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ANTICONVULSIVE ACTION OF SUBSTANCES DESIGNED AS INHIBITORS OF γ-AMINOBUTYRIC ACID-α-KETOGLUTARIC ACID TRANSAMINASE*

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Abstract—5-Methyl,5-phenyl-2-pyrrolidinone (MPP) and 5-ethyl,5-phenyl-2-pyrrolidinone (EPP) were designed and synthesized to penetrate the blood–brain barrier and to be hydrolyzed there to yield γ-aminobutyric acid analogs (GABA-analogs) that will inhibit γ-aminobutyric acid-α-ketoglutaric acid transaminase (GABA-T).

Both EPP and MPP exhibited significant inhibiting action upon GABA-T activity of mouse and rat brain homogenates. This inhibition was markedly reduced or even disappeared when GABA concentration was doubled, indicating its competitive type. γ-Ethyl-γ-phenyl-γ-aminobutyric acid (EPGABA) caused a stronger inhibition of GABA-T activity of rat brain homogenates, which favors the idea that EPP is hydrolyzed by the homogenates to yield EPGABA, the active substance.

The brain levels of glutamic acid, aspartic acid, glutamine, and GABA were not modified by intraperitoneal injection of 160 mg of either EPP or MPP/kg.

EPP at doses of 80 and 120 mg/kg exhibited a strong protective action in mice against death caused by thiosemicarbazide: EPP at 80 mg/kg showed an even stronger protection against pentylenetetrazol.

Doses of 25 and 50 mg of EPP/kg did not significantly impair a conditioned instrumental response in rats. A dose of 120 mg EPP/kg inhibited completely the conditioned response and also occasionally the unconditioned, but did not inhibit tne patellar, flexor, palpebral, and pupillary reflexes. MPP did not affect the conditioned response at doses up to 200 mg/kg.

Both EPP and MPP exhibited a powerful anticonvulsive action in rats against maximal electroshock, at doses from 25 to 200 mg/kg. Both the tonic and clonic phases were reduced by 50–98% and were sometimes completely abolished. Diphenylhydantoin produced similar effects in the same experimental conditions.

AN IMPAIRMENT in the biosynthesis of γ-aminobutyric acid (GABA) is apparent in a number of convulsive states[1–4]; nevertheless, the administration of large doses of GABA has a definite anticonvulsive action.[5] The need for such large doses is attributed to the low permeability of the blood-brain barrier to a polar molecule like GABA. The 2-pyrrolidinone has a stronger anticonvulsive action than GABA,[6] apparently because it is a less polar molecule that penetrates more easily to the brain where it is hydrolyzed to yield GABA.[7]

The main catabolic pathway of GABA is its transamination with α-ketoglutaric acid.[8] It has been found that the inhibition of this reaction by hydroxylamine or

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aminooxyacetic acid produces an increase in GABA levels[9–12] and counteracts the convulsive action of several agents, although the maximal anticonvulsive action does not coincide with the highest cerebral GABA level.[13,14]

This report presents data on three substances designed to inhibit GABA-T* activity. Theoretically this action would require a GABA analog with no free hydrogens on the γ-carbon, to avoid the isomerization of the Schiff base necessary for the transamination. On the other hand, the radicals to be introduced in the γ-carbon of GABA should also help the substance to bind itself to the enzyme—for example, by hydrophobic bonds of phenyl radicals. Finally, the molecule should have as small polarity as possible, in order to penetrate the blood–brain barrier easily. Three new GABA analogs were synthesized, all of them having in the γ-carbon one phenyl radical and a second radical: a methyl, an ethyl, or a second phenyl (MPP, EPP, and DPP). They were prepared in the lactam form to reduce their polarity, based on the supposition that they would be hydrolyzed in the brain as is 2-pyrrolidinone.

Experiments are described on the effects of MPP and EPP on the GABA-T activity of mice and rat brain homogenates *in vitro*, on the levels of cerebral GABA and other amino acids, and on convulsive activity and death caused by thiosemicarbazide (TS) and pentylenetetrazol (PT).

It was considered interesting to study the action of these pyrrolidinone derivatives on the convulsions induced by electroshock (ES), as well as their effects on an instrumental conditioned response, taken as an indication of physiological higher nervous activity.

