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AMINOOXYACETIC ACID: CORRELATION BETWEEN BIOCHEMICAL EFFECTS, ANTICONVULSANT ACTION AND TOXICITY IN MICE

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Abstract—The time courses of changes in brain content of γ-aminobutyric acid (GABA) and sensitivity to convulsions were studied in mice after administration of aminooxyacetic acid (AOAA) (30 mg/kg, s.c.). There was a good correlation of increase in GABA content with elevation of the thresholds for the electroconvulsion as well as for the pentetrazole convulsion, the maximal effect being after 6 hr. The activity of GABA-α-oxoglutarate aminotransferase (GABA-T) was reduced to near zero within 30 min of administration of AOAA, whereas the activity of glutamate decarboxylase (GAD) was not influenced *in vivo*. However, AOAA had a pronounced inhibitory effect on both enzymes *in vitro*. Based on the results of the threshold determinations, the anticonvulsant effect of AOAA was determined 6 hr after administration in two tests: the maximal electroshock seizure test and the pentetrazole seizure threshold test. AOAA had a definite and dose-dependent anticonvulsant effect in both tests (ED_{50} 28 and 27 mg/kg, respectively), but in a range being potentially lethal (LD_{50} 40 mg/kg). Supplementation with pyridoxine via the drinking water for 3 days (daily intake 29 mg/kg) elevated the LD_{50} significantly to 70 mg/kg and increased the anticonvulsant effect, but was without influence on the inhibition of GABA-T or the levels of GABA in brain.

The possible role of γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter in mammalian brain tissue led to the speculation that compounds which elevated the concentration of the amino acid in brain might act as anticonvulsant agents (for review see [1]). Aminooxyacetic acid (AOAA) has been shown to cause an increase of GABA in brain upon administration to various species by inhibiting the GABA-degrading enzyme γ-aminobutyrate-α-oxoglutarate aminotransferase (GABA-T) [2]. Although AOAA could be shown to possess anticonvulsant properties, complete dose–response relationships were not presented and the protection against electroshock and drug-induced seizures in most studies was not related to the AOAA-induced elevation in brain GABA level [3–6]. Besides the inhibition of GABA-T, AOAA is an effective inhibitor of a number of aminotransferase and decarboxylase enzyme systems because it complexes with the essential cofactor, pyridoxal phosphate [7]. In addition, AOAA inhibits pyridoxalphosphokinase, the enzyme that catalyses the phosphorylation of pyridoxal, pyridoxine and pyridoxamine [8]. Higher doses of AOAA result in severe side effects in several species like impairment of motor function and convulsions [5, 9, 10]. Toxicity of the compound has been associated with the production of a vitamin B_6 deficiency, however, concomitant administration of equimolar concentrations of pyridoxine resulted in an unexpected potentiation of AOAA toxicity [11].

In the present study, the time course of the biochemical and pharmacodynamic effects of AOAA should be quantitated and correlated. In further experiments, the influence of a massive pyridoxine substitution on anticonvulsant effect and toxicity was studied.

MATERIALS AND METHODS

Animals. Male mice of the NMRI-strain (Bomholtgård 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°. Mice were fed on Altromin standard food R 1224 (Altrogge, Lage, Germany), the vitamin B_6 content of which was determined by Landwirtschaftliche Untersuchungs- und Forschungsanstalt Kiel to 18.6 mg/kg, an amount considered sufficient for mice [12].

Biochemical determinations in vivo. Groups of 20 mice were injected subcutaneously with 30 mg/kg AOAA and were killed by decapitation at different times after administration of AOAA. One half of the surviving mice in each group was used for the determination of the activity of glutamic acid decarboxylase (GAD; EC 4.1.1.15) and γ-aminobutyrate-α-oxoglutarate aminotransferase (GABA-T; EC 2.6.1.19), the other half for the determination of the GABA level in brain. For the determination of the activities of GAD and GABA-T, the brains were immediately removed and homogenized in 4.0 ml icecold water (tubes immersed in a bath of methanol at −1.0°). The activity of GAD was determined by the method of Lowe *et al.* [13], that of GABA-T by the method of Salvador and Albers [14]. Details of these methods have been described in a previous paper [15]. The results of the GABA-T determinations are given in 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.

