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CONVERSION OF 1,4-BUTANEDIOL TO γ-HYDROXYBUTYRIC ACID IN RAT BRAIN AND IN PERIPHERAL TISSUE*

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Abstract—Experiments in rats demonstrated that brain, liver, kidney and heart can convert 1,4-butanediol to γ-hydroxybutyrate. The liver was by far the most active tissue studied. Although brain was capable of converting 1,4-butanediol to γ-hydroxybutyrate, it appeared that the majority of γ-hydroxybutyrate derived from systemically administered 1,4-butanediol is formed in the liver. Thus, partial hepatectomy reduced the formation of γ-hydroxybutyrate from 1,4-butanediol as well as markedly reducing the "sleep" normally produced by administration of 1,4-butanediol. Very little difference was observed in the ability of various brain regions to oxidize 1,4-butanediol to γ-hydroxybutyrate. In addition, regional brain areas did not appear to have any observed specificity for the binding or retention of newly formed γ-hydroxybutyrate when 1,4-butanediol was administered either intravenously or intraventricularly.

PREVIOUS experiments have indicated a number of similarities in the neuropharmacological action of γ-hydroxybutyric acid (GHB), γ-butyrolactone (GBL) and 1,4-butanediol (BD).[1] Although the molecular basis for the anesthetic-like action of these compounds still remains somewhat obscure,[2–5] it has been conclusively demonstrated that GHB is the active molecular species in exerting the anesthetic-like action of these three compounds.[2,6–9] In the course of studying the conversion *in vivo* of BD to GHB, it was observed that after the intravenous administration of 5·8 m-equiv./kg of BD that the blood level of GHB never exceeded about 4 μmoles/ml, while the brain concentration reached a maximum level of about 2 μmoles/g 90 min after administration of BD.[6] This finding can be contrasted to the results obtained after administration of equimolar amounts of GHB and GBL where it was observed that at 90 min, when the brain levels were only about 1 and 1·5 μmoles/g, respectively, the blood concentration of GHB was equal to or greater than that observed after treatment with BD.[7] This suggested to us that some of the brain GHB might be derived directly from local metabolism of BD rather than solely from blood-borne GHB formed extracerebrally. Thus, from this standpoint alone it was of interest to see if mammalian brain could synthesize GHB from BD. Also, in view of the findings that BD may exist among lipid-diols in the rat[10] and the evidence that BD is converted to GHB *in vivo*,[6] the suggestion must be entertained that endogenous BD might serve as a depot form of

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precursor for endogenous GHB, a natural metabolite of rat brain.[11] A general prerequisite for this assumption is that brain can convert BD to GHB. Therefore, the primary purpose for these experiments was 3-fold: (a) to determine if brain could convert BD to GHB, (b) to evaluate the formation of GHB from BD by other tissues, and (c) to determine which site in the body is most influential in producing the GHB from BD which ultimately leads to the observed behavioral depression after administration of BD.

MATERIALS AND METHODS

Preparation and utilization of tissue slices and minces. Cortical slices were prepared essentially by the method outlined by Roth and Giarman[2] except that a "bow-cutter" (cf. McIlwain and Rodnight[12]) was used instead of a Stadie blade. Subcortical slices were made by removing all cortical tissue and the two hippocampi from a chilled brain and slicing horizontally across the upper surface of the subcortical tissue. No more than six to eight slices per cortex or subcortex were made from each brain. The ventricle of the heart and the kidney and liver were sliced in the same manner as the cortex except no concern was given for the number of slices taken from a given organ.

All other regions of the brain used were minced with a Stadie blade. These regions included the cerebellum, caudate nucleus, hippocampus, brain stem and thalamus. Care had to be taken not only to maintain a constant thickness, but also to maintain a relatively constant amount of tissue, since the conversion of BD to GHB appeared to vary in some instances and was not directly proportional to the weight of the tissue used. Minces and slices were weighed on a torsion balance and the incubations carried out at 37° in oxygenated Krebs–Henseleit medium of the following composition (mM): NaCl, 118·07; KCl, 4·75; $CaCl_2$, 2·54; KH_2PO_4, 0·93; $MgSO_4$, 1·19; $NaHCO_3$, 25·00; glucose, 11·10; EDTA, 0·027. Prior to the start of the incubation 0·94 μc of 2-^{14}C-BD (9·4 μc/μmole, obtained from ICN Chemical and Radioisotope Division, Irvine, Calif.) was added to each sample.

