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IN VIVO CONVERSION OF γ-AMINOBUTYRIC ACID AND 1,4-BUTANEDIOL TO γ-HYDROXYBUTYRIC ACID IN RAT BRAIN

STUDIES USING STABLE ISOTOPES

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Abstract—The formation of 4-[1,4-^{13}C]hydroxybutyric acid ([^{13}C]γ-hydroxybutyric acid; [^{13}C]GHB) in rat brain was studied following intracerebroventricular (i.c.v.) administration of either 4-[1,4-^{13}C]aminobutyric acid ([^{13}C]GABA or 1,4-[1,4-^{13}C]butanediol ([^{13}C]1,4-BD) to awake, freely moving animals. GHB and [^{13}C]GHB were measured with a gas chromatographic mass spectrometric (GC/MS) technique designed to detect the lactone derivative of GHB with the acid or lactone being determined by conditions of tissue extraction. [^{13}C]GHB was detected following i.c.v. administration of [^{13}C]GABA with a turnover rate of 2.04 nmol/g tissue/hr and [^{13}C]1,4-BD with a turnover rate of 1.4 nmol/g/hr. The formation of [^{13}C]GHB from [^{13}C]GABA was blocked by an inhibitor of GABA-transaminase, but this drug had no effect on the formation of [^{13}C]GHB from [^{13}C]1,4-BD. The latter pathway was also unaffected by alcohol dehydrogenase inhibitors, compounds which block this pathway in the periphery. Further, in the course of these experiments, naturally occurring endogenous γ-butyrolactone (GBL) was detected in rat brain in a concentration of 200 pmol/g tissue weight, but lactonization *in vivo* of [^{13}C]GHB formed from either labeled GABA or 1,4-BD was not demonstrated. These data confirm two separate pathways of synthesis for GHB in brain, demonstrate the presence of GBL in brain, and illustrate the utility of a new GC/MS technique for analysis of GHB and for GBL which does not involve extensive derivitization.

γ-Hydroxybutyric acid (GHB) is a four-carbon compound which occurs naturally in mammalian brain [1, 2] and possesses a number of properties which suggest a role of neuromodulation or neurotransmission [3]. The main parent compound of GHB in brain is thought to be γ-aminobutyric acid (GABA) [4–7]. The evidence for this hypothesis consists of tracer studies in which the formation of [^{3}H]GHB was followed after intracerebroventricular (i.c.v.) administration of [^{3}H]GABA.

GABA is formed from glutamic acid by L-glutamic acid decarboxylase (GAD; EC 4.1.1.15) and then transaminated by GABA-α-oxoglutarate aminotransferase (GABA-T; EC 2.6.1.19) to succinic semialdehyde (SSA) which is then reduced to GHB by a "specific" NADPH-dependent oxidoreductase [8–11]. GHB can be metabolized back to SSA and then to succinic acid which enters the Krebs cycle. The GHB→SSA reaction appears to be catalyzed by a cytosolic NADPH-dependent oxidoreductase called "GHB dehydrogenase", an enzyme which appears also to be a D-glucuronate reductase (EC 1.1.1.19) [12–17]. Alternatively, Vayer *et al.* [18, 19] have reported that GHB formed from SSA can be rapidly converted back to GABA in the presence of both GHB-dehydrogenase and GABA-T.

Another, less well characterized pathway for the formation of GHB may involve the putative GHB precursor, 1,4-butanediol (1,4-BD). The evidence for such a pathway is indirect. Intracerebroventricular administration of 1,4-BD is associated with an increase of GHB levels in brain [7, 20]. In addition, exogenously administered 1,4-BD is known to be converted rapidly to GHB [21–23], probably by alcohol dehydrogenase [24–27]. Until recently one problem with the hypothesis that 1,4-BD is a GHB precursor in brain has been a lack of evidence for the presence of 1,4-BD in brain. However, this compound has now been demonstrated to be a normal constituent of mammalian brain [27].