For the determination of GABA a newly developed gas chromatographic method was used. After decapi-

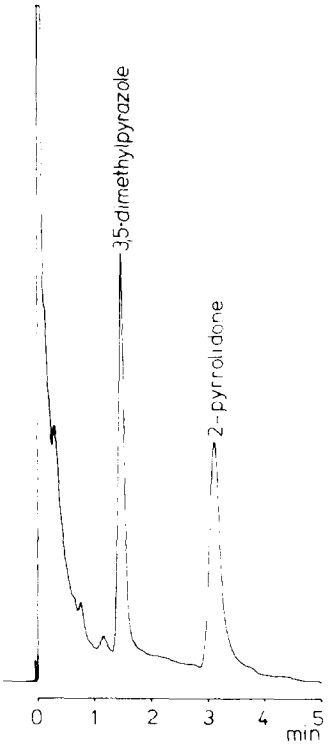

Fig. 1. Gas chromatogram of an extract of brain of an unpretreated mouse on 10% Carbowax 6000. The first peak is that of the internal standard used, the second peak results from GABA by dehydration on the column and was used for the calculation of the GABA concentration. Attenuation was 0.1 × 128.

tation, brains were immediately removed and homogenized in 10 ml ice-cold 70% aqueous ethanol (tubes immersed in a bath of methanol at −1.0°). The time from decapitation to homogenation never exceeded 30 sec. Under these conditions, GABA levels were identical to those obtained after freezing of the brains in liquid nitrogen and, by extrapolation to zero time, the postmortal rise was calculated to about 15 per cent only [15]. This is in agreement with results of Alderman and Shellenberger [16]. After homogenization, the tubes were centrifuged at 4000 rev./min for 15 min at 0°. The supernatants were transferred to separating funnels and the residues were resuspended twice in 5 ml 70% ethanol and again centrifuged; the combined supernatant (20 ml) was shaken for 2 min with 20 ml chloroform. The aqueous phase was centrifuged and the clear supernatant was evaporated to dryness in a rotating evaporator at a maximum temperature of the waterbath of 40°. The residue was dissolved in 1 ml distilled water containing 50 μg/ml 3,5-dimethylpyrazole as internal standard and 5 μl were injected into the gas chromatograph. Analysis was carried out with a Varian 1200 gas chromatograph equipped with a glass column (6 ft., 2 mm I.D.) packed with 10% Carbowax 6000 on Chromosorb WAW (80/100). The temperature of the column was 160°, injector block and FID were kept at 240°. Nitrogen, hydrogen and air flow rates were 46, 50 and 300 ml/min, respectively. When calculating the concentrations of GABA, the peak area was compared with that of the internal standard. Peak areas were calculated as the product of peak height and width at half height. Standard curves for the quantitation of GABA were prepared by analysing a series of aqueous standard solutions of GABA.

The peak in the gas chromatogram after injection of standard solutions of GABA as well as of brain extracts was kindly identified by K. Jakobs (Department of Pediatrics, Free University) using a gas chromatograph mass spectrometer system consisting of a Varian 2740 gas chromatograph connected to a double focussing mass spectrometer (Varian MAT, type 311 A). Gas chromatographic conditions were the same as described above. The temperature of the molecular separator and the ion source was 250°, and the ion source had an electron energy of 93 eV.

A sample gas chromatogram of a brain extract obtained from an unpretreated mouse is shown in Fig. 1. The peak was identified by mass spectrometry as 2-pyrrolidone, the lactam of GABA, resulting from GABA by dehydration on the column (Fig. 2). For the final estimation of the structure, synthetic 2-pyrrolidone was also examined by g.c. and g.c.-m.s. tech-

