Biochemical Pharmacology, Vol. 19, pp. 3013–3019. Pergamon Press, 1970. Printed in Great Britain

FORMATION AND REGIONAL DISTRIBUTION OF γ-HYDROXYBUTYRIC ACID IN MAMMALIAN BRAIN*

ROBERT H. ROTH

Departments of Pharmacology and Psychiatry, Yale University School of Medicine, New Haven, Conn. 06510, U.S.A.

(*Received* 19 *February* 1970; *accepted* 15 *May* 1970)

Abstract—Studies *in vitro* with rat brain cortical slices revealed that, similar to observations *in vivo*, γ-aminobutyric acid can serve as a precursor of γ-hydroxybutyric acid (GHB). Endogenous GHB had a discrete distribution in guinea pig brain with the highest concentration found in the hippocampus, midbrain and diencephalon and cerebellum. Subcellular investigations of sucrose homogenates of guinea pig brain demonstrated that very little GHB was found associated with the particulate fraction. Administration of ethanol, an agent known to alter tissue redox potentials, caused a marked increase in tissue levels of GHB, presumably by facilitating the reduction of succinic semialdehyde.

IN 1963 Bessman and Fishbein[1,2] reported the natural occurrence of γ-hydroxybutyric acid (GHB) and of γ-butyrolactone (GBL) in mammalian brain in a combined concentration of 1–3 m-moles/g. However, the isolation of GHB or GBL from mammalian brain by paper chromatography in various solvent systems has not been subsequently verified by Fishbein[3] and others.† Furthermore, by means of a more specific and sensitive gas chromatography assay procedure, Giarman and Roth,[4] Fishbein[3] and Guidotti (personal communication) could not detect GHB or GBL in rat or cat brain at concentrations originally reported by Bessman and Fishbein to occur endogenously. More recently, experiments in our laboratory have indicated that GHB is a natural metabolite found in rat, cat, guinea pig and bovine brain, but at a concentration in the range of 1–4 nmoles/g.[5,6] It has also recently been demonstrated that exogenous administration of GHB causes a marked and rather selective increase in brain dopamine, which seems to be temporally correlated with the behavioral "sleep" produced by this agent.[7,8] This increase appears to be due at least in part to an antagonism in the utilization or release of this monoamine.[9] By means of a fluorescence histochemical method, the GHB-induced increase in brain dopamine content was further shown to be highly localized within discrete sites that are presumed to represent the nerve endings or terminals of the dopamine-containing neurons.[10] However, at the present time the biological significance of the presence of GHB, an agent which causes behavioral depression, in mammalian brain is unknown. It was therefore considered of interest to learn more about its mode of formation, its distribution and possible ways to alter its endogenous levels in the central nervous system.

* This work was supported in part by a grant from the United States Public Health Service (MH-14092).

† A. Guidotti, personal communication; R. H. Roth and N. J. Giarman, unpublished data.

Some preliminary investigations have already indicated that in rat brain *in vivo* γ-aminobutyric acid (GABA) can serve as a precursor for the formation of GHB, although under normal conditions of wakefulness this pathway does not appear to be exceptionally active.[11] The purpose of this present report is 3-fold: first, to further investigate the formation of GHB from GABA; second, to investigate the natural occurrence and the regional distribution of GHB in guinea pig brain; and third, to determine the effect of ethanol, an agent known to alter tissue redox potentials,[12] on tissue levels of GHB.