MATERIALS AND METHODS

The details of the methods for the synthesis of MPP, EPP, and DPP will be the subject of another paper;[15] only a concise summary will be given here. The compounds were prepared by a Stobbe condensation between the appropriate ketone and the diethyl ester of succinic acid, using sodium hydride as the condensating agent. The products of this condensation were transformed into the corresponding lactones by hydrobromic or sulfuric acid. Finally, the lactones were transformed into the lactams with liquid ammonia under pressure. The compounds were characterized by elementary analysis of carbon, hydrogen, and nitrogen and by their infrared spectra compared with that of 5,5-dimethyl-2-pyrrolidinone, an analog already known in the chemical literature.[16] EPGABA was obtained from EPP by alkaline hydrolysis in boiling, saturated $Ba(OH)_2$ solution, eliminating the Ba with CO_2 and SO_4H_2. The amino acid was separated from the inorganic salt impurities by extracting it with absolute ethanol. EPGABA showed much greater solubility than EPP, gave positive $-COOH$ (HCO_3^-), positive N (Nessler), positive $-NH_2$ (ninhydrin), and produced a single spot in thin-layer[17] and paper chromatography, developed with 60% H_2SO_4 and ninhydrin respectively. Its infrared spectrum (Beckman IR-5) was different from that of EPP and was compatible with that of an amino acid.

Thiosemicarbazide and pentylenetetrazol were obtained from commercial sources (Eastman Kodak and Parke Davis). Diphenylhydantoin (DPH) and glutamic acid-γ-hydrazide (GAH) were obtained from Parke Davis and Calbiochem respectively.

* Abbreviations used are: GABA-T, γ-aminobutyric acid-α-ketoglutaric acid transaminase; MPP,5-methyl,5-phenyl-2-pyrrolidinone; EPP,5-ethyl,5-phenyl-2-pyrrolidinone; DPP,5,5-diphenyl-2-pyrrolidinone; EPGABA, γ-ethyl-γ-phenyl-γ-aminobutyric acid.

Over 300 adult mice (weighing 22–32 g) and 200 rats (weighing 220–300 g) from a local strain were used in the different experiments of this study.

Determination of GABA-T activity in vitro *and* in vivo

The activity was estimated according to the conditions established by Bessman *et al*[18] and Baxter and Roberts[19] adapted for homogenates.[20] The brains were homogenized with 10 vol of cold (0°) 0·05 M borate buffer (pH 8·2). The procedure is based on the measurement of the glutamic acid produced in the transamination between GABA and α-ketoglutaric acid induced by the homogenate (incubation period 1hr at 37°). The pyrrolidinone derivatives were introduced at a final concentration $7{\cdot}0 \times 10^{-3}$ M. The reaction was stopped by the addition of absolute ethanol. Extracts free from proteins and lipids were obtained by Awapara's method.[21] These extracts were dried and concentrated 10-fold. The glutamic acid was separated by unidimensional paper chromatography (phenol 80%), eluted, and measured by one of the variants of the ninhydrin reaction[22] in a Beckman B spectrophotometer at 570 mμ. A blank tube with neither of the substrates was run in parallel in order to compensate for the possible overlapping of other amino acid spots with the glutamic acid spot. The values given in the tables are the differences between the problem and blank tubes. Recovery tests gave an error not greater than 10% by this method.

For the determinations *in vivo* EPP was injected i.p. into the rats, and the animals were killed by decapitation half an hour later. The determinations was carried out in the same way as in the experiments *in vitro*.

Determination of the free amino acid concentration

The MPP and EPP were dissolved in distilled water at a concentration of 20 mg/ml. The mice were injected i.p. with 160 mg of these substances/kg and killed by decapitation after 2 or 6 hr. The mice brains were dissected and immediately immersed in liquid air for 1·5 min. Each brain was weighed while frozen and homogenized in Potter–Elvehjem homogenizers with 10–20 vol. 80% ethanol. A protein- and lipid-free extract was obtained by Awapara's method[21] and, after concentrating it 10-fold, it was run in duplicate series of bidimensional chromatograms (phenol 80%; acetic acid–*n*-butanol–water, 1:4:1 by vol.). The glutamic acid, aspartic acid, GABA, and glutamine were eluted and measured by a ninhydrin reaction, as described above. The recoveries obtained by this procedure are around 95% for GABA, glutamic acid, and aspartic acid and 90% for glutamine.[23]

One half the brains from the rats used in the GABA-T determinations *in vivo* were frozen in liquid air and their glutamic acid, aspartic acid, GABA, and glutamine concentrations were measured after the procedure described above.

