for which brain concentrations were found to be maximal 1.5 hr after oral administration. Mice not showing clonic seizures with loss of righting reflexes were considered protected. In addition, as far as not known from previous studies, anticonvulsant activity was also determined in the maximal electroshock seizure test [21] using an A-615-B shocker of Lafayette Instruments Co. and eye electrodes. Stimulation was by 250 V and 50 cycles/sec for 0.2 sec. The serial resistance of the apparatus was set to 10 KΩ. The tonic extension of the hind limbs was used as endpoint.

2. Aminooxyacetic acid and gabaculine. Aminooxyacetic acid (AOAA, doses expressed as the hemihydrochlorid) was tested for an anticonvulsant effect against the CD 97 of MP and administered subcutaneously 6 hr before injection of the convulsant, the time of predetermined peak drug effect [15].

The anticonvulsant activity of gabaculine (doses expressed as the hydrochlorid) was determined 4 hr after i.p. injection against the CD 97 of MP and pentetrazole and in the MES-test. This time interval was chosen on the basis of a study by Rando and Bangerter [22] who determined a maximal inhibition of GABA-T 4 hr after i.p. injection of gabaculine. For gabaculine also the LD 50 was determined after i.p. injection. Both drugs were dissolved in water and administered in vol. of 10 ml/kg.

3. Ketamine. Ketamine (doses expressed as the hydrochlorid) was examined for an anticonvulsant effect against the CD 97 of MP, pentetrazole and in the MES test. The commercial 5% solution was diluted with water and injected i.p. in a vol. of 10 ml/kg 1 min before the injection of MP, 4 min before pentetrazole and 7 min before the electroshock was delivered, respectively, the different time intervals being due to the different latency of the seizures.

Biochemical determinations

Groups of 12 mice were injected subcutaneously with the CD 97 or 2 times and 3 times this dose of MP and killed by decapitation at the time of convulsions. Six mice were used for the determination of the activity of glutamic acid decarboxylase (GAD, EC 4.1.1.15) and γ-aminobutyric-α-oxoglutarate aminotransferase (GABA-T, EC 2.6.1.19), and the other six for the determination of the GABA level in brain. In order to test the antagonistic effect of anticonvulsant drugs as well as of AOAA, gabaculine and ketamine on the MP-induced alterations of the activities of GAD and GABA-T, mice were injected with the CD 97 of MP, either without or after pretreatment with fixed doses of the individual drugs, these doses being the anticonvulsant ED 84 against MP except in the case of AOAA and gabaculine (ED 50 instead of ED 84), ethosuximide (ED 84 against picrotoxin) and dimethadione (ED 84 against picrotoxin and the dose equimolar to the ED 84 of trimethadione against MP).

The times of pretreatment were the same as used for testing for anticonvulsant activity and mice were killed 5.6 min after administration of MP (mean latency of first fully developed clonic seizure). Six determinations were done for each treatment and six untreated controls were used for each experiment. For determination of the activities of GAD and GABA-T the brains were immediately removed, weighed and homogenized in 2 ml icecold water (tubes immersed in a bath of methanol at −1°).

GAD activity was determined by the method of Lowe *et al.* [23] as previously described [4]. However, the dilution of the homogenate had to be minimized, since the degree of the reversible inhibition of GAD by MP proved to be very dependent on the final concentra-

