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THE METABOLISM OF TREMORINE

IDENTIFICATION OF A NEW BIOLOGICALLY ACTIVE METABOLITE, *N*-(4-PYRROLIDINO-2-BUTYNYL)-γ-AMINOBUTYRIC ACID

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Abstract—A new metabolite of tremorine, *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid (Pybu-Gaba), has been identified in the urine of rat. Pybu-Gaba is cyclized to oxotremorine *in vivo* but this reaction does not occur at physiological pH *in vitro*, suggesting an enzymatic mechanism. The pharmacological action of Pybu-Gaba closely resembles that of oxotremorine and probably is mediated by the latter.

SINCE the discovery by Everett *et al.*[1] of the unusual biological effects of tremorine (1,4-dipyrrolidino-2-butyne) (TMN) much interest has been focused on the relationship between effects and metabolism of this compound. Two groups of investigators[2–4] independently demonstrated that TMN was bioactivated in the body and the metabolite oxotremorine (1-pyrrolidino-4(2-oxopyrrolidino-2-butyne) (OT) was subsequently identified.[4] All known effects of TMN such as tremor, ataxia, hypothermia, salivation and lacrimation are produced by OT[5] and are antagonized by drugs which inhibit the metabolism of TMN.[6, 7]

Cho *et al.*[8] separated a number of metabolites by paper chromatography of urine extracts from rats given ^{14}C-TMN and ^{14}C-OT. None of these metabolites was identified; however, suggestive evidence for the formation of tremorine-N-oxide from TMN was later presented.[9]

In this paper we report the identification of a new metabolite of TMN, N-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid (Pybu-Gaba), which produces marked hypothermia and tremor in animals.

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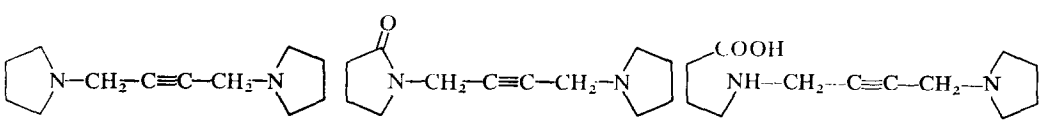


TMN OT Pybu-Gaba

Abbreviations used: tremorine = TMN; oxotremorine = OT; *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid = Pybu-Gaba.

MATERIALS AND METHODS

Preparation of urine samples and separation of TMN metabolites by thin layer chromatography (TLC)

Male Sprague–Dawley rats, weighing 300–400 g were injected repeatedly i.p. with TMN dihydrochloride (10 mg/kg). The injections were spaced in such a way that the rats recovered well from the previous administration. Urine was collected free of faeces in a flask containing 1 ml of 0·1 N HCl. The urine from several rats was pooled, adjusted to pH 9 with 1 N NH_4OH and the amines were adsorbed on Dowex 50 (H^+). The column (10 ml bed vol.) was washed with 10 ml of 0·1 N NH_4OH and 50 ml of deionized water. The adsorbed material was eluted with a 1 N solution of $Ba(OH)_2$. Fractions of 1 ml were collected until Ba^{2+} appeared in the eluate. All Ba^{2+}-free fractions were pooled and freeze-dried. The residue was extracted three times with 10 ml of methanol. The combined extracts were concentrated *in vacuo*, applied to thin layer plates 20 × 20 cm coated with silica gel G, and developed in a system consisting of heptane, chloroform, ethanol, diethylamine (12:2:1:1). In this system OT moved 4 cm (R_f = 0·30). An area, 2–5 mm from the origin containing Dragendorff positive material was scraped off and eluted with methanol. The extract was concentrated *in vacuo* and used for gas chromatography as described below.

Gas liquid chromatography (GLC)

Gas chromatographic analysis was performed with an Aerograph 204 apparatus equipped with a hydrogen flame ionization detection system.