METHODS

Brain slice experiments

Male Sprague–Dawley rats, obtained from Charles River, were killed by decapitation and the brains quickly excised according to the procedure outlined by McIlwain and Rodnight.[13] The brains obtained in this fashion were immediately chilled in ice-cold Krebs–Ringer bicarbonate medium. To avoid undue anoxia, the brains were then sliced as rapidly as possible by means of a Stadie blade and a microscopic glass slide, ground on one side. The initial slice and any slices that were too thick to be transparent were discarded. Unless otherwise indicated, incubations were carried out in normal Krebs–Ringer bicarbonate medium to which were added 5 μmoles/ml of glucose, 10 μc/ml of GABA (New England Nuclear Corp.; sp. act., 2·0 c/m-mole) and GHB, 1 μmole/ml. In some cases incubations were carried out in Krebs–Ringer bicarbonate with high potassium (50 mM KCl in place of NaCl). Control incubation (to correct for any nonenzymatic conversion) consisted of tissue slices initially precipitated with 0·4 M perchloric acid. All incubations were carried out in a final volume of 5 ml at 37° in an atmosphere of 97 per cent O_2 and 3 per cent CO_2 with constant shaking.

After incubation of the brain slices for 30 min at 37°, the reaction was terminated by the addition of 0·5 ml of 4 M perchloric acid. An additional 50 μmoles of unlabeled GHB and 25 μmoles of unlabeled GABA were added as carrier; the samples, including the media and slices, were homogenized and centrifuged and the supernatant was adjusted to pH 2·0 with potassium hydroxide. The samples were chilled, recentrifuged to remove the perchlorate precipitate, and the supernatant was applied to 3 × 0·4 cm columns of Amberlite CG-120, which selectively retains the unchanged GABA. The columns were washed with distilled water to a final effluent volume of 10 ml. The effluent was acidified to pH 1 with HCl, heated to 90° for 10 min to convert the GHB to the lactone GBL, then adjusted to pH 6 and the lactone extracted with 2 vol. of redistilled benzene twice. The benzene phase was removed and concentrated to a convenient volume and the extracts were analyzed by gas chromatography as described previously.[11,14] Initially the samples were analyzed to determine the concentration of GBL recovered. Then the column effluent was conducted to a heated collection port and the GBL peak trapped by bubbling the effluent gas through a scintillation mixture composed of 4 g 2,5-diphenyloxazole plus 50 mg 1,4-bis-2-(5-phenyloxazolyl) per liter of toluene. Control collections were also made before and after the emergence of the GBL peak. At the end of the collection period, the Teflon tubing was rinsed with scintillation fluid to remove any GBL which might have condensed in the tubing. The samples were then counted to a constant error of 1·5 per cent on a Beckman scintillation spectrometer.

GHB analysis

The labeled GHB used in these experiments was obtained from Schwartz Biochemical Corp. Prior to use, this compound was assayed for purity by gas chromatography after conversion to the lactone form and extraction into benzene. About 99 per cent of the radioactivity extractable into benzene had the same retention time, when analyzed by gas chromatography, as authentic GBL. Tissue GHB was assayed as described previously by a combined technique of gas chromatography and isotope dilution.[6] The tissue samples utilized were always greater than 1 g. In order to obtain enough tissue for the determination of GHB in the hippocampus, 10–12 brains were removed from guinea pigs, the hippocampi removed and pooled, and a single analysis was run on each of the pooled groups. Brains were dissected into the following regions: telencephalon (including the cortex, hippocampus and caudate nucleus), midbrain and diencephalon and the hindbrain. In some cases the telencephalon was further subdivided into the cortex and hippocampus.

Subcellular distribution

Four guinea pig brains were pooled and homogenized in 3 vol. of 0·32 M sucrose by means of a Teflon-glass homogenizer. The crude homogenate obtained was centrifuged for 1 hr at 105,000 *g*. The supernatant was decanted off, and subsequently precipitated with 0·2 vol. of 50 per cent trichloroacetic acid. Labeled GHB was added to the supernatant and the extract was carried through the previously described assay for GHB. The particulate fraction was also analyzed for GHB after extraction with 10 ml of 15 per cent trichloroacetic acid.

