Inhibition of High Affinity Uptake of GABA by Branched Fatty Acids

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Summary. Several branched fatty acids including an antiepileptic agent nDPA were tested as potential inhibitors of high affinity uptake of GABA by brain slices and synaptosomes. Only three compounds (2-butyl-3-propylhexanoic acid, 5-propyloctanoic acid, 2-propylpenten-2-oic acid) were found to be relatively weak inhibitors of the uptake system. There was no correlation between anticonvulsant properties of the branched fatty acids and their potencies as inhibitors of high affinity uptake of GABA.

It has been reported[1] that several branched aliphatic acids inhibited 4-aminobutyrate-2-oxoglutarate aminotransferase (EC 2.6.1.19, GABA-transaminase). Some of them, including an antiepileptic agent *n*-dipropylacetate (nDPA, 2-propylpentanoic acid) were shown to have protective effects against audiogenic seizures in sensitive mice[2]. Ciesielski et al.[3,4] recently showed a good correlation between potency of these compounds as anticonvulsants in vivo and inhibitory action on GABA-transaminase in vitro. The protection against convulsions by these drugs is accompanied by an increase in levels of 4-aminobutyrate (GABA) in central nervous tissue[2]. Either of these effects may be due to factors other than inhibition of GABA-transaminase. Direct actions on postsynaptic receptors or release and uptake of GABA should also be considered. In this paper we studied the possible effects of branched fatty acids on the high affinity uptake of GABA by brain slices and synaptosomes.

High affinity uptake of GABA by brain slices or synaptosomes was studied by methods previously published[5]. Brain slices (approx. $0.1 \times 0.1 \times 2.0$ mm), prepared with a locally designed McIllwain tissue chopper, were preincubated for 15 min in fresh, oxygenated phosphate-buffered Krebs-Ringer medium (at 10 mg of wet wt. tissue per 10 ml of medium). [^{3}H]GABA (New England Nuclear, 10 Ci/mmol) was then added (0.2 μCi per 10 ml) and incubation continued for another 10 min. Conical flasks (50 ml) were used as incubation vessels and all incubations were carried out in New Brunswick Gyrotary Water Bath Shaker at 25 °C. According to previously published data, the total concentration of GABA (that is, including the endogenous GABA released during preparation of slices) in the incubation medium should not exceed 0.6 μM[6]. Solutions of fatty acids, neutralized with sodium hydroxide, were added into the medium (0.1 ml per 10 ml medium) at the beginning of the preincubation period. Final concentration was 1 mM in all screening experiments.

The reaction was terminated by rapid vacuum filtration through Whatman No. 1 filters (25 mm diameter). These were then transferred into scintillation vials and extracted with 1 ml of water for 1 h. The total radioactivity in these vials was measured using the liquid scintillation technique.

Synaptosomes, prepared according to method of JOHNSTON, VITALI and ALEXANDER[7], were preincubated and incubated in the same manner as the brain slices. The reaction was terminated by rapid vacuum filtration through Millipore HAWP 02400 filters, with a pore size 0.45 μM and a diameter 25 mm. The filters were transferred into scintillation vials, treated with 1 ml of water, and dissolved in the scintillator solution. An Intertechnique scintillation spectrometer and a dioxan based scintillation cocktail[8] were used in all experiments. Inhibitor concentrations causing 50% inhibition (IC_{50} values) were calculated by method of IVERSEN and JOHNSTON[5] and kinetic constants were determined graphically.

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	Synaptosomes			Brain slices
	Inhibition (% ± SD)	IC_{50} (μM)	K_i (μM)	Inhibition (% SD)
2-Propylpentanoic acid (nDPA)	n.s.			n.s.
2-Butyl-3-propylhexanoic acid	70 ± 1	950	620	37 ± 8
5-Propyloctanoid acid	35 ± 6			n.s.
2-Propylpenten-2-oic acid	9 ± 1	3670		n.s.
2-Methyl-2-ethylhexanoid acid	n.s.			n.s.
2,2-Dimethylpentanoic acid	n.s.			n.s.
5-Propylhexanoic acid	n.s.			n.s.
9-Methylbutyric acid	n.n.			n.s.

Slices or synaptosomes were preincubated with an inhibitor (1 mM) for 15 min, and uptake measured after incubation for a further 10 min in presence of [^{3}H]GABA. Blank (i.e. non-incubated preparation) values were subtracted and mean values ± standard deviations of quadruplicate experiments calculated. In inhibition experiments these were compared with means ± SD of the controls, and inhibition was regarded as statistically significant if the possibility of random occurrence of difference from the controls was less than 2% as calculated by Student's *t*-test. For determination of IC_{50} values and K_i, see text. n.s. = not singificant.

