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EFFECT OF ACUTE AND CHRONIC ANTICONVULSANT ADMINISTRATION ON ENDOGENOUS γ-HYDROXYBUTYRATE IN RAT BRAIN

O. C. SNEAD, III, L. J. BEARDEN and V. PEGRAM
Department of Pediatrics and the Neurosciences Program, University of Alabama in Birmingham, Birmingham, AL, U.S.A.

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Summary—The effect of acute and chronic administration of ethosuximide, trimethadione, sodium valproate, clonazepam, phenobarbital, and diazepam on brain concentrations of γ-hydroxybutyrate (GHB) was determined by gas–liquid chromatography. The dose and time to sacrifice for each drug was determined by testing for effectiveness against GHB-induced absence seizures in the rat using an automated frequency analysis for quantitation of the electrocorticogram. Acute administration of ethosuximide, trimethadione, and sodium valproate, produced an increase in whole brain GHB. Ethosuximide, trimethadione, and phenobarbital given chronically produced a decrease in whole brain GHB. All changes took place in subcortex and cerebellum. Acute ethosuximide treatment produced a greater increase in GHB concentration at higher doses. The acute changes with the drugs coincided with the onset of anticonvulsant effect, but were short-lived and, in the case of ethosuximide and trimethadione, followed by a significant depression in GHB concentrations. The anti-*petit mal* action of these anticonvulsants may be related to their effect on GHB in brain.

γ-Hydroxybutyrate (GHB) is a metabolite of gamma-aminobutyric acid (GABA) (Roth and Giarman, 1969; Doherty, Stout and Roth, 1975a; Gold and Roth, 1977) which occurs naturally in human brain (Doherty, Hattox, Snead and Roth, 1978). This substance produces marked electroencephalographic (EEG) and behavioral changes in animals which consist of paroxysmal electrical seizure activity associated with a trance-like state (Winters and Spooner, 1965; Snead, Yu and Huttenlocher, 1976; Godschalk, Dzoljic and Bonta, 1977; Snead, 1978a) that resembles *petit mal* epilepsy seen in children. Further, these GHB-induced EEG and behavioral abnormalities are reversed or blocked by administration of anticonvulsants which are specific in their action against *petit mal* seizures (Godschalk *et al.*, 1976; Snead, 1978b, c).

Since this naturally occurring substance has epileptogenic properties, it is possible that it may play a role in the pathogenesis of *petit mal* epilepsy. A study was therefore undertaken to determine the effects of a number of anticonvulsant drugs effective against *petit mal* on the level of GHB in rat brain.

METHODS

Drugs

Clonazepam and diazepam were supplied by Roche Laboratories, ethosuximide by Parke-Davis & Co., trimethadione and sodium valproate by Abbott Laboratories and phenobarbital by Lilly Laboratories. Gamma-hydroxybutyrate was obtained from commercial sources.

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The GHB, ethosuximide and sodium valproate were dissolved in sterile water for injection to make a 20% solution of each drug. Trimethadione was prepared as a 5% solution in sterile water for injection. Clonazepam was dissolved in benzyl alcohol, propylene glycol and ethanol to a concentration of 1 mg/ml. The diazepam and phenobarbital were utilized as the commercially available injectable preparations. In control studies, the vehicle of the appropriate drug involved was used alone.

Electrical studies

Male Sprague–Dawley rats (Charles River Laboratories) weighing 200–300 g were used for all experiments. The animals were watered and fed *ad libitum* and maintained on a 12 hr light–dark cycle. Permanent epidural cortical electrodes were implanted under pentobarbital anesthesia to permit an analysis of the electrocorticogram (ECoG). The ECoG was quantified by means of a computerized frequency analysis system based on the zero crossing method. The intensity of selected frequency bands was summed and recorded out continuously at 1 min intervals. Baseline recordings were taken for 30 min. Then GHB was given intraperitoneally in a dose of 300 mg/kg, and a standard response determined in terms of time of onset, duration of action and intensity of effect on the ECoG. Recording time ranged from 150 to 180 min. Once this standard response to GHB was determined, the effect of a number of drugs on this response was studied with primary attention to time of onset of action of the anticonvulsant and the effective dose. The drugs used were clonazepam, diazepam, phenobarbital, ethosuximide, trimetha-