Ethanol treatment

Male Sprague–Dawley rats obtained from Charles River were injected intraperitoneally with 1 ml/100 g of a 50 per cent (v/v) solution of ethanol. The animals were killed 1 hr after the injection, at a time when they were still depressed, as indicated by the loss of their righting reflex. The brains were rapidly removed, weighed and homogenized in 15 per cent trichloroacetic acid and carried through the procedure for GHB analysis. Brain norepinephrine and dopamine were analyzed by previously described techniques.[15,16]

RESULTS

In order to further document the conversion of GABA to GHB previously reported *in vivo* in rat brain, we sought to determine whether this conversion would also take place in rat brain cortical slices *in vitro*. The results from brain slice experiments are illustrated in Table 1. In this case the control samples were brain slices, which were initially precipitated with perchloric acid prior to incubation to block any enzymatic conversion. These experiments illustrate that *in vitro* GABA can be converted to GHB, since upon incubation for 30 min there was about a 50-fold increase in the specific activity of the GHB isolated from this tissue. Although high potassium has a profound effect on glycolysis in isolated brain slices,[17] 50 mM potassium had no effect on the conversion of labeled GABA to GHB in these experiments.

Previous experiments have indicated that GHB is a normal metabolite in mammalian brain and therefore it was of interest to investigate the regional distribution of this substance in brain. By use of a previously described technique of gas chromatography

TABLE 1. CONVERSION OF ^{3}H-GABA TO ^{3}H-GHB IN RAT BRAIN CORTICAL SLICES

Experimental procedure	N*	Sp. act. of GHB (counts/min/μmole/100 mg tissue)†
Control	3	5·6 ± 1·9
Normal Krebs medium	3	258·5 ± 16·2
High potassium Krebs medium	3	266·9 ± 13·0

* N = number of experiments.
† Results are expressed as the mean ± the standard error of the mean.

combined with isotope dilution, it was possible to determine accurately the endogenous concentration in as little as 1–2 g brain tissue.[6] However, routine determinations of GHB in whole brain were usually carried out on two or more pooled brains. In the case of specific brain regions, enough brain tissue was pooled to obtain one or more grams of tissue for each determination of GHB. This usually required the utilization of 6–12 animals for a single analysis of a given brain region. The guinea pig brain was chosen for this study, since the normal content of GHB per g in guinea pig brain is about twice that found in rat brain.[6]

Since many bioactive substances found in brain such as acetylcholine and GABA change rapidly post-mortem, it was necessary in initial experiments to determine whether this was also true for endogenous GHB. Table 2 indicates that there is no significant difference in the GHB content of brain from guinea pigs decapitated directly into liquid nitrogen and those brains that were rapidly removed, weighed and homogenized in trichloroacetic acid. However, if the heads were immediately chilled on ice, the brains subsequently removed and allowed to remain at room temperature for 4–5 min (time necessary to dissect into various brain regions), the endogenous content of GHB was significantly increased (Table 2). This post-mortem increase is most likely due to synthesis of new GHB in the anoxic tissue.

TABLE 2. REGIONAL DISTRIBUTION OF γ-HYDROXYBUTYRATE IN GUINEA PIG BRAIN

Tissue	Treatment	N*	γ-Hydroxybutyrate (nmoles/g)†
Liver	Freezing	5	1·97 ± 0·10
Whole brain	Quick freezing	5	3·99 ± 0·22
Whole brain	Rapid dissection	8	4·07 ± 0·18
Whole brain	Chilling on ice	11	6·55 ± 0·25
Cortex	Chilling on ice	3	6·79 ± 0·18
Telencephalon	Chilling on ice	5	6·52 ± 0·47
Midbrain + diencephalon	Chilling on ice	5	8·43 ± 0·64‡
Hindbrain	Chilling on ice	4	4·91 ± 0·26§
Cerebellum	Chilling on ice	4	7·74 ± 0·47
Hippocampus	Chilling on ice	2	10·04 (9·68, 10·39)

* N = number of experiments.
† γ-Hydroxybutyrate was assayed by a combined technique of gas chromatography and isotope dilution. Results are expressed as the mean ± S.E.M.
‡ Significantly different from whole brain ($P < 0·05$).
§ Significantly different from whole brain ($P < 0·02$).