Protection against thiosemicarbazide and pentylenetetrazol

The TS in doses of 20 mg/kg was administered to groups of at least 10 mice. The control group was injected with saline solution 0·5 hr after TS; the treated groups were injected with 80 and 120 mg of MPP or EPP/kg. For comparison, diphenylhydantoin was used in another group. The unprotected animals died 1–2 hr after the TS injection.

Doses of 75, 80 and 85 mg of PT/kg were used. The controls groups were injected with saline 15 min before PT; the treated groups were injected with 80 and 100 mg of EPP/kg.

Effect on conditioned responses

Sixty rats were used in these experiments, most of which were studied for several months. An avoidance reaction produced by nociceptive electrical stimulation was conditioned to a visual stimulus. The rats were trained to jump to the other half of a cage when a light was applied. The intertrial interval was 1–3 min, and the total number of trials in a daily session was 20–30. After the necessary training the rats showed a 90–100% conditioning.

The substances were injected intraperitoneally in doses of 25–200 mg/kg. MPP and EPP were dissolved in water; DPP was dissolved in 50% polyethylene-glycol. The injection of the same volume of 50% polyethylene-glycol had no effect whatsoever upon the conditioned response. Ten trials were given before the injection, and groups of five trials were given at different intervals after the administration of the drug, covering a period from 5 min to 24–32 hr.

Effects on electroshock convulsions

Over 60 rats were used in these experiments. Maximal electroshock convulsions were induced by stimulating the animals with rectangular pulses of 10–15 mA, 1 msec, and 200/sec for 5 sec. The rats were free in a cushioned cage, with the electrodes fixed to head and tail. The duration of the tonic and clonic phases was measured in each fit. The fits were provoked at 15- or 30-min intervals, and the substances were applied after several control fits of similar duration in doses of 25–200 mg/kg. The duration of the convulsions was observed during several hours, maintaining the same interval of time between stimulations. The 50% polyethylene-glycol which was used as DPP solvent had no effect on electroshock convulsions.

RESULTS

Action on GABA-T activity

The three compounds EPGABA, EPP, and MPP ($7{\cdot}0 \times 10^{-3}$ M) exhibited a marked inhibiting action upon the transamination of GABA by brain homogenates (Table 1). The strongest inhibition was produced by EPGABA, a fact that agrees with the assumption that the lactams have to be hydrolyzed to yield the amino acids in order to inhibit GABA-T. This inhibition was reduced (EPGABA and EPP) or it even disappeared (EPP and MPP) when the concentration of GABA was doubled, suggesting that the inhibition was competitive.

The inhibition produced by EPP showed a tendency to increase with the preincubation time. The transaminating activity of brain acetone powders was not affected by EPP. These two facts agreed with the assumption made above, that the lactam had to be hydrolyzed in order to inhibit, which would take some time and would require enzymes that might be absent in the acetone powders.

The brains of rats previously injected intraperitoneally with 160 mg of EPP/kg, showed a slightly decreased GABA-T activity (Table 2). Although it was not statistically significant because of the great variability, the tendency was present in each of the five experiments performed.

TABLE 1. ACTION OF EPP AND MPP ($7{\cdot}0 \times 10^{-3}$ M) ON GABA-T ACTIVITY OF MOUSE AND RAT BRAIN HOMOGENATES, WITH HALF-HOUR PREINCUBATION*

Activity in micrograms glutamic acid/100 mg tissue per hr.

Homogenates	GABA in the medium	Controls	EPGABA	EPP	MPP
Mouse	10^{-2} M	346·3 ± 21·0 (9)		269·7 ± 11·3 (9) −22% (P < 0·01)	
		297·3 ± 21·2 (7)			285·7 ± 31·0 (7)
	5×10^{-3} M	193·9 ± 19·4 (8)		111·4 ± 17·5 (8) −43% (P < 0·01)	133·4 ± 17·5 (8) −31% (P < 0·05)
Rat	10^{-2} M	260·0 ± 18·0 (2)	219·0 ± 42·0 (2) −16% (P = 0·5)		
		310·0 ± 45·0 (3)		333·3 ± 65·0 (3)	320·0 ± 59·0 (3)
	5×10^{-3} M	190·2 ± 18·8 (5)	81·1 ± 14·0 (5) −57% (P = 0·01)		
		233·6 ± 18·5 (6)		139·1 ± 22·8 (6) −40% (P < 0·01)	141·1 ± 25·5 (6) −39% (P < 0·02)

*The results are the average value + S.E. The P values were obtained after the 't' test. Number of experiments in parentheses (4 mouse brains each; 2 rat brains each).