Table 1. Whole brain acute study

Drug	Dose	Time to sacrifice	Concentration GHB (nmol/g ± SEM)
Clonazepam	1 mg/kg	15 min	2.63 ± 0.21 (12)
Control		15 min	2.70 ± 0.21 (12)
Ethosuximide	200 mg/kg	60 min	3.34 ± 0.12 (10)**
Control		60 min	2.59 ± 0.14 (10)
Trimethadione	300 mg/kg	45 min	3.23 ± 0.29 (10)**
Control		45 min	2.52 ± 0.12 (10)
Diazepam	10 mg/kg	60 min	3.18 ± 0.2 (10)
Control		60 min	3.13 ± 0.18 (10)
Sodium valproate	200 mg/kg	30 min	3.39 ± 0.22 (10)**
Control		30 min	2.34 ± 0.2 (10)
Phenobarbital	30 mg/kg	60 min	2.14 ± 0.08 (10)
Control		60 min	2.38 ± 0.15 (10)

Parenthesis designates number of experiments.
** $P < 0.01$.

dione and sodium valproate. The drugs were given in various dosages intraperitoneally with the advent of GHB-induced electrical changes in the ECoG. The time of onset of action of the anticonvulsant was determined as that time when attenuation or abortion of the electrical changes was noted and the effective dose was the minimum dose which consistently blocked these GHB-induced abnormalities. The exception to this was diazepam where these parameters were determined in the non-GHB treated animal by observing behavior and the appearance of low voltage fast activity in the ECoG, since this drug had no effect on the GHB-induced paroxysms.

Dosing schedules

The anticonvulsant agents used were as described above. The doses used were those determined in the recording studies to be effective against GHB-induced electrical abnormalities. The time from dose to sacrifice was that determined in the electrical studies as time of onset of action of the anticonvulsant. Drugs were administered both acutely and chronically with the latter consisting of once daily injections each for 7 days. For each drug group, there was a separate control group of animals. There were 6–12 rats in both treatment and control groups for all drugs. Control animals received the appropriate vehicle only. The dose and time to sacrifice were the same for both acute and chronic studies for all the drugs except trimethadione. One-half the acute dose was used in the chronic studies of trimethadione because of the amounts of 5% solution that had to be injected daily.

Determination of GHB

Animals were sacrificed by decapitation and the brains rapidly excised and placed on ice. For regional studies, the brain was dissected into cortex, cerebellum and subcortex. The latter consisted of midbrain, pons, diencephalon and basal ganglia regions. It was necessary to pool two samples of each region for the GHB determinations.

In the whole brain studies, single brains were used for each GHB determination. The GHB was assayed by a modification of the electron capture gas liquid chromatographic method described previously (Doherty, Snead and Roth, 1975b). The modifications of this method were as follows: (1) A 0.01 N acetic acid solution was used to wash both the methyl ester and heptoflurobutyral diester; (2) the internal standard, delta valerolactone, was added to the brain homogenate rather than the initial chloroform extract; (3) the extraction time for the ethyl acetate–silica gel step was 30 min; (4) the internal diam. of the 24 ft column was 4 mm.

Dose response and timed studies

A dose–response curve was done for ethosuximide by varying the dosage from 100 to 800 mg/kg and determining whole brain GHB levels 60 min later.

The time to sacrifice was varied for ethosuximide, trimethadione, and sodium valproate utilizing the dosages outlined in Table 1. The time to sacrifice ranged from 15 to 360 min.

Statistical methods

The Mann–Whitney U-test was used to analyze experiments with an N_1 and N_2 of 10 or greater (Winer, 1962; Downie and Heath, 1965). For a smaller N_1 and N_2 the Wilcoxon–Mann–Whitney sum of ranks test (Matheson, Bruce and Beauchamp, 1974) was used; N_1 equaled N_2 in each experimental group.