The regional distribution of GHB was determined in six general areas of guinea pig brain (see Methods for details on dissection); cortex, telencephalon, midbrain, hindbrain, cerebellum and hippocampus. Of these generalized areas, the hippocampus was found to contain the highest concentration of GHB (Table 2).

Experiments on the subcellular distribution of guinea pig brain GHB indicate that the bulk (over 70 per cent) of this compound can be recovered in the supernatant fraction of sucrose homogenates (Table 3). The remainder found in the particulate fraction does not appear to be localized to any specific subcellular fraction when analyzed by differential centrifugation.

TABLE 3. SUBCELLULAR DISTRIBUTION OF γ-HYDROXYBUTYRATE IN GUINEA PIG BRAIN

Distribution	N*	% Total GHB ± S.E.M.
Supernatant	6	76·8 ± 2·7
Particulate	6	23·2 ± 2·7

* Number of analyses. Each analysis represents the determination of the distribution of GHB in a sucrose homogenate of four pooled guinea pig brains.

Experiments in which ethanol was administered to rats indicated that this agent causes a large increase (about 160 per cent) in the endogenous brain levels of GHB (Table 4). A small but significant increase (about 60 per cent) was also observed in the liver of treated rats. Ethanol in the above dosage produced no significant change in the brain levels of norepinephrine or dopamine.

TABLE 4. TISSUE LEVELS OF γ-HYDROXYBUTYRATE, NOREPINEPHRINE AND DOPAMINE IN NORMAL AND ETHANOL-TREATED RATS

Tissue	Treatment	N	Metabolite measured*	% Increase	P
			GHB (nmoles/g)		
Brain	None	6	1·81 ± 0·14		
	Ethanol (0·5 ml/100 g)	6	4·73 ± 0·85	161	< 0·02
Liver	None	7	2·78 ± 0·13		
	Ethanol (0·5 ml/100 g)	7	4·44 ± 0·45	60	< 0·02
			Norepinephrine (ng/g ± S.E.M.)	Dopamine (ng/g ± S.E.M.)	
Brain	None	5	365 ± 4	615 ± 23	
	Ethanol (0·5 ml/100 g)	6	307 ± 6	607 ± 36	

* Results are expressed as the mean ± the standard error of the mean.

DISCUSSION

The experiments *in vitro* in which cortical slices were incubated with ^{3}H-GABA confirmed our original observations that rat brain could convert GABA to GHB. However, increasing the potassium concentration in the incubation medium to 50 mM (a concentration sufficient to lead to depolarization of the tissue) had no effect on this

conversion. As in the case of the experiments *in vivo*, the extent of the conversion of ^{3}H-GABA to GHB was only minimal. It is, of course, quite possible that the normal conditions of wakefulness in an animal at the time of sacrifice do not provide a brain sample which has a maximal capacity for the conversion of GABA to GHB *in vitro*. There is also the possibility that GHB is derived biosynthetically from another source. The observation of the endogenous occurrence of GHB in the liver (cf. Table 2) makes this possibility even more plausible. It may be noteworthy that in the above experiments we cannot conclusively rule out the possibility that the radioactive GABA employed in these experiments might have contained trace amounts of impurities such as succinic semialdehyde which then could have been directly converted enzymatically to GHB. However, contamination of labeled GABA with labeled succinic semialdehyde seems somewhat unlikely, in view of the inherent instability of succinic semialdehyde in solution.