GHB assay. After incubation, tissues and media were immediately precipitated by addition of 0·5 ml of 50% trichloroacetic acid (TCA) and chilled in ice. The tissues were then homogenized and centrifuged at 9000 *g* for 15 min. The supernatant was retained and 50 μmoles of GHB and BD added to each sample as carriers. At this point, samples were usually frozen and retained overnight for analysis on the following day. Once thawed, the pH of the samples was adjusted to 11·0, the samples were saturated with NaCl and shaken with twice their volume of butanol (saturated with water and NaCl) for 20 min. The samples were centrifuged at 4000 rev/min in an International Centrifuge (swinging-bucket rotor), after which as much of the butanol as possible was removed and discarded. This extraction was repeated two more times to quantitatively remove the BD from the extract. After the third extraction, the pH of the samples was readjusted to 1·0 and the samples were heated at 85° for 10 min to convert GHB to the lactone form. The samples were then chilled in an ice bath, 0·5 ml of 0·5 M potassium phosphate buffer, pH 7·0, was added and the pH was readjusted to 6·0. Samples were then shaken with twice their volume of benzene, as with the butanol extraction, except that the benzene phase was retained. This extraction with benzene was repeated once, and the benzene phase from the second extraction was added to the first benzene extract. An aliquot of each combined benzene

extract was added to 10 ml of dioxane–toluene–ethanol (DTE) scintillation fluid (consisting of 1 l. each of dioxane, toluene and ethanol, containing 0·3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 15 g of 2,5-diphenyloxazole and 240 g of naphthalene) and counted in a Packard scintillation spectrophotometer. The remainder of the benzene extract was analyzed gas chromatographically (see below).

Gas chromatography. The benzene extracts containing the GBL were carefully evaporated at 60° under nitrogen to 0·2 ml in the case of liver slice extracts and to 0·1 ml for brain extracts. From each sample, 10 μl was removed, 5 μl of which was placed in 10 ml of DTE and counted, while the other 5 μl was injected into a Model 5000 Barber–Coleman gas chromatograph equipped with a model 5190 radioactive monitor system (10/1 splitter). Figure 1 shows a comparison between (a) the gas chromatograms of authentic GBL (in the extraction procedure GHB is quantitatively converted to its lactone form, GBL; see GHB assay) and the benzene extracts of rat liver slices incubated with 2-^{14}C-BD, and (b) the radiograms of a standard prepared from GHB-^{14}C (Carboxyl-^{14}C-GHB obtained from Schwarz Bioresearch, Inc., Orangeburg, N.Y.) and a radiogram from the benzene extract prepared from the same rat liver slices incubated with 2-^{14}C-BD as illustrated in Fig. 1.

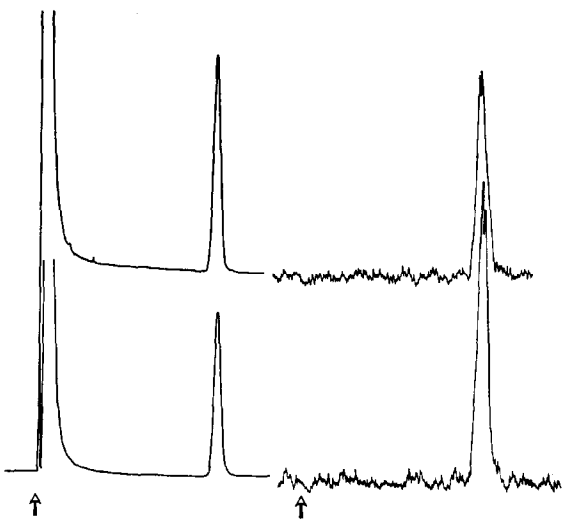

FIG. 1. Left panel (upper trace): gas chromatogram of authentic γ-butyrolactone (2·75 μg) compared with (lower trace) an extract from rat liver slices incubated with 1,4-butanediol-^{14}C. Right panel (upper trace): gas radiochromatogram of standard ^{14}C-γ-hydroxybutyrate, compared with radiochromatogram of extract from liver slices incubated with 1,4-butanediol-^{14}C.