RESULTS

Electrical studies

The effects of GHB on rat ECoG were similar to those described by Godschalk *et al.* (1977), and consisted or paroxysms of high voltage spiking which progressed to spike and slow wave activity (Fig. 1). These changes occurred within 10–15 min of administration of GHB and were associated with staring and immobility of the animal. The changes in the ECoG

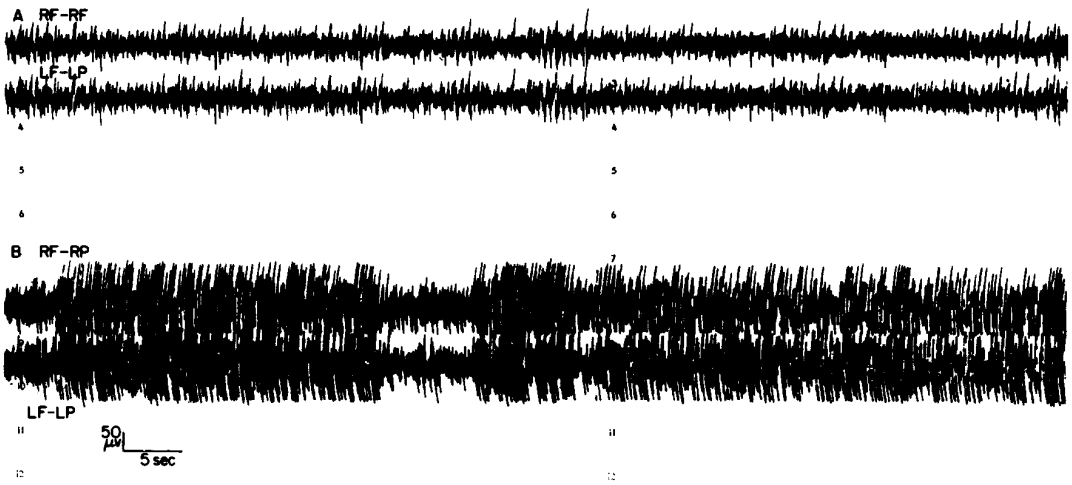

Fig. 1. Abnormalities in ECoG of rat given 300 mg/kg GHB (A) Baseline rat EEG. (B) 20 min after 300 mg/kg GHB. RF-RP is right frontal to right parietal cortical lead. LF-LP is left frontal to left parietal cortical lead. Behaviour during (B) consisted of staring, immobility and occasional myoclonic jerks.

were reflected in the frequency analysis as a dramatic increase in activity of the 3–6 Hz band (Fig. 2). The electrical and behavioral changes lasted 80–100 min. The anti-convulsants utilized against these changes were effective in the following order: ethosuximide ⩾ trimethadione > sodium valproate > clonazepam ⩾ phenobarbital. Diazepam was not effective against the GHB-induced abnormality. This was predictable since this drug exhibits no anti-*petit mal* activity. The effective dosages and time of onset of action of the drugs as reflected in time to sacrifice, are outlined in Table 1.

Effect of drugs on endogenous GHB

These results are summarized in Tables 1–3. The mean concentration of GHB for all acute studies was 2.61 ± 0.04 nmol/g. For the chronic studies, control values were significantly higher at 3.37 ± 0.07 nmol/g. Acute treatment with ethosuximide, trimethadione, and sodium valproate was associated with a 29, 28 and 43% increase respectively in whole brain GHB, which was most striking in subcortex and cerebellum (Table 3).

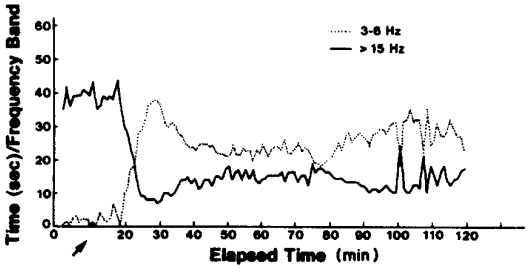

Fig. 2. Automated frequency analysis of rat given 300 mg/kg GHB (arrow). The increase in 3–6 Hz activity correlates with electrical activity seen in Fig. 1(B).