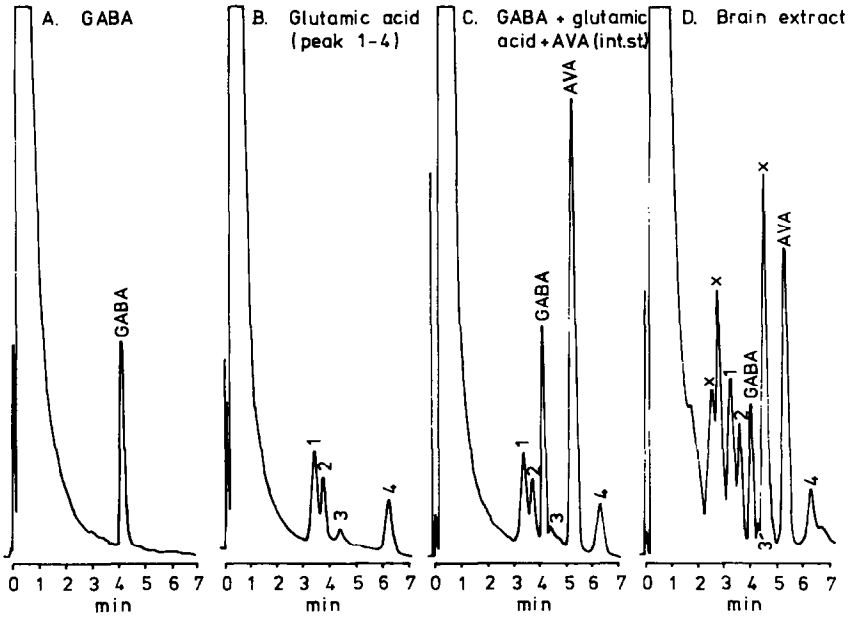

Fig. 1. Gas chromatograms obtained from the hexafluorisopropanol-pentafluorpropionic-derivatives of A. GABA (2 μg), B. glutamic acid (10 μg, peak 1–4), C. GABA (2 μg), glutamic acid (10 μg) and AVA (2 μg) and D, material obtained from mouse brain homogenized in an 80 % aqueous ethanol solution containing AVA as internal standard.

Peaks marked as "x" are attributable to brain constituents but could not be identified yet. Derivatization and gas chromatographic conditions are described under Materials and Methods.

tion of tissue. To 0.5 ml of a 20% (w/v) brain homogenate was added 0.5 ml of an incubation mixture to give final concentrations of 12 mM L-glutamic acid, 0.24 mM pyridoxal phosphate and 100 mM phosphate buffer (pH 6.4) These assay conditions were found to be suboptimal with respect to glutamate concentration which was essential because *in vitro* experiments have shown that MP is a competitive inhibitor of the enzyme [6].

The activity of GABA-T was determined by the method of Salvador and Albers [24] in which the succinic semialdehyde produced by the enzyme during incubation is measured. Details of this method have been described in a previous paper [4], the only modification was that as in the GAD assay a 20% homogenate was used. The results of the GABA-T determinations are given in arbitrary fluorescence units (f.u.) since samples of succinic semialdehyde which had been kindly prepared by Dr. Smalla polymerized too rapidly to permit the construction of a quantitatively reliable standard curve.

Determination of GABA. For the determination of GABA in mice brain the mass fragmentographic assay of Bertilsson and Costa [25] was modified for use in gas chromatography. GABA is subjected to derivate formation after extraction, the amino group being acylated with pentafluoropropionic anhydride and the carboxylic group esterified with hexafluoroisopropanol in a one-step reaction. After decapitation, brains were immediately removed, weighed and homogenized in 2 ml 80% aqueous ethanol (tubes immersed in a bath of methanol at −30°) containing 40 μg/ml δ-aminovaleric acid (AVA) as internal standard. The time from decapitation to homogenization never exceeded 30 sec. Under these conditions GABA levels were identical to those obtained after freezing of the brains in liquid nitrogen [4].

After homogenization, the tubes were centrifuged at 4000 rev/min for 10 min at −5° and 0.4 ml of the supernatant was transferred to 2 ml screw cap glass vials (Varian, Darmstadt) and evaporated to dryness by a stream of nitrogen (tubes heated to 40° in a thermoblock). 200 μl of 1,1,1,3,3,3-hexafluoroisopropanol and 100 μl of pentafluoropropionic anhydride were then added, the vials were sealed, heated for 1 hr at 60° in the thermoblock and the reaction mixture again evaporated to dryness. Just before the gas chromatographic analysis, the residue was dissolved in 50 μl of ethyl acetate and 5 μl were injected into the gas chromatograph. Analysis was carried out with a Varian 3700 gas chromatograph equipped with hydrogen flame ionization detector and linear temperature programming. The column was a silanized 6-ft glass tubing with 2 mm inner diameter packed with 3% OV 17 on 80/100 mesh Gas-Chrom Q (Bodenseewerk Perkin-Elmer Co. GmbH, Überlingen). The temperature of the column was held at 70° for 1 min, programmed from 70 to 115° at 15°/min and held at 115° for 5 min. Injector block and FID were kept at 200 and 220°, respectively. The flow rate of the carrier gas (nitrogen) was 40 ml/min, that of hydrogen and air was 40 and 300 ml/min, respectively. Because of an accumulation of nonvolatiles and extraneous material deposited at the head of the column from the extracted sample, the glass wool plug at the inlet side of the column was replaced after the column had been in use for some time.