The finding that GHB has a rather discrete distribution in the mammalian CNS is suggestive that it may play a role other than involvement in intermediary metabolism. The observation that a higher concentration of this substance occurs in the hippocampus and cerebellum than in whole brain was not completely unexpected, in view of the observed distribution of exogenous GHB administered intravenously to cats. In GHB-treated cats, the highest brain concentration of GHB was found in the temporal lobe and cerebellum.[14] However, the parallelism between the exogenous distribution and the endogenous content of GHB in cat and guinea pig brain, respectively, is not complete in that the guinea pig midbrain contains a relatively high concentration of endogenous GHB while the midbrain of the cat is not exceptionally active in taking up exogenous GHB. A possible explanation for the observed regional differences in the GHB content of guinea pig brain could be a differential tendency of various brain structures to form GHB post-mortem.

Endogenous GHB was not found to be associated to any great extent with particulate matter. However, this finding does not rule out the possibility that GHB may be associated with some particular cellular component *in vivo* and become dissociated during the process of homogenization.

Previous experiments by other investigators have indicated that ethanol can cause the "redox state" of the liver and other tissues to be shifted toward a more reduced state. This presumably results from the oxidation of ethanol in the body with a resultant increase in the NADH/NAD ratio both in the liver and in the central nervous system. It is known that GABA is normally metabolized in the mammalian CNS and liver by transamination with α-oxoglutarate to form glutamate and succinic semialdehyde. followed by oxidation of the succinic acid which then enters the Krebs cycle. Experiments in our laboratory have also indicated that rat brain *in vivo* and *in vitro* can convert labeled GABA into labeled GHB.[11] The mechanism of this conversion has not been completely elucidated, but a pathway via succinic semialdehyde appears most likely. If this is the case, it would be expected that an increase in the NADH/NAD ratio in brain would favor a greater reduction of succinic semialdehyde to its corresponding alcohol, GHB. The results expressed in Table 4 are consistent with the contention that, at least in brain, GHB can be formed from GABA, probably via transamination of the GABA and subsequent reduction of the succinic semialdehyde formed by the transamination reaction. However, since GABA is not a metabolite normally found in mammalian liver, another precursor of succinic semialdehyde or GHB or of both

must be present in this tissue, since administration of ethanol also causes an increase in GHB in this tissue although to a lesser extent.

Acknowledgement—The author is grateful to Mr. Karlis Rozitis for his competent technical assistance.

REFERENCES

1. S. P. Bessman and W. N. Fishbein, *Nature, Lond.* **200,** 1207 (1963).
2. W. N. Fishbein and S. P. Bessman, *J. biol. Chem.* **239,** 357 (1964).
3. W. N. Fishbein, Ph.D. thesis, University of Maryland (1965).
4. N. J. Giarman and R. H. Roth, *Science, N.Y.* **145,** 583 (1964).
5. R. H. Roth, Ph.D. dissertation, Yale University (1965).
6. R. H. Roth and N. J. Giarman, *Biochem. Pharmac.* **19,** 1087 (1970).
7. G. L. Gessa, L. Vargiu, F. Crabai, G. C. Boero, F. Caboni and R. Camba, *Life Sci.* **5,** 1921 (1966).
8. G. L. Gessa, F. Crabai, L. Vargiu and P. F. Spano, *J. Neurochem.* **15,** 377 (1968).
9. R. H. Roth and Y. Suhr, *Biochem. Pharmac.* **19,** 3001 (1970).
10. G. K. Aghajanian and R. H. Roth, *J. Pharmac. exp. Ther.*, in press.
11. R. H. Roth and N. J. Giarman, *Biochem. Pharmac.* **18,** 247 (1968).
12. A. K. Rawat, *Eur. J. Biochem.* **6,** 585 (1968).
13. H. McIlwain and R. Rodnight, *Practical Neurochemistry*, Little, Brown, Boston (1962).
14. R. H. Roth and N. J. Giarman, *Biochem. Pharmac.* **15,** 1333 (1966).
15. R. H. Roth and E. A. Stone, *Biochem. Pharmac.* **17,** 1581 (1968).
16. A. H. Anton and D. F. Sayre, *J. Pharmac. exp. Ther.* **145,** 326 (1965).
17. M. M. Kini and J. H. Quastel, *Nature, Lond.* **184,** 252 (1959).