The column support, 100–120 mesh Gas Chrom P, was acid-washed and silanized according to the method described by Horning *et al.*[10] The coatings were applied by the filtration technique.[10, 11] The column packings used were NGS (7%) and OV 17 (5%). Separation was carried out with 1·5 m × 1·6 mm glass columns. Carrier gas was nitrogen. The temperature of the flash heater and the detector were kept 50° above that of the column.

Gas liquid chromatography—mass spectrometry (GLC-MS)

The principles of the technique have been described in detail by Ryhage.[12] The mass spectrometric work was carried out with an LKB 9000 gas chromatograph—mass spectrometer including a fast scan system and the Ryhage "molecule separator". The ion source was kept at 290° and the ionization potential and trap current were 70 eV and 60μA, respectively. The separations were made at 220° on a 2 m × 3·2 mm glass column of 5% OV 17 on Gas Chrom Q, 80–100 mesh with helium as the carrier gas. At the outlet of the column the separated compounds were concentrated and continuously fed into the mass spectrometer. The mass spectrometer simultaneously serves as a gas chromatographic detector and for recording of mass spectra of the compounds as they emerge from the column.

Spectra of reference compounds and of components of the urinary extract were obtained under identical conditions.

High voltage electrophoresis (HVE)

The pooled urine samples from several rats which had received i.p. either 10 mg/kg of TMN dihydrochloride or 1 mg/kg of OT oxalate were worked up as described for TLC. Both drugs were specifically labelled with tritium in the pyrrolidine ring.[13]

Separation was performed with a Thermoelfores (AB Analysteknik, Vallentuna, Sweden) on Whatman chromatographic paper No. 1 at 3000 V. The buffer systems glacial acetic acid (100), formic acid (150), water q.s. 1000 ml (pH ≈ 1·9) and phenol (13 g), triethylamine (20 ml), acetone (150 ml), water q.s. 1000 ml (pH ≈ 10·8) were used.

Procedure. The paper was dipped into the buffer and blotted between dry sheets of paper to reduce the content of moisture. Ideally, the amount of buffer solution should be close to the weight of the paper. The moist paper was placed onto the apparatus and the samples were applied. At the end of the electrophoresis the paper was dried in air and the location of the metabolites determined with a Packard Radiochromatogram Scanner.

Synthesis of N-(4-*pyrrolidino*-2-*butynyl*)-γ-*aminobutyric acid* (*Pybu-Gaba*)

OT (0·5 g, 2·4 m-mole) was hydrolyzed with BaO (0·37 g, 2·4 m-mole) in 5 ml of water at 90° over night. The cold reaction mixture was filtered and the filtrate was extracted with toluene (3 × 10 ml) to remove unhydrolyzed OT. The aqueous solution was neutralized with 1 N HCl and barium ions were precipitated with carbon dioxide. The precipitate was filtered off and the water solution was evaporated under vacuum. The residual oil was dissolved in ethanol and the oxalate precipitated by means of a solution of oxalic acid in ethanol. Recrystallization from ethanol-water yielded 0·3 g (31%) of colorless crystals. Mp = 150° (Kofler bench).

Anal. Calc. for $C_{12}H_{20}N_2O_2 \cdot 2C_2H_2O_4$: C, 47·5; H, 5·98; N, 6·93.
Found: C, 47·5; H, 6·08; N, 7·06.

When the same synthesis was carried out with labelled ^{3}H-oxotremorine a dioxalate with the same melting point was obtained. TLC and HVE revealed that both the radioactive and the cold Pybu-Gaba were free from OT.

Leuckart derivative of Pybu-Gaba: N-*methyl*-N-(4-*pyrrolidino*-2-*butynyl*)-γ-*amino-butyric acid methyl ester*

The amino acid was heated with an excess of formaldehyde (40% aqueous solution) and excess formic acid in an ampoule at 100° for 2 hr. The reaction mixture was then evaporated to dryness. A methanolic solution of the residue was treated with an excess of diazomethane in ether. The ether was evaporated and the residue was dissolved in methanol and used for GLC.