Some samples were also chromatographed in a model 5000 Barber–Coleman without the radioactive monitor system. In these cases, a 10-μl aliquot of each sample was chromatographed as outlined by Roth and Giarman.[2,13] Radioactive purity of the extract was determined by injecting 5 μl of a sample into 20 ml of the DTE scintillation mixture and 5 μl into the gas chromatograph and collecting the column effluent.[13] The average recovery of benzene extractable label in the GBL peak was 95 per cent. Under the conditions of the above gas chromatographic assay for GBL, BD had a

much longer retention time on the column and thus did not contaminate the GBL peak with radioactivity.

Intracisternal injection. Rats were injected intracisternally according to the procedure of Jeffers and Griffith[14] as modified by Roth[15] with 50 μl of a solution of 2-^{14}C-BD in Krebs–Henseleit buffer (47 μc/ml, sp. act. = 9·4 μc/μmole). A series of different rats were injected with a similar amount of the same solution to which GHB ($8{\cdot}3 \times 10^{-3}$ M final concentration) had been added. The rats were sacrificed after 30 min; their brains were removed and dissected as quickly as possible and frozen on dry ice.

Five regions were separated: the cerebellum, the cortex, the two hippocampi, the two caudate nuclei and the remaining bulk of the midbrain. After being weighed, each section was homogenized in 5 ml of 15% TCA and assayed for GHB-^{14}C as described above.

Intravenous injection. Male Sprague–Dawley rats (200–250 g) were injected intravenously via the tail vein with 5·8 m-equiv./kg of a solution of 5·8 m-equiv./ml of 2-^{14}C-BD (13·48 μc/ml). Sleep induction time (loss of righting reflex; R.R.) and waking time (regaining R.R.) were noted, if possible (i.e. if rats were not sacrificed prior to their regaining the R.R.). Rats were sacrificed by decapitation at various times after injection. As much blood as possible was collected after decapitation and mixed with 200 units of heparin. The brains were rapidly removed and frozen immediately on dry ice. After being weighed, they were homogenized with 5 ml of 15% TCA and centrifuged for 15 min at 10,000 rev/min in a Sorvall refrigerated centrifuge. The blood was precipitated with 2 vol. of 15% TCA and centrifuged at 4000 rev/min in an International Centrifuge (swinging-bucket rotor). The supernatants were then assayed for total radioactivity, 2-^{14}C-BD and GHB-^{14}C. Aliquots were removed at three points in the procedure: the first after the addition of the carrier BD and GHB (0·05 ml, for estimation of total radioactivity); the second, 2 ml from the first butanol extraction for estimation of ^{14}C-BD); and the third from the pooled benzene phase (0·05 ml, for estimation of ^{14}C-GHB).

Hepatectomy. Hepatectomies were performed as outlined by Higgins and Anderson,[16] using ether as the anesthetic. Control rats were subjected to all aspects of surgery except for the actual tying off and removal of the liver. The portion of the liver removed was weighed, and the weight of the remaining liver was determined at the time of sacrifice. The percentage of liver removed was calculated by dividing the weight of the liver removed during hepatectomy by the total liver weight. The per cent of the liver removed was $73{\cdot}1 \pm 2{\cdot}2$.

Regional brain distribution. Male Sprague–Dawley rats (150–225 g) obtained from Charles River, Inc., were injected intraperitoneally with 5·8 m-equiv./kg of a solution of 2-^{14}C-BD (1·7 μc/m-mole). Sleep induction time and waking time were noted, when possible. Two rats were sacrificed at each time point, except for the 90-min interval at which four rats were used. The standard deviation was calculated for the 90-min point. The blood was removed as described previously. The brains were removed, placed on filter paper over ice, washed quickly with isotonic saline, and dissected rapidly into seven regions (cortex, cerebellum, hippocampus, striatum, reticular formation, thalamus and hypothalamus) as outlined by Glowinski and Iverson.[17] After being weighed, the tissue was homogenized with 5 ml of 15% TCA and extracted as before (see "Intravenous injection") except that the pellet was resuspended in an additional 1 ml of 15% TCA and recentrifuged, and the two supernatants were

pooled. The assay for labeled BD was not performed in this case. Two ml of the final pooled benzene phases was counted in 10 ml of a toluene scintillation fluid [containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene/liter of toluene to estimate newly formed 2-^{14}C-GHB]. Estimation of the total counts in each region was carried out as described above.