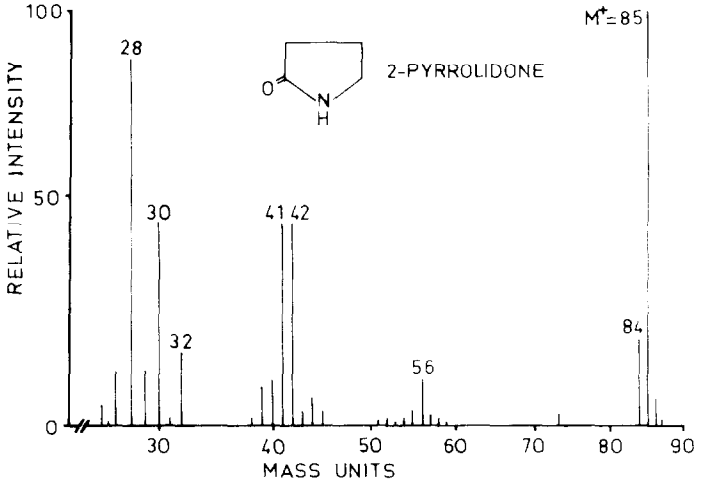

Fig. 2. Mass spectrum of the peak that occurs in the gas chromatogram after injection of standard solutions of GABA as well as of brain extracts from mice.

niques. The retention time on g.c. and the mass spectrum of the authentic compound were identical with those of the unknown.

Biochemical determinations in vitro. To study the effect of AOAA on the activity of GAD and GABA-T *in vitro*, AOAA was added in concentrations of 0.25–4 μg/ml to brain homogenates from unpretreated mice. AOAA was added in a volume of 20 μl of adequate strength as to provide the final concentration in the sample before incubation. The pH of the sample was not altered by AOAA. Six determinations were done with each concentration.

Convulsant thresholds. The electro- and chemoconvulsant thresholds were determined at the following times after subcutaneous administration of 30 mg/kg AOAA: 1, 2, 4, 6, 8 and 24 hr. Electroshock was applied by eye electrodes using an A-615-B shocker (Lafayette Instrument Co.). Stimulation data were 50 cycles/sec for 0.2 sec with the serial resistance of the apparatus set to 10 kΩ. Endpoint was the tonic extension of the hind limbs. For the determination of the electroconvulsant threshold the voltage of the shock was changed according to the "up and down" method of Kimball *et al.* [17]. The threshold for clonic and tonic chemoconvulsions was determined in freely moving mice by the method of Hint and Richter [18]. A 1% solution of pentetrazole was infused intravenously at a rate of 0.3 ml/min by means of a Sage syringe pump, model 351. Endpoints were the fully developed clonic siezure with loss of the righting reflexes and the tonic extension of the hind limbs. Twenty mice were used for each threshold determination. The thresholds are given as the voltage or the dose of pentetrazole, provoking the respective endpoint in 50 per cent of the mice.

Anticonvulsant effect and toxicity of AOAA. The LD_{50} and the anticonvulsant ED_{50}s in the maximal electroshock seizure test and the pentetrazole seizure threshold test [19] were determined after s.c. administration of AOAA with the method of Litchfield and Wilcoxon [20]. Groups of 20 mice per dose were used, however, for the dose of 30 mg/kg the number of animals used for the determination of the lethal dose was considerably higher since the threshold determinations had been performed with this dose. Based on the results of the threshold determinations, the anticonvulsant effect was determined 6 hr after administration of AOAA. Electroshock was given by an Lafayette shocker A-615-B using eye electrodes; stimulation data were 250 V at 50 cycles/sec for 0.2 sec, serial resistance 10 kΩ. The tonic extension of the hind limbs was used as endpoint. In the pentetrazole seizure threshold test, a dose of 100 mg/kg pentetrazole was injected s.c. and the mice were observed for 30 min for clonic convulsions with loss of the righting reflexes. AOAA was injected in a volume of 10 ml/kg.

In further experiments, the LD_{50} and the ED_{50} in the maximal electroshock seizure test were re-determined in mice that had received 0.1 mg/ml pyridoxine HCl with the drinking water for 3 days. The daily intake of pyridoxine was 29 ± 1.9 mg/kg in these experiments.

Statistics. Arithmetical means and S.D. are given for the biochemical determinations. Significance of differences was calculated by Student's *t*-test except in the case of ED_{50}s and LD_{50}s, in which the Chi^2-test was used.

Drugs and reagents. The following drugs and reagents were used: Aminooxyacetic acid hemihydrochloride and 3,5-dimethyl-pyrazole (Ferak, Berlin), 2-pyrrolidone, γ-aminobutyric acid, pyridoxine hydrochloride and pyridoxal 5-phosphate (E. Merck, Darmstadt), pentetrazole (Knoll AG, Ludwigshaven).