Action on cerebral amino acids

By the semiquantitative bidimensional paper chromatographic method employed, no significant deviations from controls could be detected in the brain free amino acid pattern of the animals injected with EPP (80 or 160 mg/kg) and MPP (160 mg/kg). Such a dose of EPP, as will be seen later, is much higher than those already active against convulsions.

TABLE 2. ACTION OF INTRAPERITONEAL EPP INJECTED HALF AN HOUR BEFORE, ON GABA-T ACTIVITY OF RAT BRAIN HOMOGENATES*

Dose EPP (mg/kg)	GABA in the medium	Controls	EPP	Difference (%)	P
80	10^{-2} M	260·6 ± 22·0 (5)	231·8 ± 17·0 (5)	−8	0·5
160	10^{-2} M	260·2 ± 18·4 (15)	218·7 ± 20·4 (15)	−16	0·1
	5×10^{-3} M	148·6 ± 12·8 (9)	112·2 ± 24·4 (9)	− 24·5	0·1

* As for Table I

Protection against thiosemicarbazide and pentylenetetrazol

EPP produced a marked protection against the convulsions and/or death elicited by TS (Fig. 1); MPP showed no protection against TS. DPH, a very well known *grand mal* antiepileptic, did not protect against TS death but, on the contrary, increased the death rate.

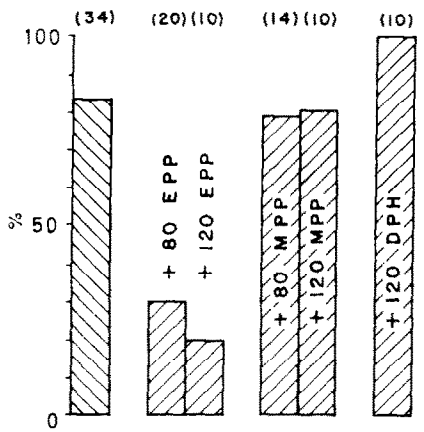

FIG. 1. Action of EPP, MPP, and diphenylhydantoin on the percentage of mortality caused by thiosemicarbazide (20 mg/kg). The number over each bar is the dose in mg/kg injected 30 min after the injection of TS. Number of mice in parentheses.

Figure 2 shows that EPP produced a complete protection against death induced by PT. The unprotected mice always died at the first tonic–clonic full-body convulsions, whereas the EPP-treated mice either had no such convulsions or survived them.

Effect on conditioned responses

EPP and DPP (120 mg/kg) produced a complete inhibition of the conditioned reaction that lasted 4–16 hr (Fig. 3). DPP at 25 mg/kg still depressed the conditioned

reaction; whereas EPP improved it when the animals did not show 100% conditioning. MPP produced only a negligible inhibition with doses up to 200 mg/kg.

The doses of EPP and DPP that completely inhibited the conditioned reaction sometimes also inhibited the unconditioned response to the electric stimulation. In spite of this fact, they did not immobilize the animals which could still walk (with a

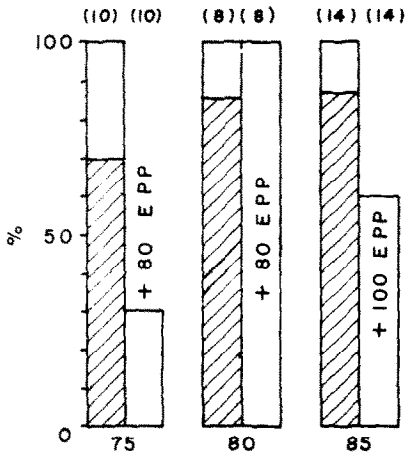

FIG. 2. Action of EPP on convulsions (total height) and death (shadowed portion) caused by penthylenetetrazol (PT). The number below each pair of bars is the dose of PT in mg/kg. The number over the second bar of each pair is the dose of EPP in mg/kg injected 15 min before the PT. Number of mice in parentheses.