RESULTS

Preliminary experiments were conducted to determine the efficiency of the extraction method for analyzing of GHB and BD. When 0·2 μc of 2-^{14}C-BD was added to 5 ml of aqueous solution, 96 per cent or more of radioactivity was recovered in the three butanol extractions. No significant amount of 2-^{14}C-BD was found in the subsequent benzene extracts. The recovery of GHB-^{14}C (0·04 μc/ml) from the same volume of aqueous phase by the benzene extraction procedure after the prior butanol extraction was 57 ± 3·5 per cent. No more than 15 per cent of the ^{14}C-GHB was extracted by the three butanol extractions. Reported values, however, are not corrected for recovery.

Formation of GHB from BD by liver slices

The first series of experiments were conducted in rat liver slices, since it seemed likely that the liver would contain sufficient enzyme activity to oxidize BD to GHB. Initial experiments indicated that the rate of GHB formation from BD was proportional to substrate concentration over a range of 10–40 μmoles/ml. Figure 2 illustrates the conversion of BD to GHB by rat liver slices incubated with 1·0 μc of the stock 2-^{14}C-BD for varying lengths of time, with and without 5 μmoles of GHB. The amount of labeled GHB recovered reached a peak at 30 min whether or not unlabeled GHB had been added, but the peak was approximately 100 per cent higher when GHB was added as a metabolic trap.

Formation in vitro *of GHB*

The majority of the regions measured for activity in the rat brain contained about

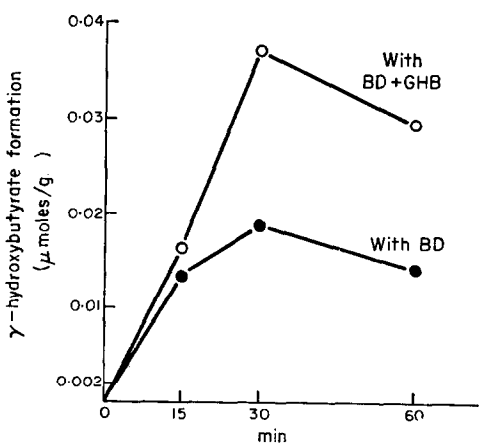

FIG. 2. Time course of γ-hydroxybutyrate formation from 1,4-butanediol in rat liver slices.

15–35 per cent of the activity found in the liver in terms of conversion of BD to GHB per gram of wet weight (cf. Table 1). Regions showing this amount of activity were the cerebellum, hippocampus, caudate nucleus, brain stem and thalamus.

The heart and the kidney were also analyzed for their capacity to oxidize BD. The ventricle of the heart showed only a slightly higher activity than the minced brain regions, whereas the kidney was comparable to the cortical and midbrain slices.

TABLE 1. FORMATION *in vitro* OF γ-HYDROXYBUTYRATE BY BRAIN SLICES INCUBATED WITH [2-^{14}C]BUTANEDIOL

	n	GHB formation (nmoles/g/hr)*
Cortex	4	8·7 ± 0·6
Midbrain	5	9·9 ± 0·8
Cerebellum	2	6·4, 7·8
Hippocampus	4	12·2 ± 1·7
Striatum	4	16·3 ± 2·0
Thalamus	2	11·1, 12·4
Brain stem	2	5·1, 8·2
Liver	4	47·6 ± 3·8

* Results are expressed as the mean ± S.E.M. or as individual values. Tissue slices or minces were incubated for 30 min at 37° in Krebs–Henseleit medium containing 0·94 μc of 2-^{14}C-BD.

Intracisternal injection

2-^{14}C-BD with and without GHB was injected intracisternally into rats (250 g). As seen in Table 2, the recovery of newly formed labeled GHB in the caudate nuclei and the cortex was especially low. The cerebellum had the highest activity, but this might merely reflect the proximity of the cerebellum to the site of injection. The amount of labeled GHB recovered in the midbrain nearly doubled with the addition of unlabeled GHB, suggesting that this region might be at least more active than other brain regions tested in the metabolism of GHB. The cortex also displayed this effect.