The object of the following experiments was to provide direct evidence for the formation of GHB from GABA and 1,4-BD and to characterize these two pathways pharmacologically.

METHODS

Drugs. γ-Vinyl GABA (GVG) was donated by Merrell Laboratories (Cincinnati, OH). 4-[1,4-^{13}C]Aminobutyric acid ([^{13}C]GABA) and 1,4-[1,4-^{13}C]butanediol ([^{13}C]1,4-BD), both approximately 99 atom%, were synthesized by Merck & Co. Inc. (St Louis, MO). All other drugs and reagents were obtained from commercial sources and were of the highest possible purity. Sterile saline was used as the vehicle for all drugs.

Animals. Male Sprague–Dawley rats

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[crl:CD(SD)BR, Charles River] weighing 250–350 g were used for all experiments. The animals were watered and fed *ad lib.* and were maintained on a 12-hr light–dark cycle. For i.c.v. administration of isotopes, cannulae were implanted stereotaxically in the lateral ventricle under halothane anesthesia; i.c.v. injections were not made until 24–28 hr after cannula implantation.

Assays. All animals were decapitated and the brains rapidly excised and immersed in liquid nitrogen. This procedure took 3–5 sec to perform. The brains were homogenized in 2 vol. of 1 N perchloric acid for all assays except when only GBL was being measured. Then deproteinization was carried out by homogenizing the brain in 2 vol. of 80% ethanol. GHB would not be expected to undergo lactonization under these conditions [28]. Ninety percent of the brain homogenate was taken for the assay of labeled and unlabeled GHB or GBL. The remaining 10% was used for measure of labeled and unlabeled GABA or 1,4-BD depending on the experiment being performed. For the GABA assay, the acidic homogenate was adjusted to a pH of 7.0 with KOH and 100% ethanol added such that the final ethanol concentration was 80%. This mixture was rehomogenized and centrifuged at 5000 rpm at 0° for 10 min, and the supernatant fraction was carried through the procedure of Bertilsson *et al.* [29]. For the 1,4-BD assay, the method of Barker *et al.* [27] was used. GHB was assayed by a modification of a technique previously described for flame ionization gas–liquid chromatography [30]. This method involves lactonization of GHB to GBL and subsequent direct measure of the lactone by GC/MS. Two micrograms delta valerolactone (DVL) was added to the brain homogenate as internal standard. The mixture was centrifuged at 15,000 rpm at 0°, and the supernatant fraction was heated in a water bath at 85° for 15 min. The supernatant fraction was then cooled in ice and extracted twice with 2 vol. of methylene chloride at 4°. The methylene chloride extract was dried with $MgSO_4$, evaporated to 100 μl and 1–3 μl placed on the GC/MS system. This technique would be expected to extract all GHB present in brain. Therefore, the concentrations obtained would represent the sum of GHB plus GBL present in brain. To determine if GBL is present naturally in brain or whether any 4-[1,4-^{13}C]hydroxybutyric acid ([^{13}C]GHB) formed from ^{13}C precursor was converted *in vivo* to [^{13}C]GBL, the brain was homogenized in 80% ethanol and the superantant fraction was extracted directly as described with methylene chloride without heating. As mentioned above, using this ethanol extraction technique, any GHB present would not be lactonized and thus not extracted into the methylene chloride. Therefore, the values obtained would represent only GBL. This hypothesis, based on known solubility constants of GHB, was proven by extracting brain homogenates spiked with known quantities of GHB with 2 vol. methylene chloride. The methylene chloride extract was then acidified with acetic anhydride to lactonize any GHB present and assayed for the resultant lactone by GC/MS. No lactone was detected in these preliminary experiments.

