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γ-BUTYROLACTONE AND γ-HYDROXYBUTYRIC ACID—I
DISTRIBUTION AND METABOLISM*

ROBERT H. ROTH† and NICHOLAS J. GIARMAN

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. U.S.A.

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Abstract—Some aspects of the distribution and metabolism of the central nervous system depressants, γ-butyrolactone and γ-hydroxybutyric acid, have been investigated. After the administration of a depressant dose of γ-hydroxybutyrate to the cat, there was a relatively higher concentration of γ-hydroxybutyrate in the cerebellum and in the lower temporal lobe of the cortex than in other areas of the brain examined. The γ-butyrolactone was found to concentrate more in lean muscle than γ-hydroxybutyrate, while there was no difference in the amount of each that appeared in the body fat. The latter finding is explained by the presence of a rapidly acting lactonase in blood and liver that catalyzes the hydrolysis of γ-butyrolactone to γ-hydroxybutyrate. ^{14}C-carboxyl-labeled γ-hydroxybutyrate and γ-butyrolactone were found to be metabolized very rapidly to $^{14}CO_2$ in the intact rat; both the brain and liver carry out this decarboxylation *in vitro*. The major pathway of metabolism does not appear to involve formation of succinic acid. These results are related to the nature of the pharmacologically active compound and its duration of action.

SOME current findings have focused attention upon the neuropharmacology and biochemistry of γ-butyrolactone (GBL) and its hydrolytic cleavage product, γ-hydroxybutyric acid (GHB). Early observations that depression of the central nervous system follows the administration of GBL[1, 2] and GHB[3, 4] to animals culminated in the demonstration that GHB is an effective anesthetic adjuvant in man.[5–7] The recent development of a senstive and specific gas chromatographic method for the differential estimation of GBL and GHB in tissues made possible the observation that when GBL is administered to the rat, it is rapidly hydrolyzed to GHB, which accounts for the subsequent depression of the central nervous system.[8] A preliminary report of the enzyme responsible for this conversion has also appeared.[9]

Within the context of investigating the distribution and metabolism of GHB and GBL, the purpose of this communication is twofold: (1) to offer some explanation for the finding that, although GHB is the active form of the drug, GBL has the longer duration of action; (2) to examine the distribution of GHB in specific regions of the brain.

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METHODS

1. *Assay for* GBL *and* GBH

The method used to identify and estimate amounts of GBL and GHB was essentially the same as that reported earlier,[8] with a few minor modifications as follows: the supernatant fraction was extracted twice with two volumes of benzene (fractionally distilled twice) instead of one volume; the benzene extract was passed over a dry-packed column of Dowex-2-chloride (2·5 × 1 cm) in order to remove small amounts of trichloracetic acid (TCA), collected, and evaporated as described, to a volume of 0·1–0·5 ml; about 3 μl of this extract was then placed on a gas chromatographic column packed with 12% ethylene glycol succinate on Anakrom ABS solid support. A flame ionization detector was employed to detect GBL under the following routine conditions: detector temperature = 220°; injector temperature = 230°; column temperature = 115°; nitrogen flow rate = 110 ml/min (inlet pressure = 32 lb); zero air flow rate = 450 ml/min (inlet pressure = 46 lbs); and zero hydrogen flow rate = 48 ml/min (inlet pressure = 21 lb). Recoveries with this method ranged from 80% to 95% depending upon the tissue under investigation. In all cases, the values reported below are corrected for recovery from the particular tissue studied.

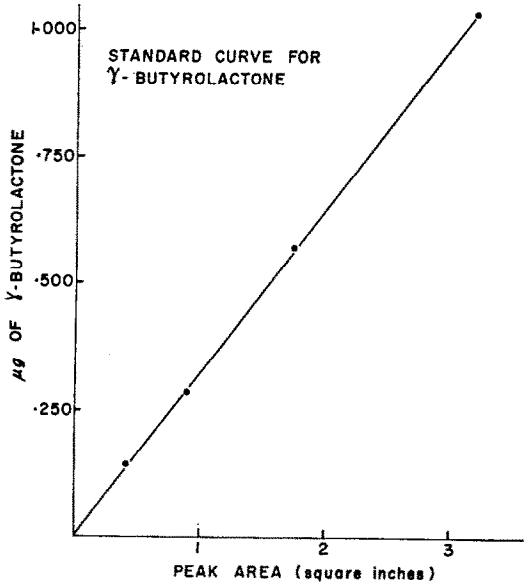

FIG. 1. Standard curve for the gas chromatographic assay of GBL with a flame ionization detector. Routine conditions as described in Methods were used. Peak areas were determined by means of a planimeter. Samples (1μl) containing the appropriate concentrations of GBL dissolved in benzene were used.

Authentic GBL was shown to have essentially the same retention time on the column by this technique as extracts of the brains of rats anesthetized with GBL.[8] Both the argon ionization and the flame ionization detectors were found to have a linear response to GBL over a wide range of concentrations. A standard curve for varying amounts of GBL obtained with a flame ionization detector is illustrated in Fig. 1.

2. *Regional brain distribution of* GHB

For this experimental series, mature cats of either sex, weighing at least 2 kg, were used. These animals were given sodium GHB in a dose of 350 mg/kg (expressed as free acid) via the right saphenous vein. The animals were sacrificed by decapitation 30 min after the drug. Each carotid artery was perfused for 10 sec with 15 ml of ice-cold isotonic sodium chloride solution to flush out residual blood in the cerebral vasculature. This procedure was found to remove very little GHB from brain. The brain was then isolated and sectioned into the desired areas, which were immediately frozen in liquid nitrogen at −196° and stored, if necessary, in dry ice subsequent to drying. Tissue sections prior to drying were broken into small pieces in a cold mortar containing powdered dry ice, and these pieces of brain were then dried at −40° for 3 days by means of a phosphorus pentoxide trap with a vacuum of about 0·001 to 0·005 mm Hg. The dried tissues were ground into a fine powder, and 100 mg homogenized in 5 ml of 10% TCA and rinsed into a centrifuge tube with distilled water. The suspension was centrifuged at 33,000 *g* for 7 min in a Sorvall refrigerated centrifuge at 0°; the supernatant fraction was decanted, heated to convert GHB to GBL, and extracted with benzene as described above. The amount of GBL in a 3-μl sample of the final extract was then determined by means of gas chromatography.