Intravenous injection with BD-^{14}C

Normal rats. Twenty-four rats were injected with 5·8 m-equiv. of BD-^{14}C as described in Methods. Four were sacrificed at each interval designated on Fig. 3. The sleep induction time was 23 ± 3·5 min and the waking time was 127 ± 12 min after injection. Total radioactivity in the blood dropped sharply after 15 min, but the brain level remained fairly constant. The brain level of BD increased slightly up to 15 min and then decreased proportionally with the blood. Both brain and blood GHB peaked at 60 min, the blood concentration maintaining a level about twice that of the brain. It appeared that a level of 0·76 mM in brain GHB (corrected for recovery) is needed to achieve and maintain a loss of the righting reflex. This is similar to the brain level of GHB reported by Giarman and Roth[7] which was necessary to cause a loss of the righting reflex in rats treated intravenously with either GHB or GBL. It can be seen in Fig. 3 that the blood level of GHB does not follow the anesthetic state of the rat as closely as does the brain level.

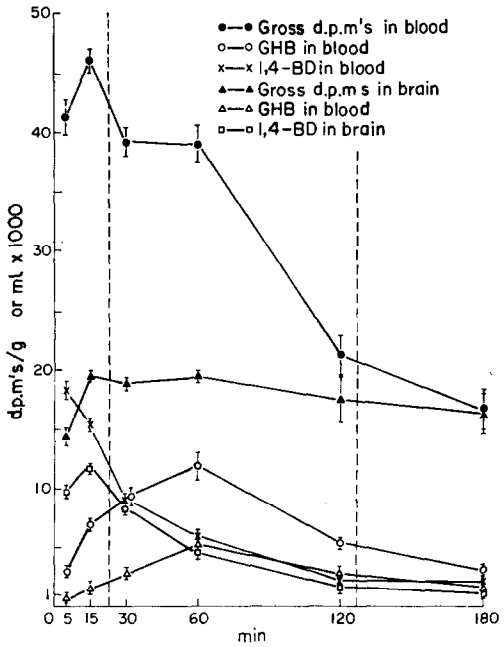

FIG. 3. Distribution in blood and brain of total radioactivity, ^{14}C-1,4-butanediol and ^{14}C-GHB after i.v. administration of 1,4-butanediol (5·8 m-equiv./kg, 13·5 μg/kg). Dashed line on left indicates sleep induction time (loss of righting reflex) and dashed line on right indicates regaining of righting reflex. Each point is the mean of four experiments. The vertical bars depict the S.E.M.

TABLE 2. FORMATION *in vivo* OF γ-HYDROXYBUTYRATE IN VARIOUS BRAIN REGIONS AFTER INTRACISTERNAL INJECTION OF [2-^{14}C]BUTANEDIOL*

Brain section	Newly formed GHB (pmoles/g)	Newly formed GHB (pmoles/g) (GHB added as trap)
Cerebellum	168	205
Striatum	35	42
Hippocampus	89	89
Cortex	29	69
Midbrain	84	158

*Each value is the mean of two or more determinations.

Hepatectomized rats. Male rats (200 g) were hepatectomized 3–5 hr prior to the administration of the BD. Two rats were sacrificed at each time indicated in Fig. 4, except that three rats were used at the 30-min point. Four sham-operated rats were prepared as indicated in Methods and sacrificed accordingly—2 at 30 min, 1 at 60 min, and 1 at 180 min. The sham-operated controls lost their righting reflex at an average of 18·8 min ± 0·9. Only two hepatectomized rats lost their righting reflex—one at 60 min, the other at 66 min after injection. The rest appeared to be highly sedated but never quite lost their righting reflex. GHB levels in both the brain and