Animal experiments

Male Swiss albino mice (20–22 g) and male Sprague–Dawley rats (200–220 g) were used. All drugs were administered i.p. in saline. The rectal temperature was recorded with an electrothermometer at intervals of 30 or 60 min. Tremor recording was performed with the technique described by Holmstedt and Lundgren.[14] All experiments were run at 18–20°.

RESULTS

Identification of N-(4-*pyrrolidino*-2-*butynyl*)-γ-*aminobutyric acid* (*Pybu-Gaba*)

The polar urinary metabolite of TMN, purified by preparative TLC, upon gas chromatography gave a single peak, with the same retention time as OT (Fig. 1, top). Synthetic Pybu-Gaba also yielded a single peak with the same retention time (Fig. 1,

middle). The mass spectra obtained from these peaks were identical with that of OT. These findings indicate an immediate ring closure of Pybu-Gaba to OT in the gas chromatograph, analogous to that reported by McKennis *et al.*[15] for γ-(3-pyridyl)-γ-methylaminobutyric acid, a metabolite of nicotine.

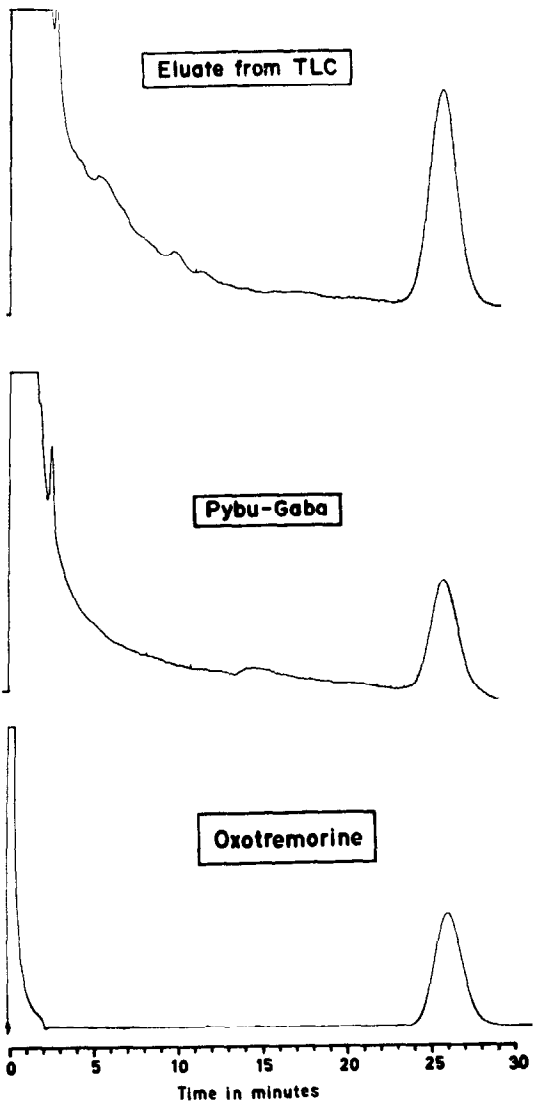


FIG. 1. Gas chromatographic analysis of extract from urine and of reference compounds. GLC conditions: 7% NGS at 200°. Top: Eluate of urine extract separated by TLC. Middle: Authentic *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid. Bottom: Oxotremorine.

The urinary metabolite and synthetic Pybu-Gaba were treated with diazomethane prior to gas chromatography. Two peaks appeared in both cases, the second peak having the same retention time as OT (Fig. 2). The mass spectrum of the second peak was identical with that of OT, while that of the peak with the shorter retention

time indicated the presence of a methyl ester and of a pyrrolidine ring system similar to that of TMN. The spectra from the urinary metabolite and from synthetic Pybu-Gaba were identical.

Ring closure was inhibited by methylation of the secondary amino group by a Leuckart reaction and esterification with diazomethane. On gas chromatography the derivatives of both the urinary metabolite and of the authentic Pybu-Gaba gave rise to a single peak with the same retention time (Fig. 3). Identity of the compounds in the two chromatogram peaks was established by MS (Fig. 4).