RESULTS

Normal GABA level and enzyme activities. The GABA level was determined to 226 ± 20 μg/g ($n = 27$), GAD activity to 48 ± 6.1 μmoles/g/hr ($n = 18$) and the activity of GABA-T to 59 ± 5.3 f.u./g/hr ($n = 19$) in untreated control mice.

Effect of AOAA on the activities of GAD and GABA-T in vitro. AOAA had a pronounced inhibitory effect on GABA-T (IC_{50} 0.43 μg/ml) and GAD (IC_{50} 1.8 μg/ml). This effect was not reversible when the concentration of pyridoxal phosphate was raised by 300 μg/ml.

Effect of AOAA on the activities of GAD and GABA-T and the level of GABA in the brain in vivo. A dose of 30 mg/kg of AOAA was without influence on the activity of GAD, but that of GABA-T was reduced to near zero within 30 min of administration (Fig. 3). GABA levels increased to a maximum of about 1 mg/kg at 6 hr and were still considerably above control levels at 24 hr (Fig. 3, Table 1). There was no difference in GABA levels and enzyme activities when mice dying of AOAA were compared to others showing no severe side effects at the same time. Pyridoxine supplementation was without influence on GABA levels and enzyme activities (Table 1).

Convulsant thresholds. The thresholds for the electroconvulsions as well as for the clonic and tonic component of the pentetrazole convulsion were clearly elevated after administration of AOAA, the time course showed a nice parallelity to the central GABA-levels (Fig. 3). The maximal effect was after 6 hr in the pentetrazole thresholds, whereas the threshold for electroconvulsions seemed identical at 2, 4 and 6 hr.

Toxicity of AOAA. Approximately 5 to 10 min after mice had been injected with AOAA, severe side effects were observed, beginning at doses of 10–15 mg/kg: The mice seemed excited at first, showed then ataxia and loss of muscular tonus. Later, such animals occupied a prone position with their legs extended spastically and in bizarre positions. They were apparently unable to perform any coordinated motor activity. After clonic and tonic convulsions, the mice lay finally 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 (mean ± S.D. of 160 mice), LD_{50} was 40 mg/kg (34–47). In mice supplemented with pyridoxine via the drinking water the LD_{50} was significantly elevated (70 mg/kg, $Chi^2 = 19.2$, $n = 2$, $P < 0.001$) (Table 1, Fig. 4).

Since some anesthetized rats developed hyperthermia after the injection of AOAA in blood pressure experiments, we followed the body temperature of mice after the s.c. administration 30 mg/kg of AOAA.