3. *Hydrolysis of* GBL

Samples of rat and guinea pig blood were obtained from adult male animals by decapitation and exsanguination. Cat blood was obtained by cardiac puncture of animals lightly anesthetized with ether; dog, rabbit, and human blood was obtained by aseptic venopuncture. In all studies with plasma, the blood was heparinized to prevent clotting, and plasma was obtained by centrifuging the blood at 27,000 *g* for 10 min at 2°. In studies of serum, no heparin was used. The clot was removed from the blood, kept in an ice bath, and the remaining fluid was centrifuged to remove residual erythrocytes.

All incubations were carried out at 37° in a Dubnoff metabolic incubator. The usual concentration of GBL employed in the studies *in vitro* was $1{\cdot}3 \times 10^{-2}$ M, although a wide range of concentrations was used with the technique that made use of a pH-stat. This high level was chosen to approximate the pharmacological levels present in rat blood *in vivo* after intravenous administration of anesthetic doses of GHB or GBL. Routinely, 2-ml aliquots were taken from the incubation vessel and carried through the standard gas chromatographic procedure for the separation and estimation of GHB or GBL. In one case the Angeli-Rimini reaction as used for the quantitative determination of esters by Hestrin[10] was adapted for estimation of the amount of GBL present. The optical density was read in a Klett photometer with a No. 54 filter. In this case only the disappearance of GBL was followed, whereas with the gas chromatographic method both the disappearance of the lactone and the formation of the acid were followed. Plasma was diluted 1:10 with isotonic sodium chloride–phosphate buffer (0·05 M) at pH 7·4.

After it was established that plasma could hydrolyze GBL to GHB very rapidly and that further metabolism by this tissue was negligible, a more rapid method was sought to follow the rate of hydrolysis. Titration of the acid formed in the reaction mixture was found to be very simple and reliable. All incubations in these studies were performed at 37°. The reactions were followed with a Radiometer Titrigraph type

SBR2/SBU1. Since no spontaneous hydrolysis of GBL was observed in sodium chloride–phosphate buffer or in saline at pH 7·4 within 60 min, and also since incubation of a 10% solution of serum in isotonic sodium chloride solution resulted in no acid production, this was considered a reliable method for estimation of the enzymic hydrolysis of the lactone.

In the analysis of "lactonase" activity, tissues were dissected as quickly as possible from male rats killed by decapitation, and a 10% homogenate was made with isotonic sodium chloride–phosphate buffer. Tissue suspensions were kept chilled until incubation. In one case a rat was anesthetized with pentobarbital (50 mg/kg), the abdomen opened, and the liver exposed. The artery to the left lateral lobe of the liver was carefully isolated, cannulated and flushed with saline to remove blood. When the liver became pale, it was quickly excised and a 10% homogenate prepared as described above.

4. *Radiorespirometric technique*

Some radiorespirometric studies were performed with a model 6000 Dynacon electrometer recording system with the DCF 250 ion chamber (ion chamber constant $= 4{\cdot}62 \times 10^{-12}$ A/μc $\pm$ 0·5%) in conjunction with a Delmar metabolism jar, equipped with a food chamber, water inlet, and ascarite trap, and adapted for separate feces and urine collection. The metabolism jar was swept with atmospheric air which was then passed through the ionization chamber of a Nuclear-Chicago Dynacon electrometer connected to a 1-mA Texas integrating linear recorder. All rats used in these experiments were 250-g male animals obtained from Charles River Co. Drugs were injected via the tail vein. In certain other radiorespirometric studies the technique was slightly modified. Rats were given labeled GHB by intravenous administration and placed in the metabolism jar for 40 min. In this case, the glass metabolism jar was swept with air at a rate of 300 ml/min, and the air was then bubbled through a Hyamine hydroxide trap (10 ml). At the end of the experimental period, 0·5 ml of the Hyamine hydroxide solution was pipetted into 15 ml of toluene PPO-POPOP and counted with a Packard Tri-Carb liquid scintillation spectrometer. Internal standards were run to avoid any erroneous effects due to quenching. The urine collection system was maintained acidic with 1 N HCl to release any $^{14}CO_2$ present in the urine. The efficiency of the method was estimated by means of $Na_2{}^{14}CO^3$ given intravenously to rats in a volume of 0·4 ml. Average recovery of respiratory $^{14}CO_2$ in 40 min was found to be about 60%.

5. *Carbon dioxide-*^{14}*C measurements*

Since it has been reported that, in a closed system, paper strips moistened with sodium hydroxide solution will quantitatively absorb carbon dioxide,[11] it seemed feasible to use this technique for measuring radioactive carbon dioxide evolved from respiring tissue slices. A simple incubation vial was constructed from a Packard polyethylene counting vial. From the cap a small piece of Whatman 3MM filter paper was hung in the center of the vial in a position such that it did not come into contact with the incubation mixture. This paper strip was moistened with 3·5 N NaOH prior to incubation and served to trap radioactive carbon dioxide produced by the tissue. When the incubation was complete, 1 ml of 20% TCA was added (by puncturing the vial cap with a 22-gauge needle) to stop the reaction as well as to release carbon

dioxide from solution. The vial was then incubated an additional 10 min at 37° to ensure complete carbon dioxide absorption by the filter paper. The vial cap with the paper strip still attached was carefully removed and screwed to the top of a new vial containing 10 ml ethanolic PPO–POPOP mixture (cf. succinic acid isolation method). The vial was stored in the cold (at − 20°) for 6 hr to ensure complete impregnation of the paper strip and then counted in a Packard Tri-Carb liquid scintillation spectrometer (window set at 35-1000, gain = 16).

The efficiency of this method to measure radioactive carbon dioxide was determined with sodium carbonate ^{14}C obtained from New England Nuclear Corp. A known amount of radioactive sodium carbonate was added to the incubation vial with the standard incubation mixture of Krebs Ringer phosphate buffer solution. The vial cover containing the sodium hydroxide-dampened filter paper was replaced, and 1 ml of 20% TCA added. The vials were allowed to equilibrate at 37° for 10 min; the filter paper was then removed and counted as described above. The average recovery of radioactive carbon dioxide was 72% ± 2·8%. This recovery value is not a reflection of lost ^{14}C-carbon dioxide but rather a decreased efficiency in the counting of radioactivity absorbed by filter paper.