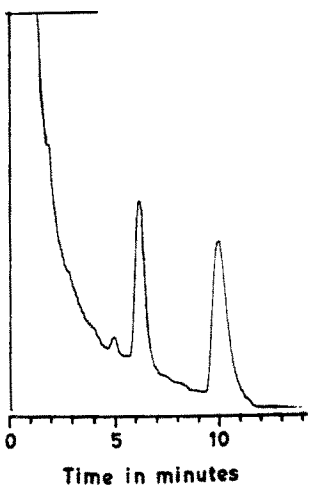


FIG. 2. Gas chromatographic analysis of extract shown in Fig. 1, treated with diazomethane. GLC conditions: 5% OV 17 at 205°.

Electrophoretic evidence that Pybu-Gaba is not a metabolite of OT

Methanolic urine preparations from rats treated with either TMN or OT were subjected to HVE at pH 10·8 (Fig. 5). Under these conditions metabolites with an acidic function move towards the anode. The extract from TMN-treated rats separated into two radioactive spots, the first having the same running distance as Pybu-Gaba. The extract from rats treated with OT showed only the second radioactive spot with the longer running distance.

Further evidence that Pybu-Gaba is not formed from OT was adduced by subjecting extracts from TMN-urine and OT-urine to HVE at pH 1·9. Only in the extract from TMN-urine was a radioactive spot present with a running distance identical to Pybu-Gaba.

Identification of OT in urine of rats and mice treated with Pybu-Gaba

Urine from rats or mice treated with tritiated Pybu-Gaba was freeze-dried. To the residue were added 100 μg of non-radioactive OT and 0·5 ml of 0·1 N NH_4OH followed by extraction with 3 $\times$ 5 ml of toluene. To the combined toluene extracts were added 0·1 ml of HCl-saturated methanol and all solvents were evaporated *in vacuo*. The small quantity of oily residue was taken up in 0·1 ml of methanol. This

solution was subjected to HVE, pH = 1·9. On scanning of the paper, radioactive material was found with the same running distance as OT. When authentic tritiated Pybu-Gaba was carried through the analytical procedure, no radioactive material was recovered.

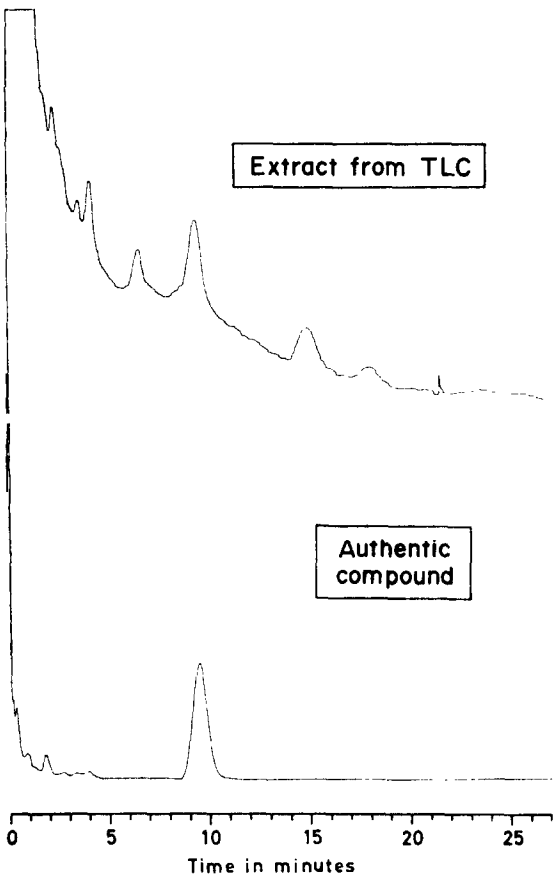


FIG. 3. Gas chromatographic analysis of extract from urine and reference substance, both methylated according to Leuckart and esterified. GLC conditions: 7% NGS at 200°. Top: Eluate of urine extract separated by TLC. Bottom: *N*-methyl-*N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid methyl ester.