Chronic treatment with ethosuximide, trimethadione and phenobarbital was associated with a 93, 63 and 31% decrease respectively in whole brain GHB, again most striking in subcortex and cerebellum. Acute and chronic diazepam, acute phenobarbital, acute and chronic clonazepam, and chronic sodium valproate produced no significant change in brain GHB levels.

Dose–response studies

The change in steady state GHB levels in brain was insignificant at an ethosuximide dose of 100 mg/kg, but rose to a maximum increase over control of 53% at a dose of 800 mg/kg of ethosuximide. This increase in GHB concentration with dose of ethosuximide was non-linear.

Timed studies (*Fig.* 3)

The effect of ethosuximide, trimethadione and sodium valproate on brain GHB concentration was

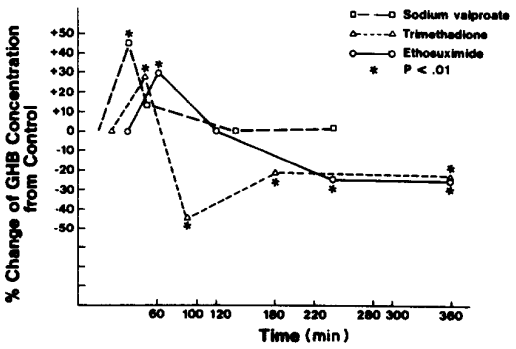

Fig. 3. Time study of the effects of ethosuximide, trimethadione and sodium valproate on whole brain GHB concentrations.

Table 2. Whole brain chronic study (7 days)

Drug	Dose	Time to sacrifice*	Concentration GHB (nmol/g ± SEM)
Clonazepam	1 mg/kg/day	15 min	2.86 ± 0.13 (12)
Control		15 min	3.18 ± 0.11 (12)
Ethosuximide	200 mg/kg/day	60 min	1.77 ± 0.14 (10)**
Control		60 min	3.43 ± 0.07 (10)
Trimethadione	150 mg/kg/day	45 min	2.10 ± 0.14 (10)**
Control		45 min	3.65 ± 0.10 (10)
Dizaepam	10 mg/kg	60 min	3.38 ± 0.07 (10)
Control		60 min	3.36 ± 0.09 (10)
Sodium valproate	200 mg/kg/day	60 min	3.36 ± 0.1 (9)
Control			3.27 ± 0.11 (9)
Phenobarbital	30 mg/kg/day	60 min	2.45 ± 0.05 (10)**
Control			3.55 ± 0.30 (10)

Parenthesis designates number of experiments.
* Time to sacrifice in chronic studies indicates time from last dose.
** $P < 0.01$.

Table 3. Regional brain studies. Dose and time to sacrifice same as in Table 1. Concentrations nmol/g ± SEM

Drug (*N*)		Study group	Regions: Cortex	Subcortex	Cerebellum
Ethosuximide	(8)	Acute	2.62 ± 0.08	4.85 ± 0.42	6.89 ± 0.17*
Control	(8)	Acute	2.57 ± 0.12	4.94 ± 0.40	5.99 ± 0.28
Ethosuximide	(6)	Chronic	2.47 ± 0.14	4.93 ± 0.43*	6.16 ± 0.15*
Control	(6)	Chronic	2.71 ± 0.20	6.11 ± 0.50	7.89 ± 0.33
Trimethadione	(8)	Acute	2.40 ± 0.19	5.60 ± 0.30*	7.50 ± 0.31*
Control	(8)	Acute	2.32 ± 0.17	4.50 ± 0.14	6.49 ± 0.09
Trimethadione	(8)	Chronic	2.54 ± 0.23	4.73 ± 0.28*	6.23 ± 0.09*
Control	(8)	Chronic	2.73 ± 0.17	5.94 ± 0.25	7.31 ± 0.14
Clonazepam	(12)	Acute	2.43 ± 0.19	5.32 ± 0.29	6.14 ± 0.13
Control	(12)	Acute	2.70 ± 0.12	4.95 ± 0.22	6.32 ± 0.17
Clonazepam	(6)	Chronic	2.76 ± 0.17	5.19 ± 0.42	6.30 ± 0.37
Control	(6)	Chronic	2.94 ± 0.20	6.23 ± 0.48	7.23 ± 0.35
Sodium valproate	(6)	Acute	2.54 ± 0.27	10.23 ± 0.22*	11.29 ± 0.46*
Control	(6)	Acute	2.69 ± 0.20	5.93 ± 0.17	6.26 ± 0.19