GC conditions consisted of an injection port temperature held at 130° and a 1-min delay before the purge valve was activated. During the delay, the oven temperature was held at 50°. Following the delay the temperature was programmed to increase at 30°/min to a maximum of 210°. The column was 30 m × 0.316 mm and coated with DB-wax at a film thickness of 0.5 mm. Use of the carbowax column minimized tailing. The mass spectrometer, an HP-598 GC/MS system, was operated in the selected ion monitoring mode under normal tuning conditions. Masses of 86, 87, 100, and 101 were scanned for 100 msec each in sequential fashion for each spectrum. Transfer lines were maintained at 270°, and the source temperature was 200°. Electron energy was 70 eV.

Kinetic experiments. The isotopes, either [^{13}C]GABA or [^{13}C]1,4-BD, were dissolved in sterile saline such that 5 μl contained 150 μg of drug. Five microliters of drug containing solution was then administered i.c.v. by hand injection over 30–60 sec. The animals were killed at intervals following i.c.v. injection ranging from 30 sec to 60 min, and whole brain concentrations of labeled and unlabeled precursor and labeled and unlabeled GHB and GBL were determined.

Pharmacological experiments. The drugs, dosages, and time of administration of drugs and isotopes in these experiments are shown in Table 4. Briefly, the effect of inhibition of GABA-T by GVG on the formation of [^{13}C]GHB from [^{13}C]GABA and [^{13}C]1,4-BD was determined. The effects of the alcohol dehydrogenase inhibitor, pyrazole, as well as that of ethanol were determined also. In addition, brains of rats that received [^{13}C]1,4-BD were assayed for [^{13}C]GABA in order to determine if labeled GABA was synthesized from the labeled GHB formed from [^{13}C]1,4-BD [18, 19]. The converse experiment was also performed. That is, the brains of animals that received [^{13}C]GABA i.c.v. were assayed for [^{13}C]1,4-BD.

Data analysis. The methods used to calculate the turnover rate (T_0) of GHB derived from i.c.v. 1,4-BD or GABA in these experiments were those described by Bertilsson *et al.* [29] and Dessort and colleagues [31]. The formula is as follows:

$$T_0 = [B] \times \frac{2(\mathrm{GHB}_{t_2} - \mathrm{GHB}_{t_1})}{(t_2 - t_1)\,[(P\text{-GHB})_{t_1} + (P\text{-GBH})_{t_2}]} \times 60$$

where T_0 = turnover; $[B]$ = mean steady-state brain concentration of GHB in nmol/g; GHB = ratio of labeled to unlabeled GHB; and P = ratio of labeled to unlabeled precursor in the brain for two time periods, t_1 and t_2, which are expressed as minutes after i.c.v. administration of isotope where $t_1 - t_2 =$ 1 min. The fractional rate constant for the time examined is expressed as $\kappa_B = T_0/B$ and the turnover time, $T_t = 1/\kappa_B$. The latter value represents the time for GHB in the pool to be synthesized. The N for each group of animals, both experimental and control, was four to six. Paired control animals were used for all experiments. Means and standard errors were calculated for all experiments. Statistical analysis was performed by the Dunnett test and by ANOVA.