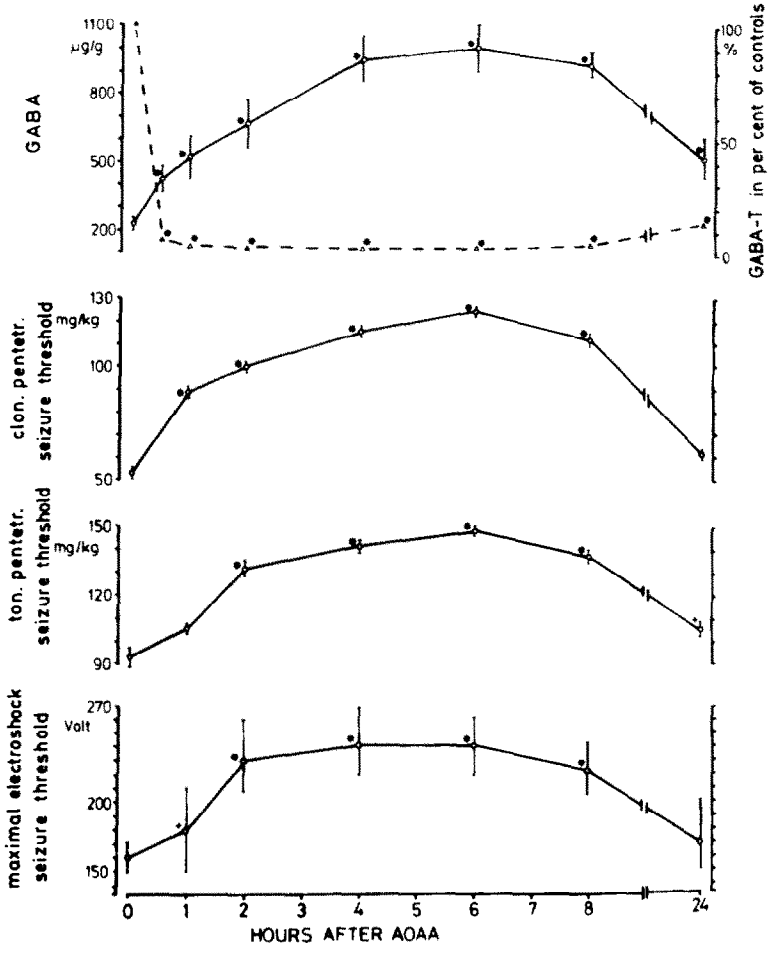

Fig. 3. Central GABA level (O——O), activity of GABA-T (Δ---Δ) and electro- and chemoconvulsant thresholds in mice as a function of time after administration of AOAA (30 mg/kg s.c.). For GABA the mean ± S.D. of 6–8 determinations is shown. The thresholds are given as the voltage or the dose of pentetrazole providing the respective endpoint in 50 per cent of the mice. The vertical bars represent the confidence limits for 95 per cent probability in the maximal electroshock seizure threshold or, respectively, the S.D. in the pentetrazole seizure threshold. Values significantly different from controls are marked by + ($P < 0.05$) or * ($P < 0.001$).

During the first hour, a considerable hypothermia developed but there was no difference between the mice dying and those surviving.

Anticonvulsant effect of AOAA. 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. Nevertheless, the results show clearly that AOAA had a definite and dose-dependent anticonvulsant effect of identical

Table 1. Toxicity, anticonvulsant and biochemical effects of AOAA in mice without or with pyridoxine supplementation via the drinking water (0.1 mg/ml for 3 days, daily intake 29 ± 1.9 mg/kg). AOAA was given subcutaneously, 6 hr before testing for anticonvulsant effect

Pretreatment	Anticonvulsant ED_{50} (mg/kg) Maximal electroshock seizure test	s.c. Pentetrazole seizure threshold test	LD_{50} (mg/kg)	GABA (µg/g) Controls	GABA (µg/g) 6 hr after AOAA (30 mg/kg)	GABA-T (f.u./g/h)* Controls	GABA-T (f.u./g/h)* 6 hr after AOAA (30 mg/kg)
none	28 (17–41)†	27 (15–46)	40 (34–47)	224‡ ±17	1010¶ ±85	58‡ ±6.3	2.48 (0–8.8)
Pyridoxine	21¶ (15–28)	—	70¶ (48–102)	230 ±21	1085¶ ±77	60 ±5.3	4.18 (1.2–4.7)

* Activity given in arbitrary fluorescence units.
† Confidence limits for 95 per cent probability.
‡ Mean ± S.D.
§ Mean and range observed.
¶ $P < 0.001$.

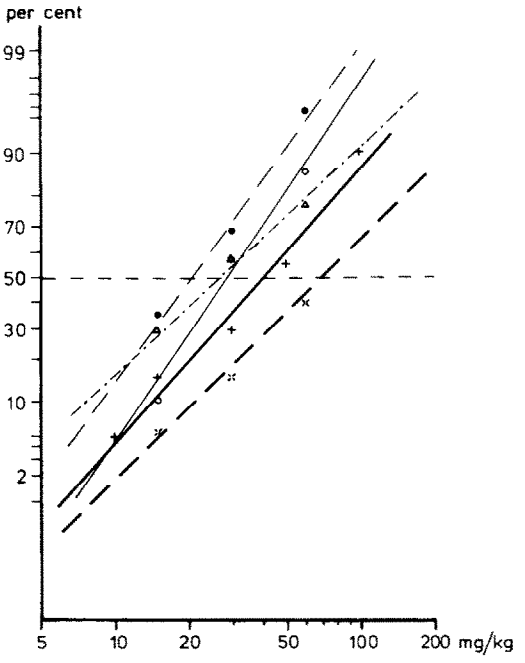

Fig. 4. Dose–effect curves of AOAA against the pentetrazole seizure threshold test (Δ—·—Δ), and the maximal electroshock seizure test before (○——○) and after (●---●) supplementation with pyridoxine. In addition, the lethal effect of AOAA before (+——+) and after (×——×) supplementation with pyridoxine is shown. The ordinate gives the per cent of mice protected or dying by AOAA, respectively, the abscissa the dose of AOAA (mg/kg s.c.).

potency in both tests employed, but in a range being potentially lethal (Table 1, Fig. 4). Supplementation with pyridoxine lowered the ED_{50} in the maximal electroshock seizure test slightly but significantly from 28 to 21 mg/kg ($Chi^2 = 16.3$, $n = 2$, $P < 0.001$) (Table 1, Fig. 4).