The results are summarized in the Table. Among the anticonvulsants 2-propylpenten-2-oic acid and 5-propyloctanoic acid were found to be relatively weak inhibitors of GABA uptake by synaptosomes. These compounds had no effect on uptake of GABA by brain slices. None of the other anticonvulsant fatty acids, including nDPA, influenced uptake by either synaptosomes or brain slices; 2-Butyl-3-propylhexanoic acid was the only compound which significantly inhibited uptake of GABA by both preparations. Kinetics of the inhibition of uptake by synaptosomes was studied at 4 different GABA concentrations (3.12 μM, 4.17 μM, 6.12 μM and 12.50 μM) and 2 different inhibitor concentrations (250 μM and 500 μM). The inhibition was found to be of a linear, non-competitive type with $K_i = 620$ μM. It is possible that a slower diffusion rate of 2-pentyl-3-propylhexanoic acid is responsible for a smaller amount of inhibition observed in brain slices as compared to that by synaptosomes. This can also explain lack of effect of 5-propyloctanoic acid and 2-propylpenten-2-oic acid on uptake by brain slices.

In summary, the results reported in this communication show no correlation between anticonvulsant properties of the compounds and their potency as inhibitors of high affinity uptake of GABA.

Formation of Lipoperoxide in Isolated Sciatic Nerve by Chinoform-Ferric Chelate

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Summary. Effects of chinoform and chinoform-ferric chelate on formation of lipoperoxide in isolated sciatic nerve were investigated. Free chinoform did not increase the lipoperoxide level, while chinoform-ferric chelate significantly increased it. Assuming that the lipoperoxide formed denatures the associated protein in the nerve, the effect of chinoform-ferric chelate could explain, at least partly, the demyelination of nerve tissues caused by massive doses of chinoform.

Epidemiological investigation in Japan revealed that massive doses of chinoform (5-chloro-7-iodo-8-quinolinol) cause a neuropathy called subacute myelo-optico neuropathy (SMON)[2,3]. Although administered chinoform is known to be absorbed in the animal body and found in nerve tissues[4,5], the mechanism of provocation of the neuropathy has not yet been fully elucidated.

Assuming that chinoform provokes the neuropathy through its direct effect on nerve tissue in situ, in vitro experiments to study the effect of chinoform on nerve tissue were considered worthwhile. It was found that the green-colored substance which appears on the tongues and in the feces of SMON patients (recognized as a characteristic symtom of this disease), is due to chinoform-ferric chelate[6]. Thus the investigation of chinoform-ferric chelate is also needed. Assuming that the morphological changes found in the nerves as a result of massive doses of chinoform are due to the denaturation of protein moiety of nerve tissues, we investigated whether chinoform or chinoform-ferric chelate has an action in denaturing the protein in the nerves. On this assumption, we have a working hypothesis that the effect of chinoform is directed primarily to lipid moiety of nerve tissue to increase its peroxide. If lipoperoxide is formed in the tissue, it would cause denaturation of associated protein, resulting in the degeneration of nerve tissue. To examine this working hypothesis, the present work deals with the measurement of lipoperoxide in isolated sciatic nerve dipped into sonicated emulsion of free chinoform or chinoform-ferric chelate in 0.9% NaCl aqueous solution.

Materials and methods. Crystalline chinoform was kindly donated by Prof. Z. TAMURA of Tokyo University. Chinoform-ferric chelate was prepared by mixing $FeCl_3$ with chinoform in dichloromethane and purified according to Tamura et al.[6].

The sciatic nerves of male adult rabbit were extracted very carefully and cut into pieces of 2.5 cm length and about 100 mg wet weight. The nerve was put into test medium except the top and the tail portions, thus forming a U-shape.

Since the level of chinoform accumulated in the sciatic nerve of mice 2 days after the injection of 100 mg/kg of chinoform was reported to be 1.6 μg/g[5], much higher concentration of chinoform was used. As test medium, 2.5 mM chinoform or the same concentration of chinoform-ferric chelate was suspended in 0.9% NaCl aqueous solution and sonicated in an ice-bath to be emulsion. The nerves were incubated at 5 °C for 4 days in the dark. Nerve pieces of the same length and weight in 0.9% NaCl aqueous solution were adopted as control.

Effects of chinoform and chinoform-ferric chelate on formation of lipoperoxide in isolated sciatic nerve

Lipoperoxide value		
control	+ chinoform	+ chinoform-ferric chelate
1.85 ± 0.45	2.06 ± 0.12	3.28 ± 0.98

Lipoperoxide value was represented by nmoles of malonaldehyde determined by TBA method (see text). Numbers of experiments are 4. Statistically significant increase was observed both between control and chinoform-ferric chelate ($p < 0.10$) and between chinoform and chinoform-ferric chelate ($p < 0.10$).

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