6. *Separation and estimation of succinic acid*

Gas chromatography was used for separation and estimation of succinic acid. A column of 12% ethylene glycol succinate coated on Anakrom ABS solid support was employed to obtain an acceptable separation. The separation of dimethylsuccinate, dimethylmalonate, and GBL achieved on this column is shown in Fig. 2. Methylation of the organic acids was accomplished with a solution of diazomethane in diethyl ether, which was freshly generated from N-methyl-N-nitroso-*p*-toluene sulfonamide, available under the trade name of Diazald (Aldrich Chemical Co.). The diazomethane was added directly to the acids or to a methanolic solution of the acids until no more nitrogen was evolved and the solution remained yellow. The excess reagents and solvents were then evaporated to produce a convenient volume, and an aliquot was placed directly on the gas chromatographic column.

For the identification and estimation of succinic acid in tissue, some preliminary purification steps had to be taken. Proteins in the tissue or tissue suspension were precipitated with a volume of 95% ethanol which gave a final concentration of 80% ethanol. The precipitate was then centrifuged at 33,000 *g* for about 7 min and the supernatant fraction passed over a Dowex 1-formate column and washed through with 20 ml 80% ethanol, followed by 10 ml distilled water. The succinic acid was then eluted with 6 N formic acid. The first 15 ml of eluate was saved and passed through a Dowex-50 column to remove interfering cations. With ^{14}C-succinic acid as a marker it was found that 95% of the succinic acid was eluted from the Dowex 1-formate column between 3 and 9 ml. Retention of the first 15 ml therefore compensated for any variation in the column efficiency and also avoided the elution of any interfering anions. The column was then washed with 10 ml distilled water. The combined eluate was lyophilized and the residue reacted with an ethereal solution of freshly prepared diazomethane. The solution of dimethylsuccinate was then identified and assayed by gas chromatography. The routine conditions used were as follows: flash heater = 220°, cell bath = 190°, column = 115°, and argon flow rate = 80 ml/min. By means of an effluent splitter, about 95%–99% of the succinate peak could be trapped in a

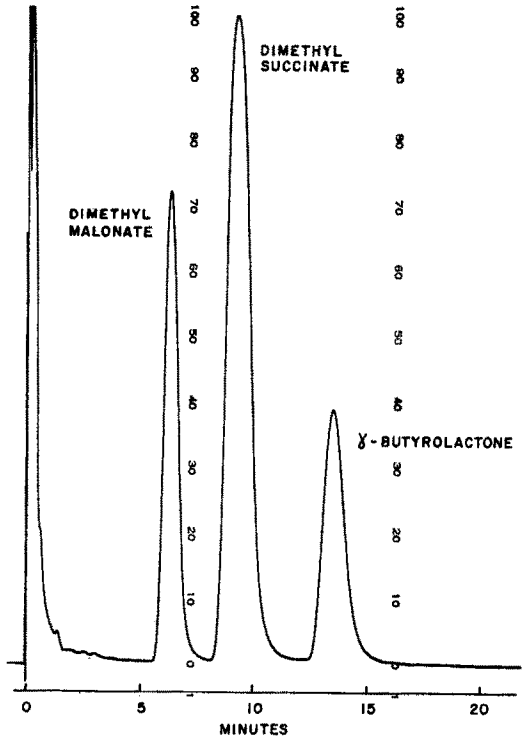

FIG. 2. Gas chromatographic analysis of a mixture of dimethylmalonate, dimethylsuccinate, and GBL. Conditions used: argon ionization detector; column of 12% ethylene glycol succinate coated on Anakrom ABS solid support, 70–80 mesh; cell temperature 190°; flash-heater temperature 220°; column temperature 115°; argon flow rate 80 ml/min; and gain of 10.

vial containing an ethanolic PPO-POPOP scintillation-counting mixture (Liquifluor) and counted with a Packard Tri-Carb liquid scintillation spectrophotometer.

7. *Metabolic studies with tissue homogenates*

Ten per cent homogenates (w/w) were routinely prepared by homogenizing 1 g tissue in 9 ml of suitable suspending medium, usually isotonic potassium chloride. The standard incubation mixture was prepared as follows:

		Final Molarity
3 ml of 10% homogenate in 0·15 M KCl		
0·6 ml	0·04 M DPN	0·004
0·6 ml	0·4 M nicotinamide	0·04
0·6 ml	0·2 M potassium malonate	0·02
0·3 ml	0·5 M phosphate buffer, pH 7·4	0·025
0·4 ml	0·1 M $MgCl_2$	0·0067
0·5 ml	^{14}C-GHB (sodium salt) spec. act. = 5·48 mc/m-mole, total conc. = 230 μg	

This mixture was incubated at 37° in a Dubnoff metabolic incubator, gassed with 100% oxygen. Incubation was carried out for 15 to 20 min; the homogenate was then

precipitated with 95% ethanol. This mixture was carried through the gas chromatographic technique for identification and estimation of succinic acid.

8. *Procedure for preparation and utilization of brain slices*

The rats used in these experiments were killed by decapitation and the brains quickly excised according to the procedure outlined by McIlwain and Rodnight.[12] The brains were transferred to a petri dish containing the incubation mixture, care being taken to remove all the dura. To avoid undue anoxia, the brains were then sliced as rapidly as possible by means of a Stadie blade. If cortical slices were to be made, the brain was placed upright on the moistened filter paper. If subcortical structures were to be studied, the whole brain was first halved by sagittal section along the longitudinal cerebral fissure. Half the brain was then returned to the incubation medium and the other half placed on moistened filter paper with its cut surface upright. Slices were made parallel to the cortical surface to obtain cortical slices (only two slices were taken from each brain). Brain slices were obtained sometimes by cutting parallel to the cut sagittal surface in order to obtain slices containing subcortical as well as cortical tissue. The slices were then washed into cold medium. Subsequently, the slices were hooked over a small wire rider, drained, weighed on a torsion balance, and transferred to the experimental vessel. Any slices that were too thick to be transparent were discarded.