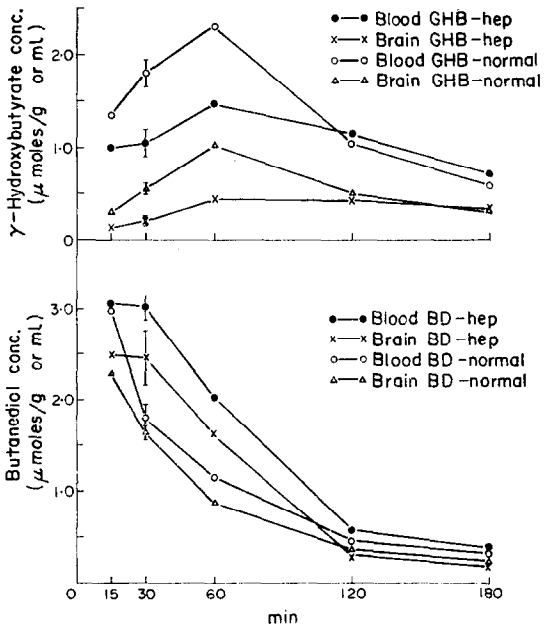

FIG. 4. (a) Upper panel: distribution of GHB at various times after i.v. administration of labeled 1,4-butanediol (5·8 m-equiv./kg, 13·5 μc/kg) in hepatectomized and sham-operated rats. (b) Lower panel: distribution of labeled 1,4-butanediol at various times after i.v. administration of 1,4-butanediol (5·8 m-equiv./kg, 13·5 μc/kg) in hepatectomized and sham-operated rats. Each point is the mean of two determinations except the 30-min interval which is the mean of four determinations. The vertical bars depict the standard deviation.

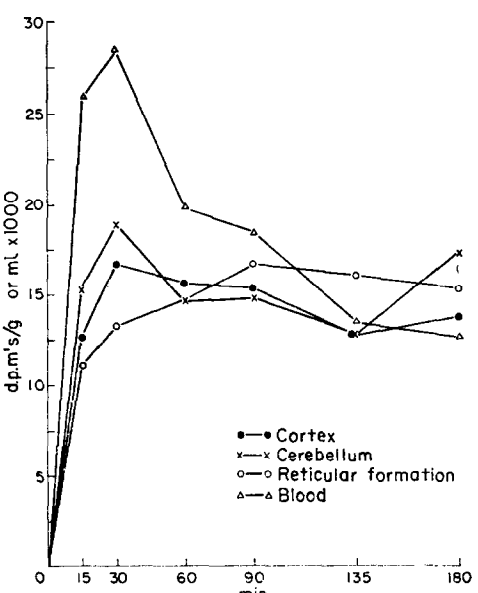

FIG. 5. Time course of the distribution of total radioactivity in blood and brain regions after i.p. administration of 1,4-butanediol. Each point is the mean of two or more experiments.

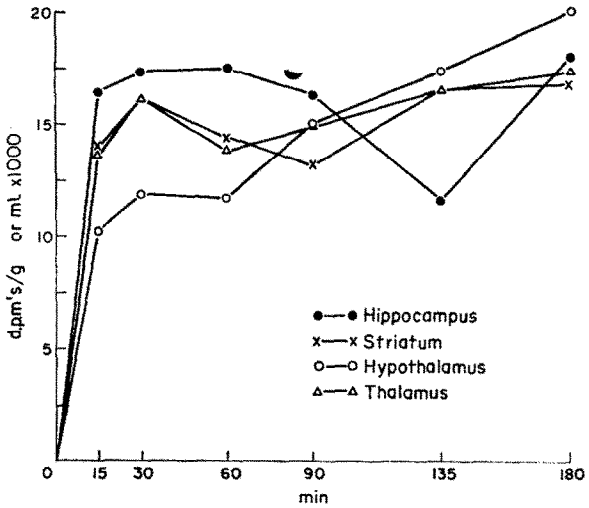

FIG. 6. Time course of the distribution of total radioactivity in brain regions after i.p. administration of 1,4-butanediol-^{14}C (5·8 m-equiv./kg). Each point is the mean of two or more determinations.

blood were greatly reduced when compared to the levels found in controls (Figs. 3, 4a). The brain level of GHB in hepatectomized rats is just barely above the level observed to cause the loss of righting reflex in normal rats. Figure 4b compares BD levels and illustrates the delay in the decrease of the BD level observed in the hepatectomized rats. Since it has been observed that the liver contained the majority of the enzymatic capacity to convert BD to GHB, this delay was not unexpected with about 73 per cent of the liver removed. One hepatectomized rat did not regain his righting reflex for 180 min, at which time he was sacrificed. As shown in Figs. 5 and 6, the levels of brain GHB in hepatectomized rats drop very slowly from a plateau between 60 and 120 min. This is most likely the result of the delayed conversion of the BD to GHB and a reduction in the rate of metabolism of GHB. The levels of BD in blood and brain of sham-operated controls were close to those of the normal rats, although slightly lower, possibly due to the shock of the operation. These results are compared in Table 3.