Quantitation was based on the ratio of the peak areas of GABA and the internal standard AVA, peak areas being calculated as the product of peak height and width at half height. Standard curves were prepared by

analysing a series of aqueous standard solutions of GABA added to mouse brain extracts in a vol. of 20 μl before evaporation. The identity of GABA in our brain extracts was kindly confirmed by C. Jakobs (Department of Pediatrics, Free University) by gas chromatography–mass spectrometry using a Varian MAT 311 A. The mass spectrum of the GABA derivative in extracts from mouse brain was identical to that shown in the original method of Bertilsson and Costa [25]. In the latter method, glutamic acid is also quantitated in brain. This was not possible in our assay since glutamic acid was found to be degraded, resulting in four degradation products. In Figure 1, gas chromatograms of GABA, glutamic acid, a mixture of both substances and the internal standard AVA as well as a gas chromatogram of a brain extract obtained from an unpretreated mouse are shown. The degradation products of glutamic acid have a much smaller detector response than the GABA derivative and do not interfere with the determination of GABA. Except for the GABA, glutamic acid and AVA peaks, three other peaks appear in the gas chromatograms of brain extracts which could not be identified but are well separated from GABA and AVA, respectively.

The endogeneous GABA concentrations determined by this method are in good agreement with those determined by other methods used previously in our laboratory [3, 15] but the present assay is more sensitive, rapid and reproducible: Standard curves were linear in the range of at least 0.01 to 2.5 μmoles of GABA, repeated assay of the same brain homogenate pooled from several mice brains was reproducible within a standard deviation of ± 3.5% ($n = 10$), and a minimum of 30 samples can be conveniently handled in a normal working day.

Determination of trimethadione and dimethadione. Both drugs were determined in serum of mice by gas chromatography as described recently [26]. Brain concentrations were determined with the same method by extracting 1 ml samples of the homogenates also used for the assay of GAD and GABA-T.

Statistics

The ED 50s were calculated by the method of Litchfield and Wilcoxon [27]. Arithmetical means and S.D. are given for biochemical determinations. Significance of differences was calculated by comparing each treated group with the control group of the same day using the unpaired *t*-test.

RESULTS

1. Convulsant properties of 3-mercaptopropionic acid (MP)

Table 1 gives the CD 50 and CD 97 of MP as well as the latency to convulsions and lethal activity in mice. For comparison, convulsant properties are also shown for picrotoxin, strychnine and pentetrazole. Convulsions induced by MP were characterized by a very sudden onset with violent running fits, followed by clonic–tonic seizures with loss of the righting reflexes. The first fully developed clonic seizure occurred between 4 and 7 min after the subcutaneous injection of the CD 97. The mice convulsed 1–2 times and only 20 per cent died, the rest recovering rapidly from convulsions.

2. Effects of MP on central GABA level and activities of GAD and GABA-T

The results after pretreatment with MP are summarized in Table 2. After the injection of the CD 97 of MP (60 mg/kg) there was a decrease of 28 per cent in the activity of GAD at the onset of convulsions coinciding with a considerable activation of GABA-T, the variations in the activities of both enzymes being highly significant. However, the GABA content was not altered by this dose.

After doubling the dose of MP, the effects on GAD and GABA-T were enhanced and there was a slight decrease in GABA concentration. The latter decrease amounted to 54 per cent after administration of the threefold CD 97 (180 mg/kg), paralleled by a strong inhibition of GAD but the activation of GABA-T was reduced.

3. Reversal of MP-induced convulsions and biochemical alterations by various agents

(a) Anticonvulsant drugs. In Table 3 the ED 50s and ED 84s of several anticonvulsant drugs against MP and, for comparison, those against picrotoxin, strychnine, pentetrazole and maximal electroshock seizures (MES), are shown. Of the anticonvulsant studied, only ethosuximide and dimethadione had no true anticonvulsant effect against MP. For ethosuximide, doses up to 1 g/kg were insufficient to provide more than 20–30 per cent protection against MP and further increase in the dose resulted in severe side effects and a reduction of the anticonvulsant effect. The animals reacted similarly to dimethadione, a dose of 1.5 g/kg protecting only 20 per cent of animals though estimation of serum

Table 1. Convulsant and lethal activity in mice of 3-mercaptopropionic acid (MP) compared with that of picrotoxin, pentetrazole and strychnine

	3-Mercaptopropionic acid	Picrotoxin	Pentetrazole	Strychnine
CD 50 (mg/kg)	44 (41–47)*	2.2 (1.8–2.7)	66 (57–77)	1.05 (1.01–1.09)
CD 97 (mg/kg)	60	4	100	1.2
Seizure type	RF/C/T	C	C/T	C/T
Latency (min)	5.6 ± 1.1	15 ± 5.8	2.8 ± 0.9	4.5 ± 2.9
% death at ED 97	20	10	40	80

The seizure type (RF means running fits, C clonic, T tonic convulsions) and the latency (mean + S.D.) of first clonic seizure with loss of the righting reflexes after administration of the CD 97 of the respective conculsant are also given.