The medium used for suspending brain slices during the incubation procedure was the standard Krebs-Ringer phosphate buffer described by Umbreit *et al.*[13] for tissue slices. This solution, after mixing was chilled and gassed with 100% oxygen. The precipitate of calcium phosphate that formed was suspended by shaking before use. The final concentration of glucose used in the incubation mixture was 5 mm. Routinely, an incubation volume of 3 ml, containing about 20 mg tissue/ml, was used.

RESULTS

1. *Distribution in blood, fat, and muscle*

When GBL or GHB (sodium salt) was administered to rats in equimolar doses, sufficient to induce anesthesia, it became apparent that initial total blood levels of GHB and GBL were about 50% lower after GBL than after GHB (Fig. 3). In addition, it was observed that the blood concentration fell more rapidly after GHB than after GBL.

In order to shed some light on these observations it appeared necessary to examine the distribution of total GHB and GBL in muscle and fat after the administration of these compounds intravenously in equivalent anesthetic doses. Figure 4 shows the results of such an experiment carried out in adult male rats. It is clear that during the entire time course studied, the levels in muscle after GBL were significantly higher than those after GHB. Since GBL is more lipid-soluble than GHB, however, it was unexpected to find that there were no differences in the levels in fat after administration of each of these compounds. This finding could be explained on the basis of our recent observation that rat blood and liver contain a rapidly acting lactonase which hydrolyzes GBL to GHB.[9] Apparently, GBL is hydrolyzed so rapidly by this enzyme that poorly perfused tissues like fat receive only limited quantities of GBL after its administration.

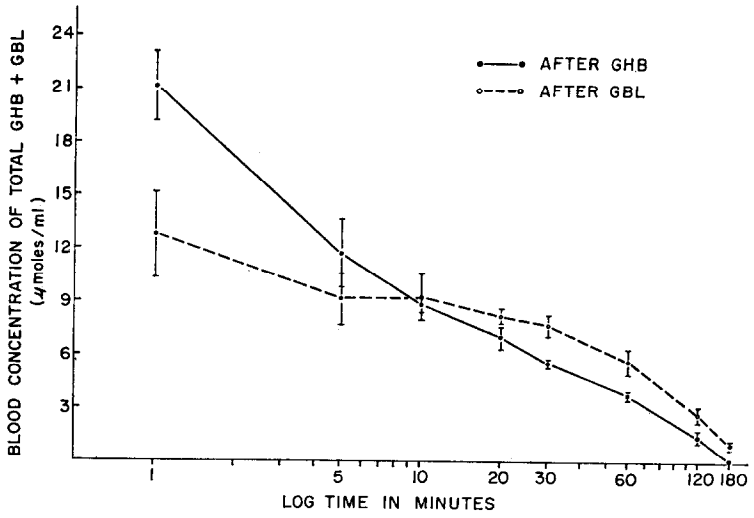

FIG. 3. Relationship of blood concentration of total GBL and GHB with time after the administration by the intravenous route of equimolar doses of GHB (sodium salt, 732 mg/kg) and GBL (500 mg/kg). Each point is the mean of at least 5 animals (male rats.) Vertical bars indicate the standard deviations of the means.

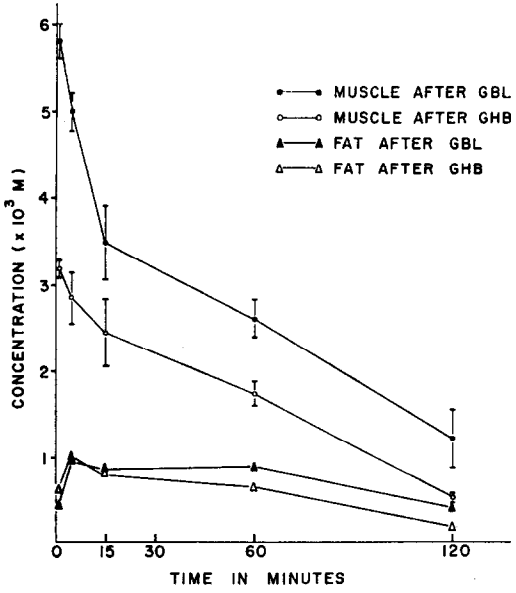

FIG. 4. Distribution of total GHB and GBL in lean muscle and fat after the intravenous administration of equimolar amounts of either GBL (500 mg/kg) or GHB (sodium salt, 732 mg/kg). Each point is the mean of at least 3 animals (male rats). Vertical bars span the standard deviations of the mean.

2. *Distribution in selected regions of cat brain*

Each of 6 cats was given GHB by the intravenous route (in a dose of 350 mg/kg) and sacrificed 30 min later, when all animals were found to be behaviorally asleep. Various regions of the brain were carefully dissected free, and determinations of GHB were carried out as described above. The results of these experiments are illustrated in Fig. 5.

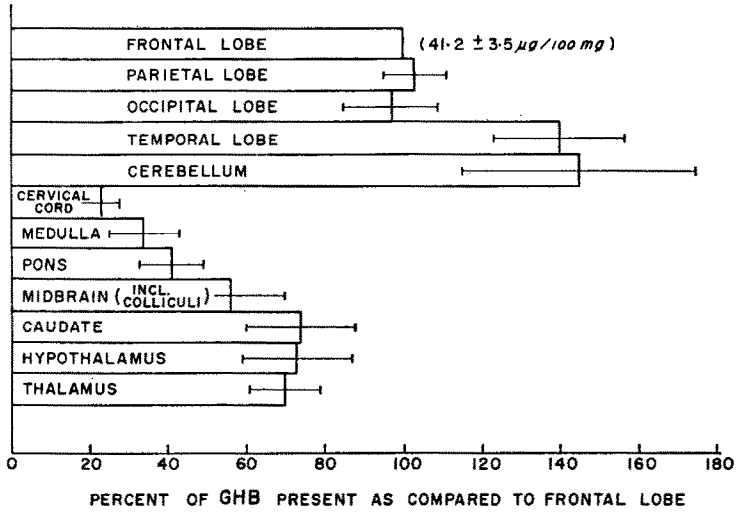

FIG. 5. Regional distribution of GHB in cat brain 30 min after the intravenous administration of GHB (sodium salt, 350 mg/kg). The results are expressed as the per cent of GHB compared to the amount found in the frontal lobe of the cortex. Each value represents the mean of at least 5 determinations, and the vertical bars represent standard deviations of the means.