Stability of Pybu-Gaba at physiological pH

Two mg of tritium labelled Pybu-Gaba were dissolved in 40 ml of 0·1 M Tris buffer pH 7·4. Duplicate samples of 2 ml were kept at 37° for up to 120 min. After addition of 0·2 ml of 5 N NaOH the samples were extracted with 2 ml of toluene containing 1·5% isoamyl alcohol. Under these conditions OT is efficiently extracted into toluene,[7, 16] while Pybu-Gaba is not. One ml of the organic phase was counted in a scintillation spectrometer. Only background activity was recorded, showing that no OT was formed.

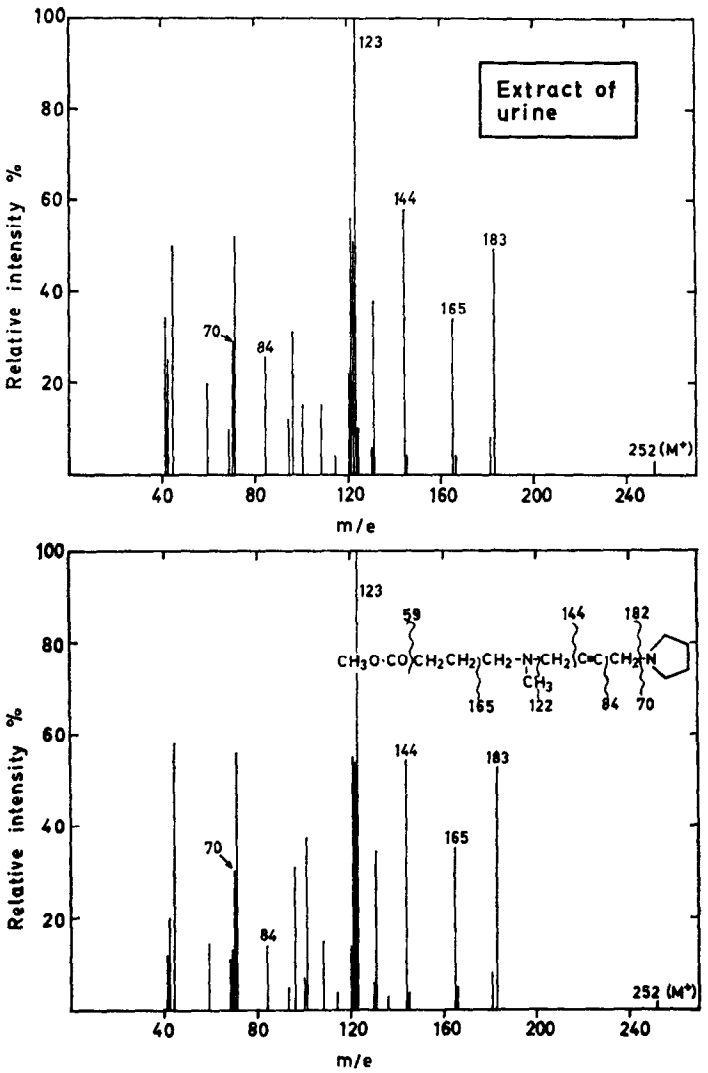


FIG. 4. Mass spectra of peaks shown in Fig. 3. Conditions as described under Material and Methods. Top: Mass spectrum of peak one in Fig. 3. Bottom: Mass spectrum of reference compound in Fig. 3.

Pharmacology of Pybu-Gaba

In doses of 1–4 mg/kg Pybu-Gaba produced a marked hypothermia in the mouse (Fig. 6) and other effects typical of OT, e.g. tremor, salivation and lacrimation. The onset and duration of hypothermia were approximately the same after i.v. and after i.p. injection (5–7 min). This is in contrast to TMN which produces a quicker and more longlasting hypothermia upon i.p. administration.