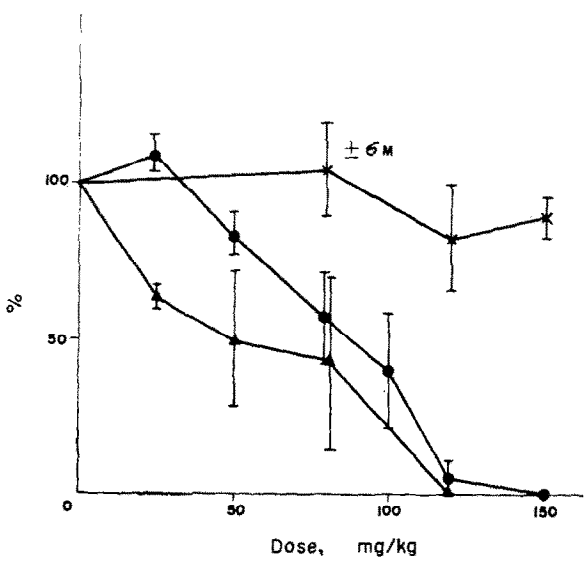

FIG. 3. Action of MPP (×), EPP (●), and DPP (▲) on the conditional avoiding reaction. Ordinate: correct responses in percentage of the control trials. The figures are average + S.E. of the maximal effects for each dosage.

certain ataxia). Even these large doses did not produce inhibition of the patellar, flexor, palpebral, and pupillary reflexes (on the contrary, the medullary reflexes seemed to be facilitated, which was shown by a *spontaneous* scratch reflex occasionally present), nor did the animals show cyanosis. DPH produced effects similar to EPP—namely, negligible effects at 50 mg/kg and complete inhibition at 120 mg/kg.

Action on electroshock convulsions

The average durations of the tonic and clonic phases of the convulsions elicited by ES were 16·9 and 10·2 respectively, giving a total duration of 27·1 sec (averages of 100 convulsions). The four substances, EPGABA, MPP, EPP, and DPP, produced on the average an 85% to 100% reduction of the duration of both tonic and clonic phases of the epileptoid convulsion (Fig. 4).

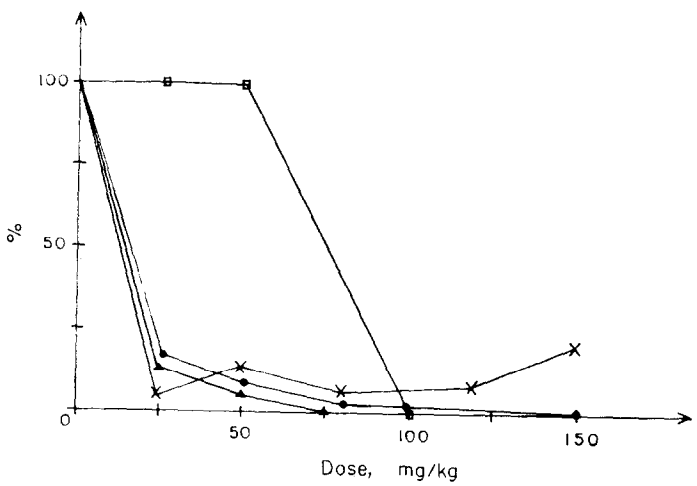

FIG. 4. Action of MPP (×), EPP (●), DPP (▲), and EPGABA (■) on the duration of convulsions produced by electroshock. Ordinate: duration of the convulsions in percentage of the control trials. The figures are average maximal effects for each dosage.

The anticonvulsive action was almost maximal at doses that did not modify significantly or even improved the conditioned responses. EPGABA showed a very delayed anticonvulsive action at 100 mg/kg and no effect at 50 or 80 mg/kg. This observation agrees with the assumption that the amino acid penetrates to the brain with greater difficulty than the lactam.

DPH produced an inhibition of the electroshock convulsion similar to that elicited by the three pyrrolidinone derivatives. On the other hand GAH, which has been shown to increase cerebral GABA level more than threefold,[24] had no effect on the electroshock convulsions when cerebral GABA was greatly increased. If at this moment EPP was injected, the full anticonvulsive effect was obtained.

The temporal pattern of the effects of EPP and EPGABA on the duration of electroshock convulsions is shown in Fig. 5.

DISCUSSION

The results obtained in mice showed that EPP exhibited a marked inhibition of GABA-T activity of brain homogenates. EPP also protected the mice against TS and PT death, in spite of the fact that it did not modify the whole-brain GABA concentration or the GABA decrease induced by TS. The lack of protecting action of MPP against TS might be correlated with its smaller inhibiting effect on GABA-T. The data obtained on rats is in general agreement with that found in mice.

