Journal of Neurochemistry
Raven Press, New York

Evidence for the Participation of a Cytosolic $NADP^+$-Dependent Oxidoreductase in the Catabolism of γ-Hydroxybutyrate In Vivo

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Abstract: The concentration of γ-hydroxybutyrate (GHB) in brain, kidney, and muscle as well as the clearance of [1-^{14}C]GHB in plasma have been found to be altered by the administration of a number of metabolic intermediates and drugs that inhibit the $NADP^+$-dependent oxidoreductase, "GHB dehydrogenase," an enzyme that catalyzes the oxidation of GHB to succinic semialdehyde. Administration of valproate, salicylate, and phenylacetate, all inhibitors of GHB dehydrogenase, significantly increased the concentration of GHB in brain; salicylate increased GHB concentration in kidney, and α-ketoisocaproate increased GHB levels in kidney and muscle. The half-life of [1-^{14}C]GHB in plasma was decreased by D-glucuronate, a compound that stimulates the oxidation of GHB by this enzyme and was increased by a competitive substrate of the enzyme, L-gulonate. The results of these experiments suggest a role for GHB dehydrogenase in the regulation of tissue levels of endogenous GHB. **Key Words:** γ-Hydroxybutyrate dehydrogenase—Succinic semialdehyde dehydrogenase—D-Glucuronate—Sodium valproate. **Kaufman E. E. and Nelson T.** Evidence for the participation of a cytosolic $NADP^+$-dependent oxidoreductase in the catabolism of γ-hydroxybutyrate in vivo. *J. Neurochem.* **48,** 1935–1941 (1987).

Studies on the metabolic fate of γ-hydroxybutyric acid (GHB), a naturally occurring compound present in both brain (Roth and Giarman, 1969; Roth, 1970) and peripheral tissues (Nelson et al., 1981), have established that this compound is largely disposed of by oxidation to CO_2 and water (Walkenstein et al., 1964). Furthermore, it has been found that most of the carbon skeleton enters the citric acid cycle as succinate (Doherty et al., 1975; Möhler et al., 1976) rather than as acetyl-CoA derived from β-oxidation as previously proposed by Walkenstein et al. (1964). The recent discovery of a metabolic disease in which GHB and succinic semialdehyde (SSA) are markedly elevated in both blood and urine due to a block in SSA dehydrogenase (Jakobs et al., 1981; Gibson et al., 1983) adds evidence to support a degradative pathway in which GHB is oxidized to SSA, which in turn is oxidized to succinate.

These findings strongly suggest that the main degradative pathway for GHB proceeds through the following series of steps.

(1) GHB ⟷ SSA
(2) SSA ⟶ succinate
(3) succinate → → → → $CO_2 + H_2O$

The reactions in step (3) are catalyzed by the enzymes of the citric acid cycle and in step (2) by SSA dehydrogenase, the enzyme that is either low or missing in patients with GHB aciduria (Gibson et al., 1983). Although step (1) is always depicted as an essential part of this scheme, until recently an enzyme or enzymes that could catalyze this reaction had not been identified.

We have reported the purification and characterization of a cytosolic $NADP^+$-dependent oxidoreductase, "GHB dehydrogenase," which can catalyze the oxidation of GHB to SSA (step 1) in vitro under conditions that approximate those in the cytosol of both brain and some peripheral tissues (Kaufman et al., 1979; Kaufman and Nelson, 1981). The physical characteristics as well as substrate and inhibitor specificity of this enzyme indicate that the ability to cata-

Received August 5, 1986; revised manuscript accepted December 15, 1986.
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Abbreviations used: GHB, γ-hydroxybutyric acid; SSA, succinic semialdehyde.

lyze the oxidation of GHB may represent a previously unreported activity for the $NADP^+$-dependent oxidoreductase (EC 1.1.1.19), commonly known as D-glucuronate reductase (York et al., 1961).

Whether this enzyme plays a significant role in the oxidation of GHB to SSA in vivo has been studied by the administration to animals of compounds that either inhibit or increase the activity of this enzyme in vitro. Determination of tissue levels of GHB or of the half-life of GHB in plasma was used to assess the effect of the inhibition of this enzyme on the catabolism of GHB in vivo. The results suggest that factors that affect the activity of this enzyme in vitro alter both tissue levels of GHB and the half-life of GHB in vivo in directions consistent with their effects on the enzyme in vitro.

MATERIALS AND METHODS

Materials and animals

Sodium α-ketoisocaproate, sodium phenylacetate, γ-aminobutyric acid (GABA), and sodium GHB were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Valproic acid was obtained from Saber Laboratories (Morton Grove, IL, U.S.A.) and salicylic acid from Fisher Scientific (Fairlawn, NJ, U.S.A.). Sprague–Dawley male rats weighing 350–450 g were obtained from Taconic Farms (Germantown, NY, U.S.A.). [1-^{14}C]GHB was obtained from Research Products International (Mount Prospect, IL, U.S.A.).