In rats, Pybu-Gaba (10 mg/kg, i.p.) evoked no hypothermia or any visible symptoms. However, after pretreatment with SKF 525 A (25 mg/kg, i.p.) or desmethylimipramine (10 mg/kg, i.p.), which both potentiate and prolong the action of OT in rats by blocking its metabolism,[6, 7] Pybu-Gaba produced tremor (Fig. 7), lacrimation and a

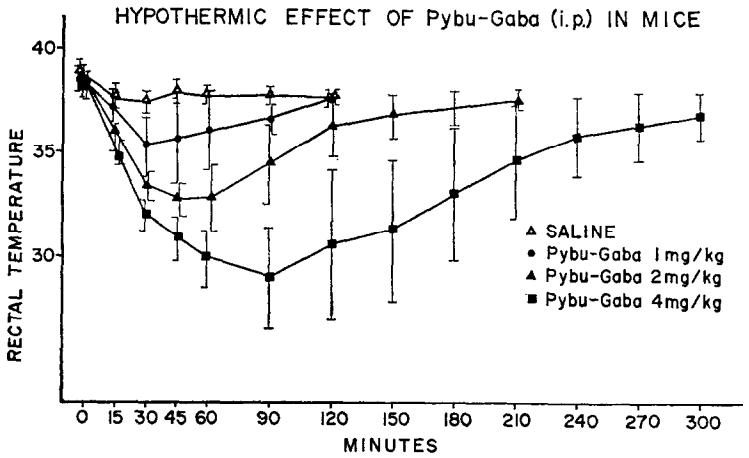


FIG. 6. Hypothermic effect of Pybu-Gaba in mice. All data are means ± S.D. (n = 5).

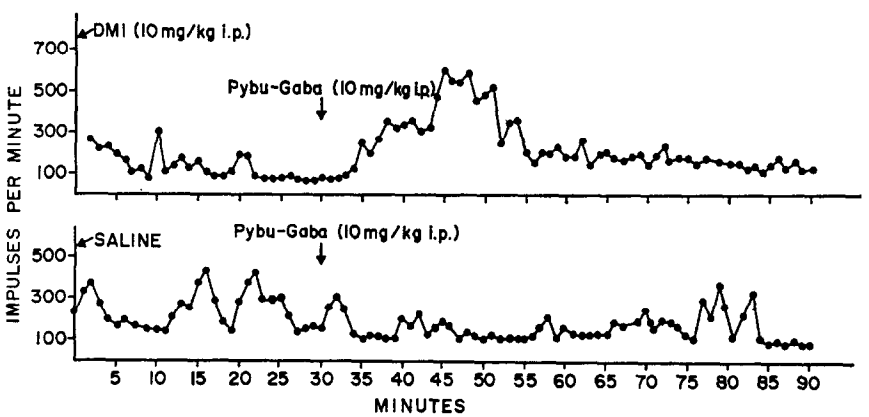


FIG. 7. Tremor recording in rats following injection of Pybu-Gaba and pretreatment with saline or desmethylimipramine.

marked longlasting hypothermia (Fig. 8). These experiments suggest that Pybu-Gaba may undergo ring closure in the body to form OT. In support of this view OT was recovered in the urine of mice and rats injected with Pybu-Gaba. By contrast, when labelled Pybu-Gaba was incubated in 0·1 M Tris buffer (pH 7·4) at 37° for periods up to 120 min and the alkalinized solutions were analysed for OT,[7, 16] only background activity was found. These data suggest that the formation of OT from Pybu-Gaba involves enzymatic processes.

DISCUSSION

The isolation and identification of *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid (Pybu-Gaba) in the urine of rats after administration of TMN shows that this compound follows, at least initially, a metabolic path similar to that of nicotine. Both compounds have in common the pyrrolidine ring, the alpha carbon of which is the preferred locus of enzymatic attack.