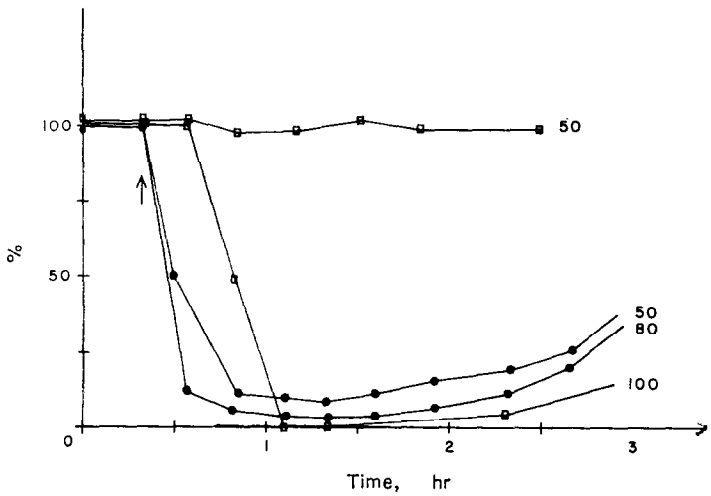

FIG. 5. Temporal pattern of the effects of EPP (●) and EPGABA (□) on the duration of electroshock convulsions. Ordinate: duration of the convulsions in percentage of the control trials. The number at the end of each line is the dose (mg/kg).

These effects of EPGABA, EPP, and MPP on GABA-T activity should be considered as preliminary findings. Such results need further confirmation by more detailed enzymological studies involving more accurate techniques for the estimation of GABA-T activity.

Other data showed that the GAH, which increased GABA levels 2-to 3-fold,[24] produced no inhibition of the electroshock convulsions or any protection against TS at the time of maximal increase in GABA level[25]; the anticonvulsive action of EPP in rats was not accompanied by any change in GABA level. Moreover, the effect of EPP on a background of high GABA level induced by GAH indicates presumably that the effect of EPP is independent of the GABA pool modified by GAH. It has been shown that an excess of brain GABA, produced by drugs (2,4-diaminobutyric acid) or by direct massive GABA administration, is associated with convulsions.[26, 27] All these results are in agreement with other findings reported in the literature,[12,14,28] pointing to the conclusion that the brain GABA level *per se* is not the determining factor for the presence or absence of convulsions.

The lack of protection against TS showed by a single dose of diphenylhydantoin, at the time when its anticonvulsive effect on electroshock convulsions is maximal, is in agreement with other observations of the lack of anticonvulsive effect showed by a single dose of this substance on drug-induced convulsions[29] and its lack of effect on *petit mal* epilepsy.[30]

In an attempt to explain the contradictions mentioned above, the following hypothesis could be considered. GABA might be only the precursor of an inhibitory transmitter, which could be β-hydroxy-γ-aminobutyric acid, as Hayashi and Nagai suggest,[27,31] or any other GABA derivative. Moreover, as Roberts has suggested[32] GABA-T could be located mainly in glial cells whereas glutamic acid decarboxylase would be in the neurons. Therefore, a substance not freely permeating the blood–brain barrier would inhibit mainly *glial GABA-T* and cause a rise in *glial GABA level*, but would not necessarily increase the active derivative in the neurons. On the other hand, EPP could penetrate to the neurons, where it could inhibit the comparatively small amount of neuronal GABA-T and/or the enzyme that catabolizes the true inhibitor, thus providing more precursor (GABA) *inside* the neuron and/or more inhibitor.

The possibility of a clinical application of MPP and EPP is suggested by their potent activity against electroshock convulsions at doses which did not impair (or even improved) an instrumental conditioned response. This leads to the expectation of a potent anticonvulsive action of both drugs against *grand mal* epilepsy, of which electroshock convulsion is considered an experimental model,[33] without an impairment of normal mental functions. The fact that even large doses which inhibited conditioned and unconditioned reflexes did not inhibit medullary and mesencephalic reflexes indicates that the effect of these substances is not due to general narcotic or anesthetic action.

Based on the protective effect of EPP against thiosemicarbazide and pentylenetetrazol, it might be expected that it will be effective on *petit mal* epilesy.[33]

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