Effects of inhibitors of GHB dehydrogenase

In studies in which the animals were infused with solutions of either α-ketoisocaproate, phenyl acetate, or 0.9% sodium chloride, a catheter was implanted in the femoral vein under light halothane anesthesia. The animals were allowed to recover from the anesthesia for at least 2 h before the experiment was started. The solutions were infused for 2 h intravenously at constant rates with a Harvard infusion pump, model number 600 (Harvard Apparatus, Dover, MA, U.S.A.). Immediately after the infusions the animals were killed by decapitation and the brains, kidneys, and quadriceps muscle were removed and quickly frozen in liquid nitrogen. The organs were stored at −80°C until assayed for GHB content as described by Nelson et al. (1981).

In the studies in which either salicylate or valproate was administered, the drug or physiological saline solution was administered intraperitoneally to the experimental and control animals, respectively. The animals were killed by decapitation 1 h after receiving salicylate and 2 h after valproate. Brain, kidney, and quadriceps muscle were rapidly frozen in liquid N_2 and assayed for GHB as described above.

Effects of D-glucuronate or L-gulonate on the $t_{1/2}$ of [1-^{14}C]GHB in plasma

Catheters were implanted in both the femoral vein and artery of Sprague–Dawley male rats under halothane anesthesia and the animals were allowed to recover from the surgery and anesthesia for at least 2 h. The experiment was started by the injection into the venous catheter of approximately 4 μCi of [1-^{14}C]GHB (sp act 4.5 μCi/μmol) in 0.4 ml of 0.9% NaCl. In one series of experiments D-glucuronate (333 mg) was administered (intravenously) as a bolus in saline just prior to the start of the experiment followed by a constant infusion (556 mg/h) during the experiment. In a second series of experiments, L-gulonate (240 mg) was given as a bolus just prior to the start of the experiment followed by a constant infusion of 320 mg/h for 3 h. Control rats received a matching bolus and constant infusion of physiological saline. Blood samples were withdrawn at various times into heparinized tubes and centrifuged. The plasma was deproteinized by the addition of 100 μl of ice-cold absolute ethanol to 50 μl of plasma. Sixty microliters of the deproteinized plasma were counted in Aquasol (New England Nuclear, Boston, MA, U.S.A.), and the remainder was used for paper chromatography. Chromatographic separation of GHB was carried out by spotting approximately 20 μl of the deproteinized plasma on Whatman 3MM Chr paper. The solvent system was ethanol/H_2O/NH_4OH, 98:2:1, by vol. The chromatogram was developed in the ascending direction for approximately 18 h, cut into 1-cm strips, and counted in a Packard Tri-carb scintillation counter, Model 3375 (Packard Instrument, Downers Grove, IL, U.S.A.) in 10 ml of Aquasol and 1 ml of H_2O. The fraction of total of ^{14}C in each plasma sample that migrated with authentic GHB was measured and used to determine the concentration of [^{14}C]GHB found in the plasma sample in the presence of radioactive metabolites of GHB. The plasma [^{14}C]GHB concentrations were plotted on semilogarithmic paper against time. The straight line that was obtained after the initial equilibration with the tissues was extrapolated to zero time and used to calculate the $t_{1/2}$ for the disappearance of GHB from plasma.

Preparation and assay of GHB dehydrogenase and of SSA dehydrogenase

GHB dehydrogenase was prepared from the livers of adult male golden Syrian hamsters as previously described by Kaufman et al. (1979). Inasmuch as the previous work on the kinetics of GHB dehydrogenase was done with the enzyme purified from hamster liver, it is important to note that the GHB dehydrogenase from rat brain and from rat kidney has been shown to cross-react with an antibody to the purified hamster liver enzyme (unpublished results). GHB dehydrogenase was assayed at 37°C in a reaction mixture containing 0.08 *M* potassium phosphate, pH 7.6, 0.0025 *M* $NADP^+$, purified enzyme, and 0.01 *M* sodium GHB. Rat brain SSA dehydrogenase was prepared according to Whittle and Turner (1978); the purification was carried out through the ammonium sulfate fractionation step, and was assayed as described by Whittle and Turner (1978). Rat brain and kidney cytosol (100,000 *g* supernatant fraction) were prepared according to the method of Sokoloff and Kaufman (1961).