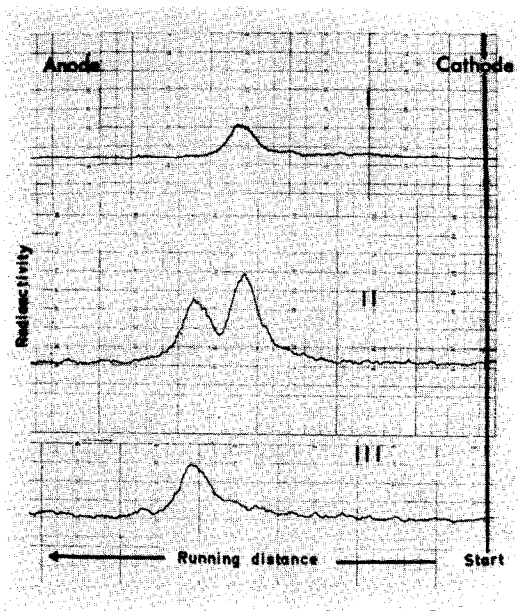


FIG. 5. High voltage electrophoresis at pH 10·8. I = authentic tritiated *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyric acid. II = urine preparations of rats treated with ^{3}H-TMN. III = urine preparations of rats treated with ^{3}H-OT. The samples were applied close to the cathode. Radioactivity was located with a Packard Radiochromatogram Scanner.

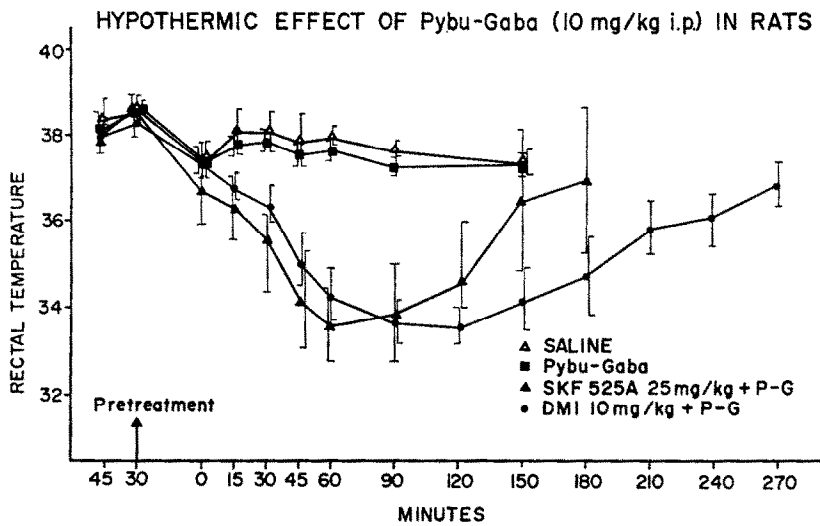
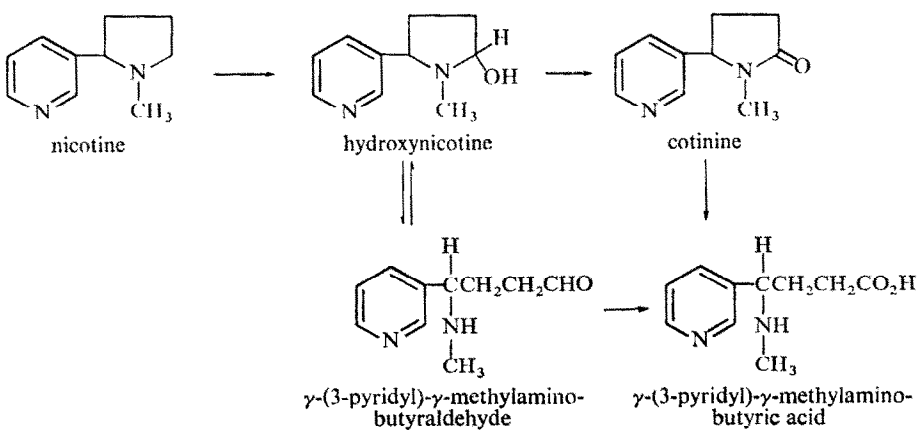


FIG. 8. Hypothermic effect of Pybu-Gaba in rats pretreated with saline, DMI or SKF 525 A. All data are means ± S.D. ($n = 5$).