* Confidence limits for 95% probability.

Table 2. Effect of MP on the level of GABA and the activities of GAD and GABA-T in mouse brain

3-Mercaptopropionic acid (mg/kg)	GABA μmoles/g/hr	GAD μmoles/g/hr	GABA-T f.u./g/hr*
Controls	1.86 ± 0.32 (18)‡	57.1 ± 3.8 (18)	59.8 ± 5.7 (18)
60	1.92 ± 0.22 (6)	40.9 ± 7.8 (6)	81.2 ± 9.5§ (6)
120	1.69 ± 0.36 (6)	36.8 ± 2.9¶ (6)	86.1 ± 8.2¶ (6)
180	0.86 ± 0.18¶ (6)	28 ± 2.2¶ (6)	70.1 ± 6.7§ (6)

The CD 97 and twice and thrice this dose were administered subcutaneously and the mice were killed at the same time of convulsions.

* Activity given in arbitrary fluorescence units.

† Mean ± S.D.

‡ Number of determinations. Values differing significantly from controls are denoted by §$P < 0.05$, $P < 0.01$ and ¶$P < 0.001$.

and brain concentrations at different time intervals after administration of dimethadione revealed that maximal brain concentrations were reached at the time of pretreatment (see Table 5). Of the other anticonvulsants tested, diazepam was most effective against MP, its ED 50 being identical to those against picrotoxin and pentetrazole. This drug, as well as phenobarbital, phenytoin carbamazepine and sodium valproate, was clearly more active against MP-induced than against strychnine-induced seizures, the ratios of the ED 50s strychnine/MP being 8.2, 3.2, 6.3, 1.8, and 1.9, respectively. Similar values were obtained for the ratio strychnine/picrotoxin. The results with pentetrazole and the MES test confirmed that drugs useful in grand mal are characterized by their marked ability to protect mice from the tonic extensor component of the MES and are less or not effective against pentetrazole (i.e. phenobarbital, phenytoin, and also carbamazepine) while drugs useful in petit mal are characterized by their relative inactivity in the MES test and by their pronounced activity against seizures elicited by pentetrezole (i.e. ethosuximide, trimethadione and its metabolite dimethadione and also diazepam). Sodium valproate was of identical potency in both tests.

In order to test the antagonistic effect of the anticonvulsant drugs on the MP-induced alterations in the activity of GAD and GABA-T, mice were pretreated with the ED 97 of MP alone or in combination with the anticonvulsants (Table 4). All anticonvulsants, except those which were without protective effect against MP-induced seizures, reversed the activation of GABA-T caused by MP. Furthermore, there was a tendency to antagonize the inhibition of GAD induced by MP. The central GABA level, not being influenced by the CD 97 of MP (Table 2, Table 5) was only determined in the case of sodium valproate and trimethadione, the anticonvulsants that decreased the MP-activated GABA-T even below the control values. Sodium valproate administered 45 min before MP caused GABA to increase from 1.82 + 0.23 μmoles/g (controls, $n = 11$) to 2.29 ± 0.19 μmoles/g ($n = 11$; $P < 0.001$), trimethadione caused an increase of 17 per cent (Table 5).

Since trimethadione is rapidly demethylated to dimethadione in the organism [28], serum and brain concentrations of both substances were determined after administration of trimethadione in order to clear whether the antagonism of MP-induced seizures and biochemical alterations was an effect of unchanged trimethadione. As shown in Table 5, at the time of this antagonism only small concentrations of dimethadione were present in brain compared to those of trimethadione, and the high dimethadione brain concentrations found after administration of dimethadione itself were without any effect of GABA, GAD and GABA-T. As opposed to trimethadione, for which brain and serum concentrations were almost identical, the brain serum ratio of dimethadione was always less than unity irrespective of the time period when measured and averaged 0.54 ($n = 12$) at the time of peak brain concentration (1.5 hr).

(*b*) *Aminooxyacetic acid (AOAA) and gabaculine.* The results with AOAA and gabaculine are given in Table 6. Both substances were found to exert a preventive effect on the seizures elicited by MP but AOAA was 5 times more potent than gabaculine. Furthermore, both drugs displayed an anticonvulsant effect against pentetrazole and MES, but in this respect gabaculine was still less effective than AOAA. However, the anticonvulsant doses of both AOAA and gabaculine were potentially (AOAA) or absolutely (gabaculine) lethal. Mice which had been injected with AOAA showed severe side effects after 5 to 10 min, beginning at doses of 10–15 mg/kg: At first they seemed excited and then showed ataxia and loss of muscular tonus. Later, such animals lay prone with legs extended spastically and in bizarre positions. They were apparently unable to perform any coordinated motor activity. After severe clonic and tonic convulsions, the mice finally lay on their sides, dyspnoic and apathetic. Mice not dying in this state slowly recovered during the next hours. The average time to death was 30 ± 9 min ($n = 160$), LD 50 was 40 mg/kg (34–47). Hence the determination of the anticonvulsant effect of AOAA had of course to be confined to the animals surviving the doses administered, so the doses quoted in Table 1 belong to a highly truncated distribution. Mice treated with gabaculine (30–300 mg/kg) became sedated with decreased spontaneous movements after approximately ½ to 1 hr, but