RESULTS

Effects of α-ketoisocaproate or phenyl acetate on tissue levels of GHB

Phenylacetate and α-ketoisocaproate, metabolic products of phenylalanine and leucine, are potent inhibitors of GHB dehydrogenase (Table 1). If this enzyme plays a role in the disposition of GHB in vivo, then these inhibitors, when administered to an animal, should decrease the rate of degradation of GHB and thereby increase the tissue level of GHB. The effects of infusions of these compounds are shown in Table 2. α-Ketoisocaproate produced a small but not

TABLE 1. *Comparison of K_i values for compounds that are inhibitors of both GHB dehydrogenase and SSA dehydrogenase*

Compound	K_i values	
	SSA dehydrogenase	GHB dehydrogenase
Valproate	4.0×10^{-3} M[a]	
	4.8×10^{-3} M[a]	5.7×10^{-5} M
Salicylate	1.2×10^{-3} M	1.15×10^{-4} M
α-Ketoisocaproate	1.7×10^{-3} M	1.7×10^{-4} M[b]
Phenyl acetate	3.5×10^{-3} M	5.0×10^{-4} M[b]

[a] The K_i of 4.8×10^{-3} M for sodium valproate for SSA dehydrogenase is taken from Whittle and Turner (1978), and the value of 4.0×10^{-3} M from Maitre et al. (1976).
[b] The K_i values of α-ketoisocaproate and phenyl acetate for GHB dehydrogenase are taken from Kaufman et al. (1983).

statistically significant increase in the level of GHB in the brain. It did, however, produce a twofold increase in the level of GHB in both kidney and muscle. Phenyl acetate, by contrast, increased the level of GHB in brain by 2.4-fold but did not increase the level in kidney or muscle.

Effects of sodium valproate and sodium salicylate on tissue levels of GHB

The drugs sodium salicylate and sodium valproate are also excellent inhibitors of GHB dehydrogenase (Table 1) and therefore might also be expected to alter tissue levels of GHB. Administration (intraperitoneal) of sodium salicylate to rats produced a twofold increase in the level of GHB in brain and a 1.5-fold increase in kidney (Table 3). Sodium valproate produced a 1.4-fold increase in the GHB level in brain and a small but insignificant decrease in the kidney. Three of the four inhibitors that were used produced statistically significant increases in the concentration of GHB in brain. The fourth inhibitor, γ-ketoisocaproate, produced a small increase that did not reach statistical significance (Tables 2 and 3). These data support a role for GHB dehydrogenase in brain.

In contrast to the effects of these inhibitors of GHB dehydrogenase on GHB levels in brain, two of these did not increase GHB levels in kidney and quadriceps muscle and therefore raise questions about the contribution of GHB dehydrogenase to the metabolism of GHB in these particular peripheral tissues. One explanation would be that there are different concentrations of valproate or phenyl acetate in the cytosol of these tissues. Secondly, the question of whether equal concentrations of these inhibitors would exhibit different magnitudes of inhibitory effects in these tissues was addressed by testing the ability of these compounds to inhibit GHB dehydrogenase activity in rat brain cytosol and rat kidney cytosol. The inhibition found in cytosol was compared to that found with the purified enzyme (Table 4). All four inhibitors, when tested at two concentrations, produced a degree of inhibition of GHB dehydrogenase activity in the cytosol from both brain and kidney that was comparable to that found with the purified enzyme (Table 4). These data provide further evidence of the identity of the enzyme in rat brain cytosol and in rat kidney cytosol with purified hamster liver enzyme, an identity previously established by titration with an antibody to the purified enzyme. Finally, it should be noted that since tissue levels represent a balance between synthesis and degradation, an effect of these compounds on the rate of synthesis could either magnify or obscure an effect on the rate of degradation.

Effects of D-glucuronate and L-gulonate on the $t_{1/2}$ of [1-^{14}C]GHB in plasma

Since the results obtained by examining the effects of inhibitors of GHB dehydrogenase on the tissue concentrations of GHB in specific peripheral tissues such as kidney were not conclusive, a method that would measure the sum of GHB metabolism occurring in the whole animal, namely, a measure of the $t_{1/2}$ of GHB in plasma, was also included.

In vitro experiments in which the oxidation of GHB was coupled to the reduction of D-glucuronate demonstrated that the addition of D-glucuronate markedly accelerates the oxidation of GHB catalyzed by GHB dehydrogenase under conditions approximat-

TABLE 2. *Effects of α-ketoisocaproate and of phenyl acetate on tissue levels of GHB*

Tissue	Saline infusion	α-Ketoisocaproate infusion		Phenyl acetate infusion	
	nmol GHB/g tissue	nmol GHB/g tissue	Percent of control	nmol GHB/g tissue	Percent of control
Brain	2.6 ± 0.3 (5)	3.2 ± 0.1 (3)	123	6.1 ± 1.0 (4)[b]	235
Kidney	27.8 ± 3.2 (5)	55.0 ± 8.2 (4)[a]	198	18.4 ± 1.3 (4)[a]	66
Muscle	22.2 ± 3.0 (6)	46.4 ± 9.9 (4)[a]	209	16.7 ± 7.1 (4)	75

Phenyl acetate (1.0 M) was given intravenously as an initial bolus of 1.5 ml followed by a constant infusion of ≅2.0 ml/h for 2 h. α-Ketoisocaproate (0.5 M) was given intravenously as an initial bolus of 0.6 ml followed by a constant infusion of ≅2.0 ml/h for 2 h. At the end of the infusion the animals were killed and the tissues were removed and assayed for GHB as described under Materials and Methods. All values are means ± SEM, numbers of animals in parentheses.
[a] $p < 0.05$.
[b] $p < 0.01$.