It is clear that among the subcortical areas studied the concentration of GHB increases as the sections progress rostrally up the brain stem from the cervical cord until a constant level is reached in the thalamus, hypothalamus, and caudate nucleus. However, the highest levels were found in the cerebellum and the lower temporal lobe.

3. *Metabolism of* GBH *and* GBL

A. Radiorespirometric studies. Investigations with ^{14}-C-carboxyl-labeled GHB (sodium salt) indicated that this compound was metabolized very rapidly in the rat. After the intravenous administration of 2 μc 1-^{14}C-GHB, respiratory carbon dioxide-^{14}C was detected within about 4 min and a peak reached in about 15 min; about 60% of the total radioactivity administered was recovered within 2·5 hr in the respired air. Similar results were obtained with 1-^{14}C-GBL. However, in this case respiratory carbon dioxide-^{14}C was not evolved quite so rapidly, and a peak was reached in slightly less than 20 min. This can be seen quite clearly by the difference in the slopes of the carbon dioxide-^{14}C evolution curves illustrated in Fig. 6. This short delay was probably due to the time required for the GBL to be hydrolyzed to GHB by an enzyme in blood and liver before GHB could be metabolized. The broader peak and somewhat reduced

slope of the falling curve following GBL may be a reflection of the sequestering of the lactone in the lean muscle mass of the body, as shown in Fig. 4. This relatively slower velocity of metabolism is seen also in the slower rate of disappearance of drug from the blood after GBL (Fig. 1).

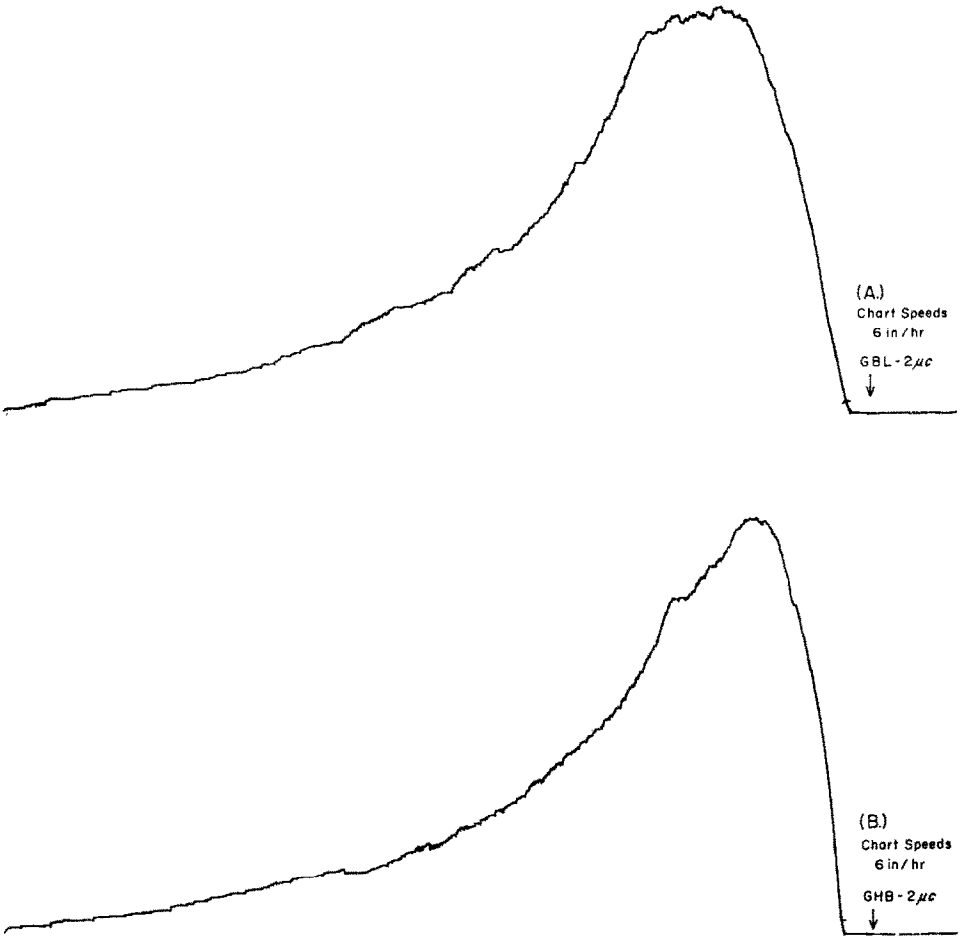

FIG. 6. Radiorespirometric curves obtained from rats after intravenous administration of 2 μc GBL-1-^{14}C (upper curve) and 2 μc GHB-1-^{14}C (lower curve). Specific activity of radioisotopic material was 5·478 mc/m-mole. Each chart division spans 10 min. Abscissa is time; ordinate is output of $^{14}CO_2$ in expired air.

B. Studies of the hydrolysis of GBL *by various tissues.* In our early investigation of the distribution of GHB and GBL it was apparent that when GBL was given by the intravenous route to the rat it was rapidly converted to GHB, which then entered the CNS and presumably caused the depression that ensued.[8] Roth and Giarman have presented evidence that an enzyme, with some cation requirement, catalyzes the hydrolysis of GBL.[9] When GBL and GHB were estimated by means of the gas chromatographic method previously described, whole rat blood was found to convert GBL

to GHB very rapidly; the half-time of conversion was less than 1 min. GBL was not hydrolyzed quite so fast by cat blood, as is illustrated in Fig. 7. Similar rates of hydrolysis were also obtained with the pH-stat method. This activity in blood was initially localized in rat plasma, hemolyzed erythrocytes being inactive.[9] Further studies showed that serum was substantially more active than plasma. Sera from rabbits, guinea pigs, cats, and humans were also active. Other tissues of the rat, such as