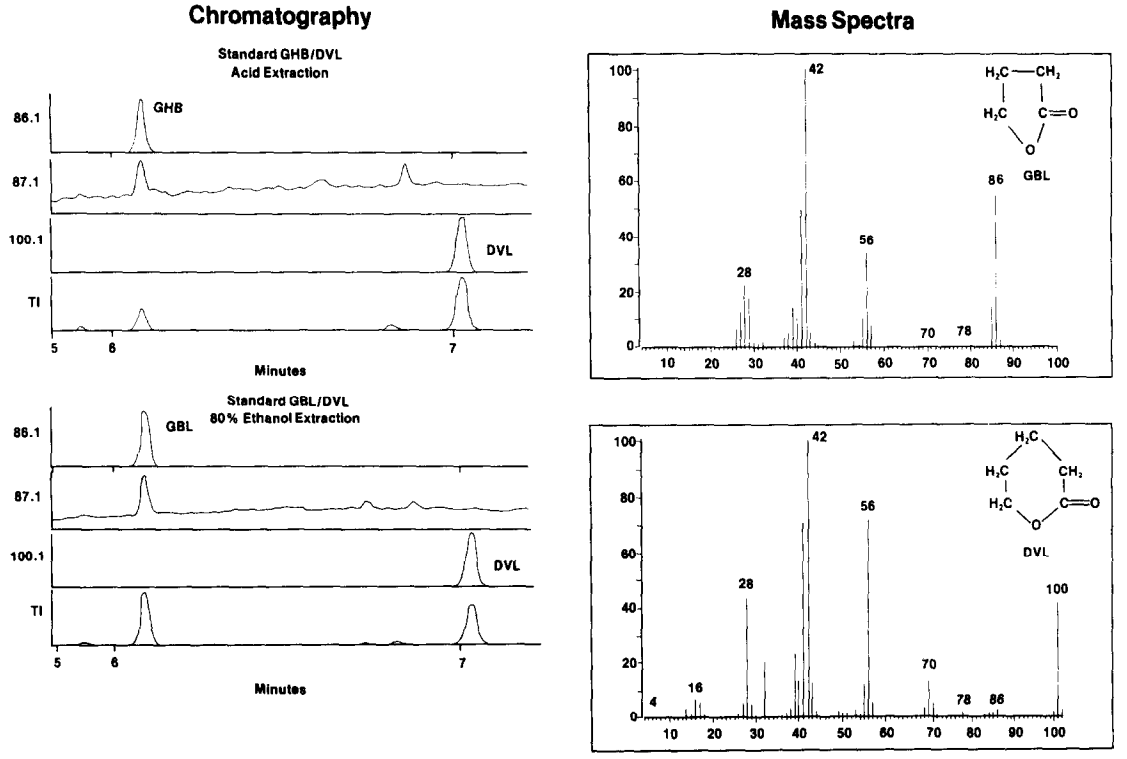

Fig. 1. Gas chromatography and mass spectra of GBL and DVL. These lactones were analyzed by extracting them from an acid medium into methylene chloride as described in Methods. The solvent was reduced in volume under N_2 to 100 μl, and 3 μl was injected into the GC/MS system. GC conditions consisted of an injection port temperature held at 130°, and a 1-min delay before the purge valve was activated. During the delay, the oven temperature was held at 50°. Following the delay, the temperature was programmed to increase at 30°/min to a maximum of 210°. The column used was 30 × 0.316 mm and coated with DB-Wax at a film thickness of 0.5 mm. The use of the carbowax column minimized tailing. The mass spectrometer, an HP-5985 GC/MS system, was operated in the selected ion monitoring mode under normal tuning conditions. Masses of 86, 87, 100 and 101 were scanned for 100 msec each, in sequential fashion for each spectrum. Transfer lines were maintained at 270° and the source temperature was 200°. Electron energy was 70 eV. T_1 refers to time.

RESULTS

The GC characteristics and mass spectra of GBL are shown in Fig. 1. The recovery of GBL using brain homogenates spiked with pure standard was 65 ± 7.1% (N = 5). The conversion of GHB to GBL under the conditions of acidification and heating used was 50%. The standard curve for the assay was linear for concentrations ranging from 50×10^{-12} to 100×10^{-2} M GHB with $r = 0.997$. The mean whole brain concentration of GHB in all control samples (N = 52) was 2.89 ± 0.25 nmol/g. GBL was also found to be present in whole brain in a concentration of 250 ± 36 pmol/g (N = 37). This is about 10% of the concentration of GHB in brain.