Table 3. Anticonvulsant activity of different anticonvulsant drugs against the CD 97 of MP, picrotoxin, strychnine, pentetrazole and in the maximal electroshock seizure test

Drugs	Dose	Time of pretreatment (min)	3-Mercaptopropionic acid 60 mg/kg	Picrotoxin 4 mg/kg	Strychnine 1.2 mg/kg	Pentetrazole 100 mg/kg	Maximal electroshock seizure test
Phenobarbital	ED 50	120	14.2 (8.9–23)*	30 (25–37)[+]	46 (40–53)[+]	28 (22–36)[+]	15 (12–19)
	ED 84		30	42	61	40	26
Phenytoin	ED 50	120	16 (13–21)	39	> 100[+]	> 100[+]	7 (5–9)
	ED 84		26	truncated			10
Carbamazepine	ED 50	60	37 (26–52)	21	65	> 200	20 (17–23)
	ED 84		66	truncated	truncated		26
Diazepam	ED 50	60	1.7 (1.1–2.7)	1.6 (1.1–2.1)[+]	14 (11–17)[+]	1.6 (1.2–2.3)	4.2 (2.6–6.7)
	ED 84		3.5	3.1	20	2.6	9.2
Sodium valproate	ED 50	45	370 (240–570)	430 (290–640)	700 (500–980)[+]	430 (310–600)	490 (420–570)[‡]
	ED 84		740	940	1350	920	710
Ethosuximide	ED 50	45	> 1000	350 (250–480)	350 (250–490)[+]	420 (350–500)	> 1000
	ED 84			580	720	620	
Trimethadione	ED 50	45	470 (350–630)	460 (360–590)	440 (360–540)[+]	480 (400–570)[+]	580 (400–840)
	ED 84		660	700	600	690	850
Dimethadione	ED 50	90	> 1500	420 (250–710)	> 1500	1100 (730–1650)	> 1500
	ED 84			1350		2500	

All anticonvulsants were administered orally, the convulsants subcutaneously. In the case of truncated dose–effect curves only the ED 50 is given (truncation occurred when increasing the dose of the anticonvulsant drug resulted in no further increase of the protective effect or in a decrease of this effect compared to lower doses).

* Confidence limits for 95% probability.

[+] From Löscher and Frey [4]

[‡] From Frey and Löscher [20]

Table 4. Antagonism of anticonvulsant drugs against the MP-induced changes in the activities of GAD and GABA-T

	Doses mg/kg	Time of pretreatment (min)	GAD μmoles/g/hr	GABA-T f.u./g/hr
Controls	- -	—	56.6 ± 4.8 (60)	60.3 ± 6.6 (73)
3-Mercaptopropionic acid (MP)	60	—	38.1 ± 4.2‡ (76)	76.4 ± 11.4‡ (70)
Phenobarbital + MP	30 + 60	120	43.2 ± 8.8+ (6)	61.4 ± 5.5 (6)
Phenytoin + MP	28 + 60	120	39.4 ± 3.5‡ (6)	61.3 ± 5.3‖ (6)
Carbamazepine + MP	66 + 60	60	42.9 ± 4.7‡ (6)	60.6 ± 4.6‖ (6)
Diazepam + MP	3.5 + 60	60	40.1 ± 3.8‡ (12)	66.1 ± 9.4§ (12)
Sodium valproate + MP	740 + 60	45	40.6 ± 5.6‡ (18)	56.7 ± 4.3‖ (18)
Ethosuximide + MP	580 + 60	45	38.4 ± 3.2‡ (6)	75.2 ± 5.3‡ (6)
Trimethadione + MP	660 + 60	45	43.9 ± 4.1‡ (6)	55.2 ± 6.1‖ (6)
Dimethadione + MP	600 + 60	90	39.7 ± 4.9‡ (6)	77.8 ± 5.8‡ (12)
Dimethadione + MP	1350 + 60	90	37.2 ± 2.6‡ (6)	76.2 ± 5.5‡ (6)

The anticonvulsants were administered orally in the ED 84 determined against MP (Table 3) except ethosuximide (ED 84 against picrotoxin) and dimethadione (equimolar dose to the ED 84 of trimethadione against MP and ED 84 against picrotoxin, respectively). The mice were killed 5.6 min after the subcutaneous injection of the CD 97 of MP (mean latency of convulsions, Table 1).