** $P < 0.01$.

maximal at the time indicated by the electrical studies as being the time of onset of anticonvulsant action of these drugs. Brain concentrations of GHB returned to normal at 120 min with ethosuximide and 60 min with sodium valproate. By 240 min, the brain levels of GHB in the ethosuximide-treated animals began to decline as they did at 90 min after trimethadione.

DISCUSSION

The control values for whole brain GHB in the acute experiments were only slightly higher than the range of 1.78–20.6 previously reported (Doherty *et al.*, 1975b; Roth and Giarman, 1969), the difference perhaps being ascribable to assay modifications and post mortem handling of brain. However, the control values for the chronic studies seem too high to be explained on that basis. These values were consistently higher than the control values of the acute studies and it is possible that this increase of GHB resulted from frequent handling and stimulation of the animals.

The anticonvulsant studies demonstrate that (1) the drugs most specific for *petit mal* epilepsy, ethosuximide and trimethadione, produced the most significant changes acutely and chronically in levels of endogenous GHB in brain; (2) the elevation of brain GHB seen with ethosuximide, sodium valproate, and trimethadione is maximal at the time when these drugs exert their anticonvulsant effect in the GHB model of *petit mal* epilepsy; (3) ethosuximide, trimethadione and phenobarbital when given chronically all produce a significant decrease in brain GHB concentration; (4) both the increase in GHB with acute anticonvulsant administration and the decrease with chronic treatment are most prominent in subcortex and cerebellum; (5) diazepam has no effect either on the GHB model of *petit mal* seizures or on endogenous levels of GHB in brain; (6) the dose–response curve of the acute effects of ethosuximide on brain

GHB is non linear; (7) the timed studies indicate that the increase in brain GHB concentration seen with ethosuximide, trimethadione and sodium valproate is short-lived and followed in the case of ethosuximide and trimethadione by a significant depression of endogenous GHB.

The mechanism by which these anticonvulsants produce an acute rise and subsequent decrease in brain GHB is not clear. γ-Hydroxybutyrate can be formed from glutamic acid (Santaniello, Manzocchi and Tosi, 1978) and from GABA (Roth and Giarman, 1969; Gold and Roth, 1977) via succinic semialdehyde (Anderson, Ritzmann and Tabakoff, 1977) and metabolized via succinic semialdehyde to succinic acid which subsequently enters the Krebs cycle (Doherty *et al.*, 1975a; Mohler, Patel and Balázs, 1976; Doherty and Roth, 1978).

Sodium valproate has been demonstrated to inhibit at least three enzymes in this metabolic pathway, GABA-amino transferase (4-aminobutyrate: 2-oxoglutarate aminotransferase) (EC 2.6.1.19), succinic semialdehyde dehydrogenase (succinate-semialdehyde; NAD oxidoreductase) (EC 1.2.1.16) (Sawaya, Horton and Meldrum, 1975) and NADPH-dependent aldehyde reductase (alcohol:NADP oxidoreductase) (EC 1.1.1.2) (Whittle and Turner, 1978). The inhibition of the latter enzyme by valproate is non-competitive and is two orders of magnitude stronger than inhibition of the first two eznymes with a K_i of between 38 and 55 μM; hence one would expect sodium valproate to be associated with a decrease in brain GHB rather than the increase observed, if these enzyme interactions accounted for its effect on GHB concentration in brain.