After administration of l-nicotine, McKennis *et al.*[17, 18] isolated and identified γ-(3-pyridyl)-γ-methylaminobutyric acid and cotinine in the urine of dogs. These authors suggested two pathways for the formation of cotinine from nicotine. The first is oxidation to the acid with subsequent spontaneous ring closure to cotinine. The second is direct oxidation to cotinine by the hydrogen peroxide-catalase system. Spontaneous hydrolysis of cotinine to the γ-amino acid was not found to occur.



Hucker *et al.*[19] presented evidence that nicotine is metabolized *in vitro* by rabbit liver microsomes mainly by the following pathway: hydroxylation at the alpha position of the pyrrolidine ring to hydroxynicotine, and dehydrogenation of this intermediate to cotinine.

On the basis of the results presented here the following transformations may be proposed (Fig. 9): (1) Hydroxylation of the pyrrolidine ring in alpha position to form hydroxytremorine. (2) Ring cleavage to yield *N*-(4-pyrrolidino-2-butynyl)-γ-aminobutyraldehyde. (3) Oxidation to yield Pybu-Gaba. (4) Ring closure to yield OT. (5) Dehydrogenation of hydroxytremorine to yield oxotremorine.

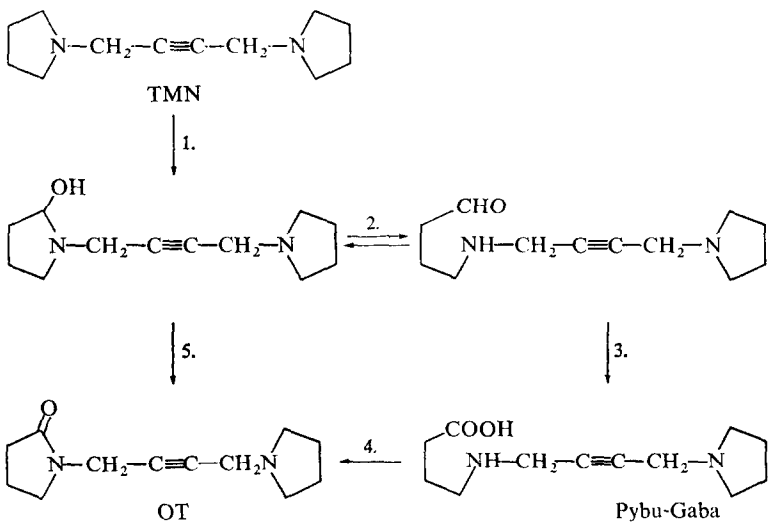


FIG. 9. Metabolic scheme for TMN.

No experimental evidence is available at present for the occurrence of reaction 1—the formation of hydroxytremorine. The formation of TMN-*N*-oxide as an intermediate step in this reaction was suggested by Cho *et al.*[9] Compounds like α-hydroxytremorine are known to exist in equilibrium with the open amino aldehyde form (Reaction 2). Reaction 4, the conversion of Pybu-Gaba to OT, was demonstrated to occur *in vivo*. Whether Pybu-Gaba is an obligatory intermediate in the formation of OT from TMN, or whether direct dehydrogenation of hydroxytremorine (Reaction 5) may also occur has not yet been determined.

The reverse of reaction 4, i.e. the hydrolysis of OT to yield Pybu-Gaba does not occur *in vivo*.

In contrast to the findings of McKennis *et al.*[17, 18] for the γ-(3-pyridyl)-γ-methyl aminobutyric acid, Pybu-Gaba does not form its lactam (OT) spontaneously. Since after i.v. administration of Pybu-Gaba the onset of the pharmacological action is delayed and since it so closely resembles that of OT, it seems probable that the syndrome is mediated by the endogenously formed OT. An enzymatic action seems therefore required for its formation *in vivo*.

To our knowledge such a mechanism has not been reported previously with regard to drug metabolism. An enzyme capable of cyclization of D-glutamate to D-pyrrolidone carboxylic acid has, however, been shown to exist in liver and kidney from mouse, rat and man.[20] Pyrrolidone carboxylic acid has also been reported in large amounts in urine from cases of burns and allergic reactions.[21] Enzymatic reactions of this type are presently under study with Pybu-Gaba as substrate.

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