* Fluorescence units.

Significant changes compared to controls are denoted + $P < 0.01$, ‡ $P < 0.001$.

Significant changes compared to mice treated with MP alone are denoted §$P < 0.01$, ‖$P < 0.001$.

showed hyperexcitability when being handled. At about 3 to 4 hr after administration, animals showed hunched posture, loss of the righting reflexes and appeared to develop muscular weakness which resulted in an inability to support themselves, the severity of the symptoms depending on the dose. Whereas mice recovered after administration of 30 mg/kg gabaculine within one day, only showing some loss in weight; mice being injected with higher doses showed severe side effects as total loss of righting reflexes, excessive urination and hypothermia for 5 to 6 days and some mice died within 24–120 hr after the injection. The average time to death was 74 hr ($n = 50$), the LD 50 62 mg/kg (54–71).

Due to the high toxicity of AOAA and gabaculine, only the ED 50s against MP were studied in their ability to antagonize the alterations in the activities of GAD and GABA-T caused by MP. As shown in Table 6, the effects of both compounds were very similar: They antagonized the inhibition of GAD, reduced the activity of GABA-T to zero and raised brain GABA to more than 500 per cent of control values.

(*c*) *Ketamine.* As shown in Table 7, ketamine was very effective in the MES test, neither the ED 50 or the ED 84 showing any side effects, but produced a marked suppression of MP and pentetrazole convulsions only at doses approaching the anesthetic level. The ketamine-induced catalepsy, with doses of 60–90 mg/kg, characterized by the loss of righting reflexes with a latency of 1 to 4 min and a duration of about 25 min, was antagonized by MP and even more by pentetrazole. However, at doses exceeding 100 mg/kg all mice lay on their sides for about 20 min and MP or pentetrazole induced convulsions in some of them when being risen from recumbent position. Furthermore, ketamine at both the ED 50 and ED 84 against MP was without influence on the alteration in the activities of GAD and GABA-T caused by MP (Table 7).

DISCUSSION

In the present study it was confirmed that seizures elicited by MP in mice are paralleled by a pronounced decrease in the activity of GAD and a considerable increase in that of GABA-T. However, the central GABA level remained unchanged after the convulsant ED 97 of MP, in spite of marked alterations in enzyme activities. This might be due to the compartmentation of GABA into at least two pools within the central nervous system [29]. It has been suggested that inhibition of GAD is the critical factor in the development of seizures since it resulted directly in a decrease of GABA released in the synaptic cleft [30, 31].

Only after increasing the dose of MP threefold, a significant decrease in GABA concentration was demonstrable which corresponds to the results of Rodriguez de Lores Arnaiz *et al.* [9, 10]. The finding that after the threefold CD 97 of MP (180 mg/kg) the activation of GABA-T was not further enhanced but reduced compared to lower doses, may be connected with *in vitro* studies of Lamar [6] and Wu [32], who showed that higher concentrations of MP inhibited GABA-T competitively, the K_i value being about 10

Table 5. Comparison of the effects of trimethadione and its metabolite dimethadione against MP-induced convulsions and biochemical alterations

Drug	Dose mg/kg	Time of pretreatment min	Brain concentration µg/ml		Mice protected %	GABA µmoles/g	GAD µmoles/g/hr	GABA-T f.u./g/hr *
			Trimethadione	Dimethadione				
Controls		—	—	—		1.72 ± 0.34 (17)	55.2 ± 5.2 (18)	61.2 ± 5.8 (18)
Mercaptopropionic acid (MP)	60	—	—	—		1.88 ± 0.23 (12)	35.8 ± 3.7** (18)	77.2 ± 9.4** (18)
Trimethadione + MP	660 + 60	45	400 [310–510]+‡ (6)	54 [27–90]§ (6)	84	2.17 ± 0.36** (6)	43.9 ± 4.1** (6)	55.2 ± 6.1++ (6)
Dimethadione + MP	600 + 60	90	—	430 [270–640]‖ (12)	0	2.0 ± 0.33 (6)	39.7 ± 4.9** (6)	77.8 ± 5.8** (12)
Dimethadione + MP	1350 + 60	90	—	880 [690–1050]¶ (5)	20	1.91 ± 0.45 (6)	37.2 ± 2.6** (6)	76.2 ± 5.5** (6)

Trimethadione was administered orally in the ED 84 determined against MP, dimethadione in the corresponding equimolar dose and in the ED 84 against picrotoxin (Table 3). The mice were killed 5.6 min after the subcutaneous injection of the ED 97 of MP (mean latency of convulsions) for determination of brain concentrations of trimethadione, dimethadione, GABA as well as the activities of GAD and GABA-T.