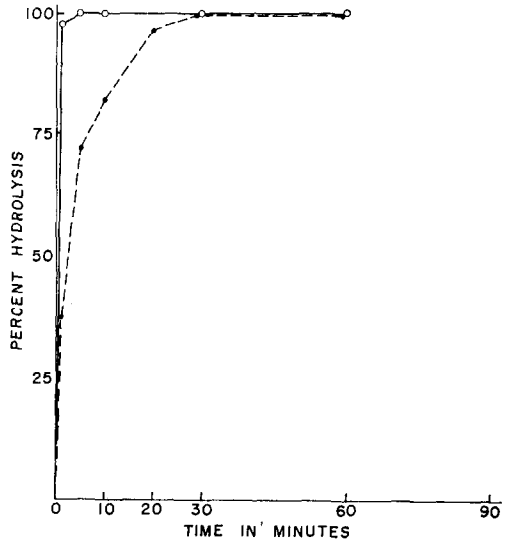

FIG. 7. Hydrolysis of GBL to GHB in the presence of blood from cat (dashed curve) and rat (solid curve) *in vitro* at 37°. Concentration of GBL used was $1{\cdot}3 \times 10^{-2}$ M.

brain, liver, kidney, heart, lung, skeletal muscle, and intestine, were examined for lactonase activity. Of these, only liver (blood removed by perfusion) was found to have any substantial activity. Human cerebrospinal fluid was also lacking in such activity.

The hydrolysis by rat and human sera was studied over a wide range of substrate concentrations, and the maximal initial rate was determined by means of the pH-stat method. With a crude enzyme concentration of 1 ml serum in 10 ml isotonic saline at pH 7·4 and a substrate concentration of $2{\cdot}6 \times 10^{-2}$ M, the maximal initial rate of hydrolysis was found to be about 40 m-equiv GBL/min/ml human serum, and the reaction rate was linear for about 2 min. A very high K_m value of $1{-}3 \times 10^{-2}$ M was found for both rat and human serum. The data of the study with the latter are plotted in Fig. 8.

C. Studies of metabolism in vitro. No direct experiments in intact tissue have been carried out to demonstrate that brain can metabolize GHB, although some studies with brain homogenates indicated this possibility.[14–16] It was of interest, therefore, to determine whether brain slices could metabolize GHB to carbon dioxide, a process

which has been shown to occur very rapidly in the whole animal.[17] Isotopically labeled compounds were used in this study to allow precise measurement of disappearance of minute quantities of substrate in the presence of large amounts of the substrate optimal for enzyme activity. With the measurement of carbon dioxide-^{14}C formation by brain slices from ^{14}C-carboxyl-labeled GHB, it was found that ^{14}C-GHB was

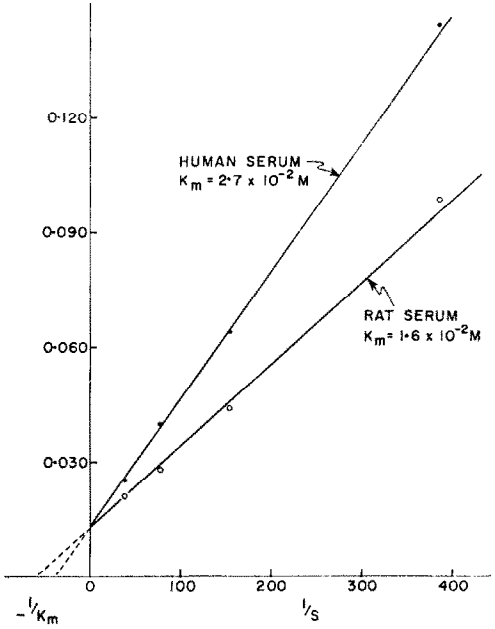

FIG. 8. Lactonase activity of human serum on GBL: reciprocal plot of velocity and substrate concentration.

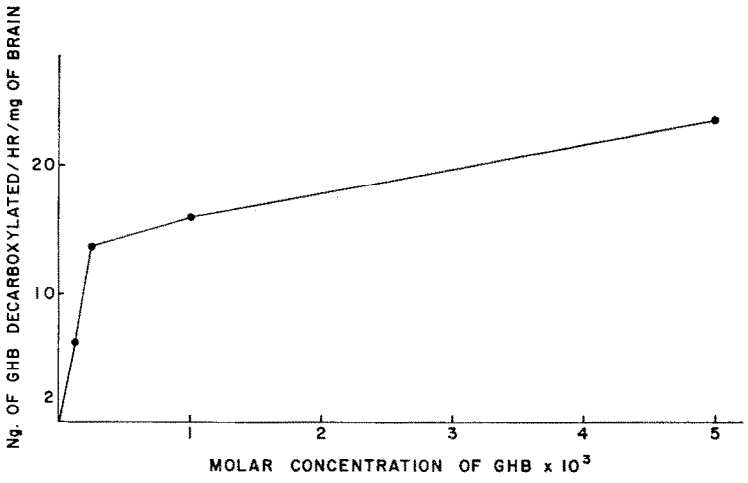

FIG. 9. Conversion of GHB-1-^{14}C to $^{14}CO_2$ by slices of rat brain cortex. Each point is the mean of at least 3 determinations.

metabolized quite rapidly. Figure 9 shows the extent of carbon dioxide-^{14}C formation by brain slices incubated in varying concentrations of labeled GHB. Liver slices were found to effect this conversion of GHB to an extent of about twice that of brain.

In view of the report by Fishbein and Bessman that GHB may enter the Krebs cycle,[15] we sought to isolate ^{14}C-labeled succinic acid after incubation of rat brain and liver homogenates and blood with GHB-^{14}C (sodium salt) in the presence of malonic acid (2×10^{-2} M), which was added to block the Krebs cycle at the succinate level. Succinic acid was isolated from possible interfering substances and analyzed by gas chromatography as described above. One to two per cent of the ^{14}C-isotope of the added GHB was found in the succinic acid from brain, and up to 6% was found in the succinate of liver; no isotope could be detected in the blood succinate. In view of the negative findings of Walkenstein *et al.*[16] with regard to labeling of succinate by GHB-^{14}C *in vivo*, the small percentage of isotope found in brain succinate in our experiments is probably an expression of a small amount of enzyme in the brain that under appropriate conditions can oxidize GHB. However, it could also be the result of random labeling of succinate due to carbon dioxide fixation. The higher percentage of the labeled succinate found in liver may be the result of alcohol dehydrogenase (ADH) activity, recently reported by Wollemann to oxidize GHB to succinic semialdehyde.[17]