DISCUSSION

The question of an anticonvulsant action of AOAA has always been subject to controversy: the effect seemed most convincing when convulsions elicited by agents interfering with the central metabolism of pyridoxine were concerned [3, 21–23], but the effect in conventional models of epilepsy, as f.e. electroconvulsions or pentetrazole-induced convulsions, was less clear, possibly because dose-effect curves never have been presented. Also the relation between elevation of GABA levels in brain and anticonvulsant effect remained an open question: the maximum of the latter has been reported to occur considerably before maximal GABA levels were reached in the brain [1, 3, 4]. In a previous study, isoniazid-induced elevations of the GABA levels in the brain of up to 170 per cent of the control level had been devoid of any effect on convulsive thresholds in mice [24], and we have considered an elevation of the "metabolic pool" of GABA by inhibition of GABA-T to be of minor importance for seizure susceptibility than alterations in the "transmitter pool" mainly governed by the activity of GAD [15].

It was therefore rather unexpected to find a close parallelity between the increase in cerebral GABA concentrations and the convulsive thresholds for electroconvulsions and both components of the pentetrazole convulsion in the present study (Fig. 3). This must not be an absolute contradiction to our previous conclusions: during complete inhibition of GABA-T by AOAA, GABA levels rose by a factor of 5, and, under these conditions, the surplus GABA might be taken up into the "transmitter pool" located in the presynaptic nerve endings.

Six hr after AOAA, when GABA levels were highest, dose-effect curves for the anticonvulsant effect of the drug against maximal electroconvulsions and pentetrazole-induced convulsions could be constructed; the effective doses were, however, in a range potentially lethal for mice.

Supplementation with high doses of pyridoxine enhanced the anticonvulsant effect but was without influence on the degree of inhibition of GABA-T and on the levels of GABA in the brain. This might point to some effects of AOAA unrelated to the well known action of the compound on the metabolism of GABA.

The other interesting feature of the present study was the high toxicity of AOAA for mice, an effect previously mentioned by DaVanzo *et al.* [3] and van Gelder [10]. The LD_{50} was in fact only slightly higher than the anticonvulsant ED_{50}s, but the variation in lethality from group to group was considerable: In 30 groups of 10 mice receiving a dose of 30 mg/kg, the death rate varied from 10 to 50 per cent, averaging 29 per cent in a total of 300 mice. The diet was not deficient in pyridoxine, but supplementation of the vitamin with the drinking water nevertheless reduced toxicity significantly. At least the lethal effect of AOAA seemed not to be related to the alterations in the metabolism of GABA since the deaths occurred during the first hour after administration, i.e. long before GABA levels had reached their maximum. A comparison of GABA levels and enzyme activities in mice dying from the drug and in others behaving quite normally at the same time did not reveal any differences. Thus, the acute toxicity of AOAA in mice must be considered as rather independent of the effect on the metabolism of GABA, it may, however, be related to an inhibition of other pyridoxal phosphate-dependent enzymes as already suggested by DaVanzo *et al.* [11]. The effect of pyridoxine treatment points to a certain reversibility of the inhibition of these latter enzymes. The inhibition of GABA-T must be considered as irreversible, normal activity of this enzyme did not recur before 20 days after the administration of AOAA in the experiments of Rubinstein and Roberts [25] and high concentrations of pyridoxal phosphate did not antagonize the inhibition of GABA-T *in vitro*.

The inhibitory effect of AOAA on GAD activity was rather strong *in vitro* but could not be reproduced *in vivo* though adequate concentrations should have been reached with the doses used. Roberts and Simonsen [7] have tried to explain these findings by the different localisation of GAD and GABA-T.

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