* Fluorescence units.

+ Mean and range.

‡ Mean serum concentration 490 µg/ml. § 120 µg/ml. ‖ 800 µg/ml. ¶ 1900 µg/ml.

** $P < 0.001$ when compared with controls.

++ $P < 0.001$ when compared to mice treated with MP alone.

Table 6. Toxicity and anticonvulsant activity of AOAA and gabaculine against the ED 97 of MP, pentetrazole and in the maximal electroshock seizure test and antagonism of both drugs against the MP-induced biochemical changes

Drug	Dose	Time of pretreatment (hr)	Mercaptopropionic acid (MP) 60 mg/kg	Pentetrazole 100 mg/kg	Maximal electroshock seizure test	LD 50 mg/kg
AOAA	ED 50	6	27 (18–41)*	27 (15–46)†	28 (17–41)†	40 (34–47)†
	ED 84		46	70	50	
Gabaculine	ED 50	4	135 (80–230)	260 (180–380)	200 (110–360)	
	ED 84		250	560	480	62 (54–71)
	mg/kg		GABA μ moles/g	GAD μmoles/g/hr	GABA-T f.u./g/hr‡	—
Controls	—	—	1.92 ± 0.15 (13)	5.7 ± 4.2 (12)	59.8 ± 5.5 (12)	
MP	60	—	1.88 ± 0.23 (12)	38.5 ± 6.2§ (12)	91.4 ± 6.4§ (12)	
AOAA + MP	27 + 60	6	9.9 ± 1.2§‖ (6)	57.2 ± 3.5‖ (6)	no activity detectable	
Gabaculine + MP	135 + 60	4	10.8 ± 2.1§‖ (6)	56.2 ± 9.5‖ (6)	no activity detectable	

For evaluation of the latter effect, both drugs were administered subcutaneously (AOAA) or intraperitoneally (gabaculine) in the ED 50 against MP and mice were decapitated 5.6 min after subcutaneous injection of the CD 97 of MP (mean latency of convulsions).

* Confidence limits for 95% probability.

† From Löscher and Frey [15].

‡ Fluorescence units.

§ $P < 0.001$ when compared with controls.

‖ $P < 0.001$ when compared with mice treated with MP alone.

Table 7. Anticonvulsant activity of ketamine against the ED 97 of MP, pentetrazole and in the maximal electroshock seizure test

Drug	Dose	Time of pretreatment min	Mercaptopropionic acid (MP) 60 mg/kg	Pentetrazole 100 mg/kg	Maximal electroshock seizure test
Ketamine	ED 50	1	73 (56–95)* [110]		
	[ED 84]	4		71 (47–100) [120]	
		7			5.9 (5.5–6.3) [6.9]
	mg/kg		GAD μmoles/g/hr		GABA-T f.u./g/hr
Controls	—	—	57.3 ± 2.9 (6)		61.2 + 5.3 (6)
MP	60	—	37.2 + 3.2§ (6)		72.2 ± 3.6‡ (6)
Ketamine + MP	73 + 60	1	38.2 ± 2.5§ (6)		72.9 ± 2.3§ (6)
Ketamine + MP	110 + 60	1	40.9 ± 5.3§ (6)		74.9 ± 1.5§ (6)

In order to determine an antagonism of ketamine against the MP-induced changes in the activities of GAD and GABA-T, ketamine was administered intraperitoneally 1 min prior to the subcutaneous injection of the ED 97 of MP and mice were killed 5.6 min after the latter injection (mean latency of convulsions).

* Confidence limits for 95% probability.

† Fluorescence units.

§ $P < 0.001$ when compared with controls.

‡ $P < 0.01$.

fold higher than that for inhibition of GAD. Phenobarbital, phenytoin, carbamazepine, diazepam, sodium valproate and trimethadione were shown to protect mice from seizures induced by MP. The ED 50s of these drugs against MP were similar to those determined against picrotoxin, but, except in the case of trimethadione clearly below those against strychnine-induced convulsions. These results pointed to differences in the mechanism of action of the anticonvulsants against MP and picrotoxin on the one side and strychnine on the other. However, ethosuximide and dimethadione, the active metabolite of trimethadione, were devoid of a true protective effect against MP-seizures, though both drugs were active against picrotoxin.