The possibility of a block of metabolism in the Krebs cycle by GHB through the formation of glyoxalate by means of a mechanism suggested by Walkenstein *et al.*,[16] prompted us to seek a depression in the metabolism of uniformly ^{14}C-labeled glucose by brain tissue (in view of the relatively inactive pentose phosphate shunt in brain). By means of uniformly labeled glucose, the evolution of carbon dioxide-^{14}C by brain slices (cortical and subcortical) was followed (cf. Methods), in the presence and absence of 10^{-3} M GHB. Only a slight depressant effect was observed on the metabolism of the labeled glucose to carbon dioxide; radioactive carbon dioxide formation was depressed about 10% in cortical and about 16% in subcortical slices. No greater depressant effect was observed when rats were pretreated with GBL (500 mg/kg) 30 min before sacrifice and preparation of brain slices.

Since the initial studies were carried out in an incubation medium of normal Krebs-Ringer phosphate buffer, the experiments were repeated with Krebs-Ringer phosphate buffer containing high potassium (100 mM) in order to stimulate neurons in the brain slices. This procedure was followed because it is known that the respiration of unstimulated brain cortical slices in the presence of glucose is only slightly affected by the presence of malonate, a potent inhibitor of the Krebs cycle.[18] On the other hand, potassium-stimulated brain respiration is highly sensitive to malonate.[19] In addition, stimulated respiration of isolated brain tissue approaches the magnitude of brain respiration *in vivo*, and possesses some of the characteristic features of brain *in vivo*, such as response to anesthetics and depressant drugs.[20] With potassium-stimulated brain cortical slices it was found that 10^{-3} M GHB inhibited the oxidation of pyruvate-2-^{14}C only about 20%. This relatively small inhibition of pyruvate oxidation was not impressive enough to warrant any conclusion concerning the mechanism of central depression for GHB.

D. Alteration of metabolism by β-hydroxybutyrate. Since β-hydroxybutyrate (βHB) is well tolerated by animals in high doses [21] and does not appear to produce marked sedation or loss of righting reflex in doses of 2 g/kg, the effect of this structurally similar

compound on the metabolism of GHB was examined. An interference with metabolism seemed likely, because Walkenstein *et al.* had postulated that GHB was metabolized in the rat via β-oxidation through the intermediate 3,4-dihydroxybutyric acid.[16] It was found unexpectedly that preadministration of βHB markedly *decreased* the sleep time of rats treated with either GHB or GBL (Table 1).

TABLE 1. REVERSAL WITH β-HYDROXYBUTYRIC ACID OF SLEEP INDUCED BY GBL AND GHB

Treatment (dose)	Duration of anesthesia (mean)		Mean brain level GHB* (μg/g ± S.D.)	Mean blood level GHB (μg/ml ± S.D.)	% ^{14}C-GHB metabolized to $^{14}CO_2$ ± S.D.
	Injection to RR† return	Duration RR lost			
GBL (350 mg/kg i.v.)	78	72	95 (4)‡ ± 7·6	254 (4) ± 36·2	
GHB (350 mg/kg i.v.)	54	46	68 (4) ± 12·4	155 (4) ± 13·1	11·2 (3) ± 0·7
β-OH-Butyric Acid (2 g/kg i.p.) followed by GBL*	46	37	45 (4) ± 14·8	139 (3) ± 29·7	
β-OH-Butyric Acid (2 g/kg i.p.) followed by GHB*	33	28	32 (4) ± 6·3	99 (4) ± 12·2	6·8 (3) ± 0·3

Animals sacrificed 50 min after GBL or 40 min after GHB treatment.
* Corrected for residual blood volume in cerebral vasculature and expressed as GBL equivalents.
† RR = righting reflex.
‡ Number of experiments shown in parentheses.
§ Interval between treatments, 20 min.

In addition, these studies showed that pretreatment with βHB caused significantly lower levels of GHB in both brain and blood to appear 50 min after the intravenous administration of GHB. Since both brain and blood levels were about halved, this suggested that βHB must be acting in some manner to stimulate the metabolism of GHB. However, experiments with liver slices in which carbon dioxide evolved from 1-^{14}C-GHB was measured showed that 10 mM βHB had a slight inhibitory effect rather than a stimulatory effect on GHB metabolism. This inhibitory effect of βHB on the metabolism of GHB was seen also with rat liver *in vitro*.

DISCUSSION

The observation of Benda and Perles[3] and of Jouvet *et al.*[22] that GBL has a longer duration of action in depressing animals than have equivalent amounts of GHB seems inconsistent with our finding[8] that GHB is the form of the drug associated with depression of the central nervous system. This greater duration of action of GBL, which we have confirmed,[8] has been used by others[23] to support the contention that GBL is the active form of the drug. In the present communication, two pieces of evidence are presented which bear upon this problem: (1) there is a lactonase in blood serum and liver of the rat that catalyzes the conversion of GBL to GHB at a high velocity; and (2) after the administration of GBL there is a higher concentration of total GBL and GBH in lean muscle than there is after the administration of GHB, but the levels in adipose tissue are the same after either compound. From these data it would appear

that richly perfused muscle can sequester a large part of an initial dose of GBL, thereby retarding its metabolism and prolonging its duration of action. On the other hand, the rate of hydrolysis of GBL by the liver and blood lactonase is so rapid relative to the poor rate of perfusion of fat that this tissue receives only a limited amount of the intact lipid-soluble GBL. The net result of these distribution phenomena is that blood levels of total GBL and GBH reach a lower peak and fall more slowly after the administration of GBL than after GBH.