TABLE 3. BLOOD AND BRAIN LEVELS OF γ-HYDROXYBUTYRATE AND BUTANEDIOL AFTER INTRAPERITONEAL ADMINISTRATION OF 1,4-BUTANEDIOL TO NORMAL AND SHAM-OPERATED RATS*

(μmoles/g or ml)	Blood BD	Brain BD	Blood GHB	Brain GHB
30-min normal	1·79	1·64	1·82	0·56
Sham-operated	1·58	1·33	2·17	0·63
60-min normal	1·16	0·88	2·33	1·02
Sham-operated	1·70	0·64	2·20	1·02
180-min normal	0·33	0·26	0·61	0·32
Sham-operated	0·17	0·19	0·26	0·19

* Male rats were injected intravenously with 5·8 m-equiv./kg of a solution containing 5·8 m-equiv./ml of 2-^{14}C-BD; sp. act. = 2·3 μc/m-mole.

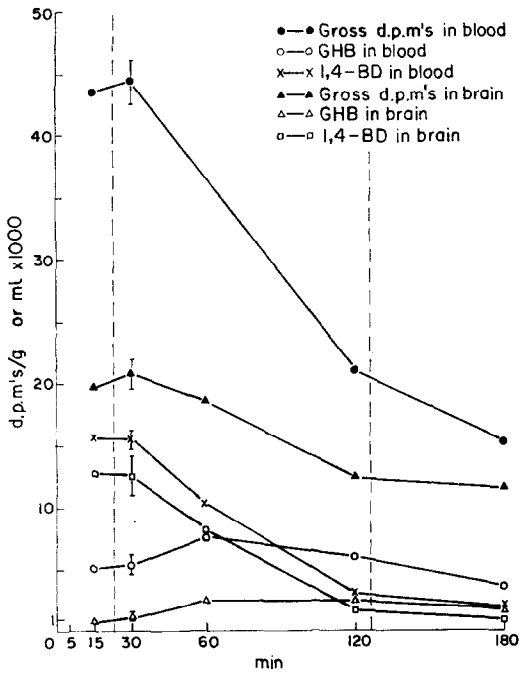

FIG. 7. Time course of the distribution of total radioactivity, GHB and 1,4-butanediol in blood and brain after i.p. injection of 1,4-butanediol-^{14}C (5·8 m-equiv./kg). Each point is the mean of two determinations, except the 90-min interval which is the mean of four determinations. The vertical bars depict the standard deviation.

Regional brain distribution

Fourteen rats were injected intraperitoneally with 2-^{14}C-BD and sacrificed as described in Methods. The sleep induction time was 24·5 ± 2·8 min and is comparable to that obtained after intravenous injection of 2-^{14}C-BD (Fig. 3). However, after

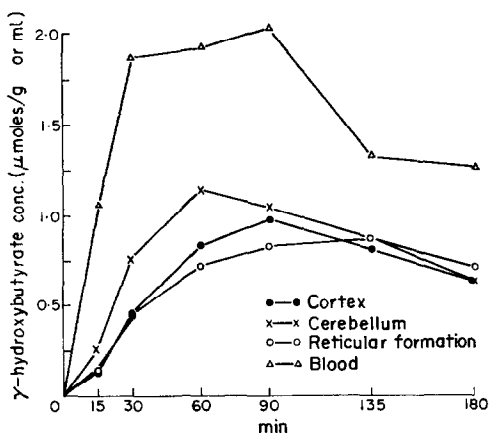

FIG. 8. Time course of the distribution of γ-hydroxybutyrate after i.p. administration of 1,4-butanediol-^{14}C (5·8 m-equiv./kg). Each point is the mean of two or more determinations.

intraperitoneal injection the sleeptime was somewhat longer than that observed after intravenous injection (Fig. 7).