Another anticonvulsant which is reported to produce an increase in brain GABA levels, diazepam (Saad, 1972) had no effect on endogenous GHB. Also mitigating against GABA involvement is the fact that the two drugs which produced the most profound changes of brain GHB concentrations in acute and chronic studies, ethosuximide and trimethadione, have no effect on brain GABA levels (Nahorski, 1972; Ferrari and Arnold, 1961). The evidence for an effect of phenobarbital on the concentration of GABA in brain is contradictory (Horton, Meldrum, Sawaya and Stephenson, 1976; Sutton and Simmonds, 1974; Tzeng and Ho, 1977), while there are no data concerning clonazepam levels of GABA in brain. The use of more specific GABA aminotransferase inhibitors such as aminooxyacetic acid (Löscher and Frey, 1978) or ethanolamine-*o*-sulfate (Fowler and John, 1972) in conjunction with the anti-*petit mal* anticonvulsants utilized in the present study might help to elucidate the role of GABA, if any, in the changes in GHB levels associated with these drugs. Another system which might be involved in the anticonvulsant-induced changes in brain GHB concentrations is the glycolytic cycle in brain. Ethosuximide, trimethadione, and phenobarbital have all been demonstrated to produce increased brain–blood glucose ratios with concurrent changes in the Emden–Myerhoff and Krebs cycle intermediates presumably reflecting a decreased rate of glycolysis in brain (Fleming and LaCourt, 1965; Gilbert, Gray and Heaton, 1971; Nahorski, 1972). Although there are no data along these lines concerning clonazepam or sodium valproate, treatment with sodium valproate is associated with a significant decrease of brain aspartate (Perry and Hanson, 1978).

Those drugs that produce an acute elevation in GHB could conceivably exert this action by producing a decreased glycolytic rate in brain with decreased activity of the Krebs cycle. This could result in accumulation of GHB as its metabolism via the cycle, was slowed. The decreased levels of GHB noted at longer time intervals after treatment could result from decreased synthesis of GHB in response to increased concentrations. Such a hypothesis needs to be substantiated by kinetic studies of the metabolic pathways involved using the anticonvulsant utilized in the present study.

A final possibility which is purely speculative is that the changes in GHB concentration observed in these experiments result from involvement of an as yet unelucidated metabolic pathway of GHB synthesis which may involve some precursor other than GABA or glutamic acid.

The biological significance of the 30–90% change of steady state endogenous brain GHB concentrations that were observed is difficult to assess for a number of reasons. First, although there are some data that provide a rough correlation of brain levels of exogenously administered GHB with aberrent behavior (Lettieri and Fung, 1979) and EEG changes (Shumate and Snead, 1979), those experiments are not really relevant to the current studies because of species difference, dosage, and behavioral parameters measured, as well as the fact that any comparison of exogenous pharmacokinetics with endogenous steady state levels is fraught with error. This is especially true with GHB since GABA-derived GHB has a half life of 0.44 hr (Gold and Roth, 1977) which is much shorter than whole brain dopamine, serotonin or norepinephrine. Hence, a change in steady state levels of GHB in the range of 30–90% which the present authors have demonstrated may be associated with an even more significant corresponding change in turnover rates of GHB. Further kinetic experiments assessing the effect of ethosuximide, trimethadione and sodium valproate on formation of GHB from GABA (Gold and Roth, 1977) and its catabolism via succinic semialdehyde (Doherty and Roth, 1978) would help to clarify this question.

Another problem in speculating on the significance of the changes observed and their possible relation to altered CNS function, is that the function, if any, of GHB in brain remains a mystery, although a number of possibilities have been suggested (Snead, 1977) because of the protean pharmacological and physiological properties of this substance. γ-Hydroxybutyr-

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