[^{13}C]GHB was demonstrable in whole brain following the i.c.v. administration of either [^{13}C]GABA or [^{13}C]1,4-BD. The kinetics of the formation of GHB from GABA and 1,4-BD under these conditions are shown in Tables 1–3 and Fig. 2. In those experiments where [^{13}C]GABA was given i.c.v. and the brain assayed for both [^{13}C]GBL and [^{13}C]GHB by varying the extraction conditions, only [^{13}C]GHB was found. This indicates that the [^{13}C]GHB formed did not lactonize *in vivo* under the conditions used. Similarly, no [^{13}C]GABA was detected in those animals who received [^{13}C]1,4-BD i.c.v. nor was any [^{13}C]1,4-BD found after i.c.v. administration of [^{13}C]GABA, indicating that the [^{13}C]GHB formed did not go on to form either one of these compounds.

Table 1. Kinetic data for the formation of GHB from GABA in whole brain for sequential 1-min periods of time from 5 to 10 min after i.c.v. administration of isotope

t_1-t_2	GHB_1	GHB_2	P_1	P_2	κ_B	T_0
5–6	0.063	0.077	0.59	0.75	1.30	3.90
6–7	0.077	0.089	0.75	0.86	1.10	3.30
7–8	0.089	0.103	0.86	1.46	0.73	2.19
8–9	0.103	0.117	1.46	2.65	0.40	1.20
9–10	0.117	0.129	2.65	2.80	0.30	0.9

κ_B = fractional rate constant; T_0 = turnover rate of GHB; P = ratio of labeled to unlabeled GABA; GHB = ratio of labeled to unlabeled GHB; subscripts refer to t_1 or t_2 (t_1 and t_2 represent minutes after i.c.v. injection of isotope).

The results of the pharmacologic experiments are shown in Table 4. The conversion of GABA to GHB was blocked completely by GVG, but this drug had no effect on the formation of GHB from 1,4-BD. Neither pyrazole nor ethanol, compounds which decrease the formation of GHB from 1,4-BD in liver [25, 26], had any effect on this pathway in brain.

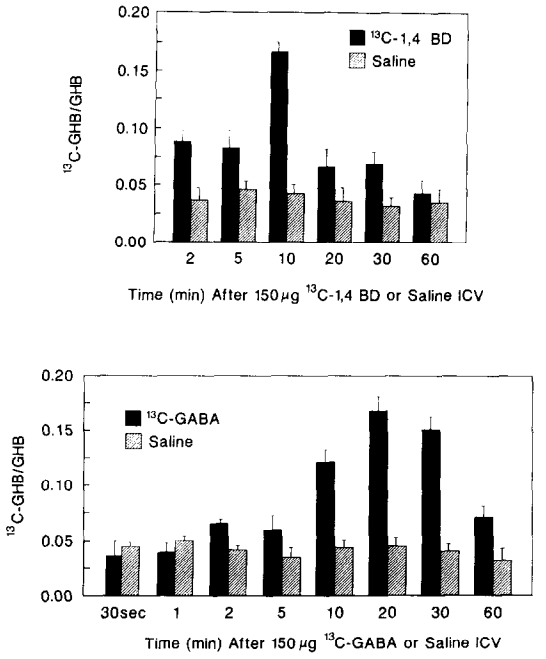

Fig. 2. Time course of conversion of [^{13}C]1,4-BD (top) or [^{13}C]GABA (bottom) to [^{13}C]GHB after 150 μg i.c.v. of the respective isotope. Each time point represents four animals run in triplicate. The vertical lines represent SEM. All ratios were significantly greater than control ($P < 0.05$) except the 30-sec and 1-min GABA and the 60-min time point in the 1,4-BD experiments.

Table 2. Kinetic data for the formation of GHB from 1,4-BD in whole brain for sequential 1-min periods of time from 5 to 10 min after i.c.v. administration of isotope

t_1–t_2	GHB_1	GHB_2	P_1	P_2	κ_B	T_0
5–6	0.075	0.096	1.06	2.07	0.85	2.55
6–7	0.096	0.115	2.07	2.07	0.64	1.92
7–8	0.115	0.139	2.07	2.98	0.48	1.44
8–9	0.139	0.162	2.98	5.19	0.32	0.96
9–10	0.162	0.186	5.19	6.60	0.22	0.66

κ_B = fractional rate constant; T_0 = turnover rate of GHB; P = ratio of labeled to unlabeled 1,4-BD; GHB = ratio of labeled to unlabeled GHB; subscripts refer to t_1 or t_2 (t_1 and t_2 represent minutes after i.c.v. injection of isotope).