Total radioactivity in blood and whole brain appeared to be very similar to the values obtained after intravenous injection of BD. Blood GHB concentrations (Fig. 7) were also similar to those obtained after intravenous injection of BD (Fig. 3).

TABLE 4. LEVELS OF γ-HYDROXYBUTYRATE IN BLOOD AND BRAIN REGIONS 90 min AFTER INTRAPERITONEAL INJECTION OF BUTANEDIOL*

Tissue	GHB (μmoles/g ± S.D.)
Blood	2·04 ± 0·23
Cortex	0·98 ± 0·16
Cerebellum	1·05 ± 0·11
Reticular formation	0·84 ± 0·17
Hippocampus	1·00 ± 0·17
Striatum	0·96 ± 0·08
Hypothalamus	0·89 ± 0·14
Thalamus	0·93 ± 0·17

* Rats were injected intraperitoneally with 5·8 m-equiv./kg of 2-^{14}C-BD; sp.act. = 1·7 μc/m-mole and the labeled GHB formed and retained in various brain regions determined 90 min later.

Additional experiments were performed to see if the newly formed GHB was localized in any specific areas within the brain. However, the regional distribution of GHB in the brain (Figs. 8, 9 and Table 4) also appeared to be fairly similar to that observed in the whole brain. The cerebellum had slightly higher levels, but at 90 min, the point at which four animals were used instead of two, they differed by only 6 per cent from those of the cortex.

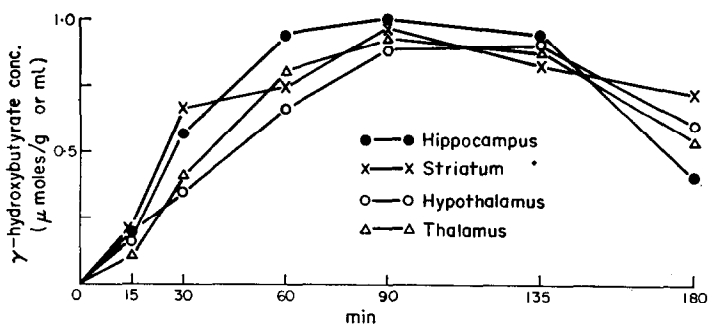

FIG. 9. Time course of the distribution of γ-hydroxybutyrate in hippocampus, striatum, hypothalamus and thalamus after i.p. injection of 1,4-butanediol-^{14}C (5·8 m-equiv./kg). Each point is the mean of two or more determinations.

DISCUSSION

The studies *in vitro* with tissue slices or minces demonstrated that certain peripheral tissues including liver, kidney and heart have the enzymatic capacity to oxidize BD to GHB. In addition, various regions of the central nervous system also possess this ability but to a lesser extent. Of all the tissues tested, liver was the most active in oxidizing BD to GHB. In the central nervous system, the striatum and hippocampus appeared to have the greatest capacity for formation of GHB from BD. However, the enzymatic activity possessed by these areas is less than that found in the liver.

When BD is administered intracisternally, bypassing the blood–brain barrier, significant amounts of GHB are recovered from various brain structures 30 min after injection. The cerebellum and midbrain appeared to be the most active in forming and retaining GHB. These experiments also lend support to the belief that brain tissue can convert BD to GHB. Thus, these observations do not rule out the possibility that endogenous GHB could be derived from an endogenous source of BD in the central nervous system. However, the observations that the brain is neither unique in possessing this enzymatic capacity nor the most active tissue in converting BD to GHB would tend to bias one somewhat against this hypothesis.

The studies *in vitro* suggest that the bulk of the GHB formed from exogenous administered BD is probably formed outside the central nervous system. This contention is further supported by the observation that partial hepatectomy markedly reduced the formation *in vivo* of GHB from BD and also tended to lessen the sleep-inducing properties of BD. It is evident that lesser centers of conversion cannot supplement the decreased role of the liver in hepatectomized rats. These observations do not directly disprove the idea that conversion of BD to GHB at only specific centers in the brain might be responsible for the "sleep" inducing properties of the drug, but this possibility tends to be weakened both by the very close correlation between the anesthetic state of the rat and the levels of converted GHB recovered from the whole brain, as well as by the apparent lack of any particular brain region to selectively accumulate newly formed GHB.

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