The relatively higher concentrations of GHB found in the cerebellum and lower temporal lobe than in other parts of the brain that were studied provoke some interest. Low doses of GHB produce ataxia and incoordination, motor disorders which may arise in the cerebullum. The localization of GHB in the lower temporal lobe may be of significance in relation to the finding that GHB prolongs amygdaloid and hippocampal seizure activity.[24] Although such physiologic factors as blood supply undoubtedly exert an influence on drug distribution to certain areas of the brain, it seems reasonably clear from these data that other factors may also be important. Thus, the hypothalamus is one of the most richly vascular areas of the brain, yet this area showed no particular localization of GHB. This failure to be concentrated by the hypothalamus has been observed with phenothiazines[25] and mescaline.[26]

Our investigations of the metabolism of GBL and GBH established that these compounds are metabolized very rapidly in the whole animal to carbon dioxide, and that, for nonvolatile depressants of the CNS, they are relatively rapidly cleared from the body. In marked contrast to the barbiturates, which tend to accumulate in body fat and persist long after the end of a barbiturate-induced anesthesia, GHB is virtually absent from all body tissues by the time an animal recovers from a depressant dose. While it might have been expected that the liver would metabolize GHB to CO_2, it was of interest to find that brain carried out this conversion to a substantial extent —about half that of liver.

The possible enhancement by βHB of the clearance of GHB and the resulting reduction in the duration of central nervous system depression produced by GHB requires further study. Since it is known that βHB is metabolized very rapidly by the rat to acetyl CoA, and further that CoA transfers very well from acetyl CoA to butyrate,[27] it is conceivable that βHB antagonizes the effects of GHB by stimulating a transferase system that can remove GHB from the circulation by forming, e.g., GHB-CoA. Other possibilities for explaining the βHB interaction exist: (1) βHB may interfere with attachment of GHB at receptor sites in nervous tissue and thereby facilitate metabolism of GHB; (2) βHB may in some way promote a more rapid excretion of GHB from the body, the net result being a lower blood level of GHB and a shorter sleeptime.

Our data also indicate that GBL is rapidly hydrolyzed to GHB in blood and liver. The biological half-life of GBL is so short, in fact, that it is hardly likely that this molecular form of the pair would assume any importance in eliciting the pharmacological actions observed, especially in view of the relatively long delay in onset of action and the duration of action of 2 – 3 hr which have been reported. Data to the contrary[23–28] are best explained on a methodological basis; i.e. they are derived from a colorimetric assay technique based on the Hestrin reaction,[10] which is highly nonspecific and with which the following substances are likely to interfere: choline esters, noncholine esters, thioesters, anhydrides, lactides, sugar lactones, and even glucose.

In fact, Bessman and Skolnik reported that the color which developed in control extracts was due to the presence of glucose, but they discounted the significance of this on the basis that glucose does not vary in blood or tissues after the administration of either GHB or GBL.[23] This, however, is at variance with the finding of Fleming and LaCourt,[29] who have reported that GHB given in anesthetic doses to mice increases blood glucose about 35% and brain glucose about 250%.

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REFERENCES

1. B. A. RUBIN and N. J. GIARMAN, *Yale J. Biol. Med.* **19**, 1017 (1947).
2. N. J. GIARMAN, Ph. D. dissertation, Yale University (1948).
3. P. BENDA and P. PERLES, *c.r. hebd Séanc Acad. Sc., Paris* **251**, 1312 (1960).
4. H. LABORIT, J. M. JOUANY, J. GERARD and F. FABIANI, *Presse méd.* **68**, 1867 (1960).
5. H. F. LABORIT, G. BUCHARD, G. LABORIT and B. WEBER, *Agressologie*, **1**, 549 (1960).
6. M. BLUMENFELD, R. G. SUNTAY and M. H. HARMEL, *Anesth. Analg. Curr. Res.* **41**, 721 (1962).
7. J. BLAISE, *Anesth. Analg. réanim.* **21**, 677 (1964).
8. N. J. GIARMAN and R. H. ROTH *Science* **145**, 583 (1964).
9 R. H. ROTH and N. J. GIARMAN *Biochem. Pharmac.* **14**, 177 (1965).
10. S. HESTRIN, *J. biol. Chem.* **180**, 249 (1949).
11. I. A. MIRSKY, *Packard Tech. Bull.* **3**, 3 (1961).
12. H. MCILWAIN and R. RODNIGHT, *Practical Neurochemistry*. Little, Brown, Boston (1962).
13. W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, *Manometric Techniques*. Burgess, Minneapolis (1964).
14. M. W. NIRENBERG and W. B. JAKOBY, *J. biol. Chem.* **235**, 954 (1960).
15. W. N. FISHBEIN and S. P. BESSMAN, *J. biol. Chem.* **239**, 357 (1964).
16. S. S. WALKENSTEIN, R. WISER, C. GUDMUNDSEN and H. KIMMEL *Biochem. biophys. Acta* **86**, 640 (1964).
17. W. WOLLEMANN and T. DEVENYI, *Sixth Int. Congress of Biochemistry*, Abstr. V-E, no. 124, p. 419 (1964).
18. J. H. QUASTEL, in *Neurochemistry* (Eds. K. A. C. ELLIOTT, I. H. PAGE and J. H. QUASTEL), pp. 226. Thomas, Springfield, Ill. (1962).
19. M. H. KINI and J. H. QUASTEL, *Nature, Lond.* **184**, 252 (1959).
20. J. J. GHOSH and J. H. QUASTEL, *Nature, Lond.* **174**, 28 (1954).
21. F. E. SAMSON, N. DAHL and D. R. DAHL, *J. clin. Invest.* **35**, 1291 (1956).
22. M. JOUVET, A. CIER, D. MOUNIER and J.-L. VALATX, *c.r. hebd. Séanc Acad. Sc. Paris* **155**, 1313 (1961).
23. S. P. BESSMAN and S. J. SKOLNIK, *Science* **143**, 1045 (1964).
24. A. B. DRAKONTIDES, J. A. SCHNEIDER and W. H. FUNDERBURK, *J. Pharmac. exp. Ther.* **135**, 275 (1962).
25. G-A. V. DEJARAMILLO and P. S. GUTH, *Biochem. Pharmac.* **12**, 525 (1963).
26. N. NEFF, G. V. ROSSI, G. D. CHASE and J. L. RABINOWITZ, *J. Pharmac. exp. Ther.* **144**, 1 (1964).
27. E. R. STADMAN, *J. biol. Chem.* **203**, 501 (1953).
28. M. HELRICH, T. C. MCASLAN, S. SKOLNIK and S. P. BESSMAN, *Anesthesiology* **25**, 771 (1964).
29. M. FLEMING and S. LACOURT, *Biochem. Pharmac.* **14**, 1905 (1965).