Table 3. Kinetic parameters of GHB derived from either GABA or 1,4-BD in whole brain

	κ_B (hr)	T_0 (nmol/g tissue/hr)	T_t (hr)
[^{13}C]GABA	0.766 ± 0.246	2.21	1.31
[^{13}C]1,4-BD	0.502 ± 0.113	1.45	1.99

κ_B = mean ± SE fractional rate constant determined from the data in Table 1 and 2; T_0 = turnover rate of GHB; T_t = turnover time or the time required for GHB in the pool to be synthesized.

DISCUSSION

The use of the GC/MS assay procedure for GHB and GBL described herein has several advantages. It is simple and gives good yields since it does not require derivitization of the GHB molecule [32, 33] nor a series of washings and reextractions [7]. In addition, this procedure allows one to differentiate clearly between GHB and GBL by altering the conditions of extraction of the brain. The separation of GHB and GBL for purposes of analysis has long been a problem particularly in pharmacologic experiments where GBL is often used as a prodrug of GHB [34]. These data confirm that GBL does occur as a natural constituent of rat brain in a concentration which is 10% that of GHB. The biological significance of this finding is uncertain since GBL is thought to have no direct effect on neuronal function [35] and has no affinity for the GHB binding site in brain [36, 37]. Further, we were unable to demonstrate the formation of [^{13}C]GBL from the [^{13}C]GHB produced from either precursor used. The explanation for this may be either that no lactonization occurred *in vivo* or that the amount of [^{13}C]GBL formed was so small as to escape detection.

Although we utilized the method of Bertilsson *et al.* [29] in calculating the kinetics of GHB synthesized in these experiments, there are problems with this method as described by those authors. The kinetic values arrived at should be considered valid only for the strict experimental conditions imposed in the current experiments. They should be interpreted in light of the fact that this method of calculation does not take into account the problem of compartmentation or biodistribution after i.c.v. administration of isotope. Moreover, the turnover time of GHB, as determined by measuring this compound varying periods of time after valproate administration, has been shown to vary from region to region [28]. Therefore, the mean whole brain turnover time of GHB in our experiments would be a function of biodistribution of labeled precursor, regional turnover differences, compartmentation, and recycling of the label. It should also be emphasized that this method is more useful for making pharmacological comparisons than establishing absolute rates [29]. However, even with these reservations, the turnover time resulting from the current experiments correlates well with that reported in previous experiments where [^{3}H]GABA was administered i.c.v. and [^{3}H]GHB measured [6].

Since the use of ^{13}C-labeled compounds in conjunction with GC/MS is qualitatively more exact than ^{14}C- [10–12] or ^{3}H- [1–6] labeled drugs, these studies provide conclusive evidence for the formation of GHB from GABA. The results of the GVG studies also confirm that GABA-T is involved in this pathway.

The failure to identify [^{13}C]GABA in animals that received [^{13}C]1,4-BD indicates that the GHB formed from the labeled 1,4-BD was not converted to GABA. Our inability to confirm a pathway by which GHB is converted to GABA [18, 19] may be explained in one of four ways. First, the amount of [^{13}C]GABA formed may have been too small to be detected by the techniques used. Second, the [^{13}C]GHB formed from labeled 1,4-BD may be in a pool separate from that where the GHB→GABA conversion normally takes place. The third possibility has to do with postmortem changes in GABA