All anticonvulsants except those which were ineffective against MP-induced seizures, i.e. ethosuximide and dimethadione, reversed the activation of GABA-T caused by MP but hardly affected the inhibition of GAD.

It was recently shown that diazepam, sodium valproate, ethosuximide and trimethadione antagonized the inhibition of GAD caused by isoniazid in doses which protected mice from the seizures induced by high doses of this drug [4]. However, the effects of the anticonvulsants on both GAD inhibitors can not easily be compared, because isoniazid acting as a pyridoxal antagonist must be regarded as a rather unspecific enzyme inhibitor and is in this respect only of weak potency when compared with MP *in vitro* [4, 6, 7, 33].

The antagonism of the MP-induced activation of GABA-T by the respective anticonvulsants contrasts with previous studies dealing with the effects of these drugs on the normal activity of GABA-T: an inhibition of the enzyme has been demonstrated only for phenobarbital, carbamazepine and sodium valproate in high concentrations [34–36].

AOAA and gabaculine displayed a definite and dose-dependent anticonvulsant effect against MP, but, compared to AOAA, gabaculine was markedly less potent on a mg/kg basis. However, it has to be considered that the time of maximum anticonvulsant effect was not reached 4 hr after administration of gabaculine.

Both drugs antagonized the inhibition of GAD caused by MP completely, reduced the activity of GABA-T to zero and raised brain GABA by more than 500 per cent.

However, the anticonvulsant doses of both AOAA and gabaculine were potentially (AOAA) or absolutely (gabaculine) lethal. Whereas the high toxicity of AOAA has been described by several authors [15, 37–38] that of gabaculine has not been mentioned before, probably due to the fact that, contrary to AOAA, the animals did not die before 24–120 hr after administration. The side effects of both drugs were somewhat similar and some of these signs, especially those of impaired motor function, have been reported with other agents increasing brain GABA level by inhibition of GABA-T, i.e. γ-acetylenic GABA [39] and ethanolamine-*O*-sulphate [40], and with GABA itself [38, 41]. Thus, the correlation between changes in motor behaviour and inhibition of GABA-T by different agents suggests that at least in part acute neurotoxicity may be related to the altered GABA metabolism.

Ketamine, which has been reported to possess anticonvulsant activity against MP [16] was in this respect only effective at doses approaching the anaesthetic level and failed to affect the biochemical alterations caused by MP.

In conclusion, these data could be interpreted as follows: provided that GAD is the rate limiting enzyme in the synthesis of presynaptically located GABA [42] and the action of GABA released into the synaptic cleft is terminated by a high affinity uptake system followed by metabolism by a sequence of reactions beginning with GABA-T [43] which regulates GABA turnover [44–45], one can assume that after GAD is rapidly inhibited by administration of MP, the activity of the uptake system and the rate of metabolism of GABA determine when (and if) the concentration of GABA within GABAergic synaptic terminals drops below a "convulsive level". It has been suggested that the GABA within the "transmitter pool" has a half-life of about 15 to 30 min whereas that within the large "metabolic pool" has a half-life in the order of hours [44, 46]. So activation of GABA-T by MP might be of significance in depressing the half-life of GABA within the neuronal pool. By antagonizing this activation by anticonvulsants it either takes a longer time before the concentration of GABA falls below the "convulsive level" or this will not occur at all, since inhibition of GAD by MP has been shown to be readily reversible within 30 min after administration [9, 12]. AOAA and gabaculine blocked the metabolism of GABA completely and antagonized the inhibition of GAD by MP. Furthermore, with respect to the uptake system, gabaculine has been demonstrated as a moderately potent inhibitor (IC 50 = 69 μm) [47] which may also contribute to its anticonvulsant action.

Drugs which were without influence on the activation of GABA-T and inhibition of GAD by MP were devoid of a true anticonvulsant effect (ethosuximide, dimethadione, ketamine).

However, this concept has to be further elaborated and we are aware that the protective activity of the respective anticonvulsants against MP-seizures may only be a consequence of more unspecific mechanisms by which all of these drugs act to a certain extent, e.g. stabilisation of neuronal membranes or prevention of the spread of seizure discharge. Nevertheless our results with MP and picrotoxin suggest that the anticonvulsant effect of phenobarbital, phenytoin, carbamazepine, diazepam, sodium valproate and trimethadione against these convulsants are at least in part mediated through an influence on the GABA system, whereas such an action for ethosuximide and dimethadione seems questionable. In this context MP proved to be a valuable addition to the range of convulsants which can be used in investigations into the mechanisms of anticonvulsant drug action.

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