Table 4. Pharmacologic profile of the formation of GHB from 1,4-BD and GABA

Drug	Dose	Time*	Isotope†	[^{13}C]GHB/GHB‡	P
Saline		30 min	Saline	0.031 ± 0.002	—
Saline		60 hr	[^{13}C]GABA	0.138 ± 0.009	<0.05§
GVG¶	1200 mg/kg	60 hr	[^{13}C]GABA	0.052 ± 0.004	<0.05‖
Saline		60 hr	[^{13}C]1, 4-BD	0.080 ± 0.007	<0.05§
GVG	1200 mg/kg	60 hr	[^{13}C]1, 4-BD	0.084 ± 0.007	NS**
Saline		10 min	[^{13}C]1, 4-BD	0.082 ± 0.008	NS**
Pyrazole	250 mg/kg	10 min	[^{13}C]1, 4-BD	0.097 ± 0.010	NS**
Alcohol	3 g/kg (36% v/v)	20 min	[^{13}C]1, 4-BD	0.095 ± 0.009	NS**

* Time between i.p. drug administration and i.c.v. administration of isotope.

† One hundred and fifty micrograms i.c.v.

‡ These values (means ± SE, N = 5–6 for all data points) represent 10-min values, but the time course in the saline-[^{13}C]GABA and saline-[^{13}C] 1,4-BD experiments is seen in Fig. 1; the time course in all NS experiments was unchanged from the appropriate control experiment.

§ Significantly increased compared to saline–saline control.

‖ Significantly decreased compared to saline-[^{13}C]GABA control, but no significant difference from saline–saline control, indicating a complete blockade of the formation of [^{13}C]GHB from [^{13}C]GABA under these conditions.

¶ GVG = γ-vinyl GABA.

** No significant difference from saline-[^{13}C]1,4-BD control.

and GHB systems in brain. Although microwave would have been a preferable method of sacrifice in these experiments, it was not possible in animals with indwelling ventricular cannulae. Further, postmortem changes that occur with GHB [33] and GABA have been shown to be negligible with the method of sacrifice and quick freezing of brain employed in the current experiments [1, 38, 39]. The data in support of a GHB→GABA pathway have been generated using either purified enzyme preparations [17] or aerated brain slices [18], preparations where postmortem changes are not an issue. Those experiments suggest that both GHB dehydrogenase and GABA-T are required for the pathway to be operative. Since GABA-T is very sensitive to asphyxia, it may be that decreased activity of this enzyme postmortem in whole animals in relation to that of GHB dehydrogenase, however slight, would be sufficient to render the GHB→GABA pathway inoperative. If this hypothesis is correct, one would expect to see evidence of such a pathway only in animals killed by microwave or in *in vivo* experiments where asphyxia is not a problem. On the other hand, since labeling of the GABA would have occurred before death, the postmortem effects of asphyxia may not have any effect on this phenomenon. A final possibility is that there is negligible conversion of GHB→GABA *in vivo* with the bulk of GHB being oxidized to $CO_2 + H_2O$.

Although a pathway from 1,4-BD to GHB was postulated 20 years ago, evidense has been indirect until now. These experiments provide conclusive proof that the brain is capable of synthesizing GHB from 1,4-BD. However, whereas in the periphery the formation of GHB from 1,4-BD is catalyzed by alcohol dehydrogenase [25, 26], the reaction in brain appears to be insensitive to either pyrazole or ethanol. The failure to demonstrate [^{13}C]1,4-BD in animals given [^{13}C]GABA suggests that 1,4-BD is not formed from GABA nor does a reverse GHB→1,4-BD pathway exist. However, the same possible explanations outlined above apply here as well, i.e. compartmentation, sensitivity of assay techniques, and/or postmortem effects, however slight, that could mask the presence of such a pathway.

In summary, these experiments have utilized a simple GC/MS technique for measuring GHB and GBL to confirm and characterize two separate pathways in brain by which GHB can be formed from GABA and 1,4-BD and to demonstrate the presence in brain of GBL. The 1,4-BD→GHB pathway remains a mystery in terms of its regional distribution, enzymology, conditions under which it is normally operative, and the source of 1,4-BD.

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