TABLE 3. *Effects of salicylate and of valproate on tissue levels of GHB*

Tissue	Saline (control)	Salicylate		Valproate	
	nmol GHB/g tissue	nmol GHB/g tissue	Percent of control	nmol GHB/g tissue	Percent of control
Brain	2.9 ± 0.5 (6)	5.8 ± 0.5 (4)[b]	200		
	2.6 ± 0.2 (4)			3.7 ± 0.2 (4)[b]	142
Kidney	34.5 ± 5.7 (6)	52.8 ± 2.5 (5)[a]	153		
	30.0 ± 4.1 (4)			23.7 ± 3.8 (4)	79

Sodium valproate (100 mg/kg, i.p.) was given 2 h prior to decapitation; sodium salicylate (500 mg/kg, i.p.) was given 1 h prior to decapitation. Tissues were removed and assayed as described under Materials and Methods. All values are means ± SEM, numbers of animals in parentheses.
[a] $p < 0.05$.
[b] $p < 0.01$.

ing those existing in the cytosol of brain, kidney, and muscle (Kaufman and Nelson, 1981). L-Gulonate, the product of D-glucuronate reduction, competes with GHB as a substrate for GHB dehydrogenase and therefore inhibits GHB oxidation. We have examined the effects of both of these compounds on the $t_{1/2}$ of [1-^{14}C]GHB in plasma and found that the administration of D-glucuronate in vivo decreases the $t_{1/2}$ by 33% whereas administration of L-gulonate increases it by 33% (Fig. 1). The decrease in the $t_{1/2}$ is consistent with an increased rate of oxidation of GHB by GHB dehydrogenase in the presence of D-glucuronate; similarly, the increase in $t_{1/2}$ caused by L-gulonate suggests a decrease in the rate of oxidation of GHB. These results are consistent with our in vitro findings.

Inhibition of SSA dehydrogenase and of GHB dehydrogenase by valproate, salicylate, α-ketoisocaproate, and phenyl acetate

Since an increase in tissue levels of GHB produced by phenyl acetate, α-ketoisocaproate, valproate, and salicylate could be attributed to either an increase in the rate of synthesis of GHB or to a decrease in its rate of degradation or to a combination of both, we examined the inhibition of both SSA dehydrogenase and GHB dehydrogenase by valproate, salicylate, α-ketoisocaproate, and phenyl acetate. Inhibition of SSA dehydrogenase could theoretically lead to higher tissue levels of SSA and, therefore, an increased rate of synthesis of GHB, whereas inhibition of the GHB dehydrogenase activity could increase GHB levels directly. Valproate, salicylate, α-ketoisocaproate, and phenyl acetate are all competitive inhibitors for GHB dehydrogenase with GHB as the variable substrate (Kaufman et al., 1983). K_i values for SSA dehydrogenase were similarly determined for salicylate, phenyl acetate, and α-ketoisocaproate with SSA as the variable substrate. All of these compounds were competitive inhibitors of SSA dehydrogenase (data not shown). Whittle and Turner (1978) have reported that sodium valproate is a noncompetitive inhibitor of SSA dehydrogenase.

The K_i values for these compounds with both enzymes are given in Table 1. In every case the K_i of these inhibitors for GHB dehydrogenase was one to two orders of magnitude lower than that for SSA dehydrogenase.

TABLE 4. *Effects of inhibitors of purified NADP$^+$-dependent GHB dehydrogenase on the NADP$^+$-dependent oxidation of GHB in rat brain cytosol and in rat kidney cytosol*

Inhibitor	Concentration (mM)	Percent inhibition		
		Purified GHB dehydrogenase	Rat brain cytosol	Rat kidney cytosol
α-Ketoisocaproate	2	82	100	76
α-Ketoisocaproate	5	96	100	93
Valproate	2	93	87	91
	5	100	100	100
Salicylate	2	92	90	84
	5	94	100	91
Phenyl acetate	2	67	51	47
	5	93	72	84

GHB dehydrogenase activity was assayed as described in Materials and Methods. Inhibitors were added to the reaction mixture in the concentrations indicated in the table.

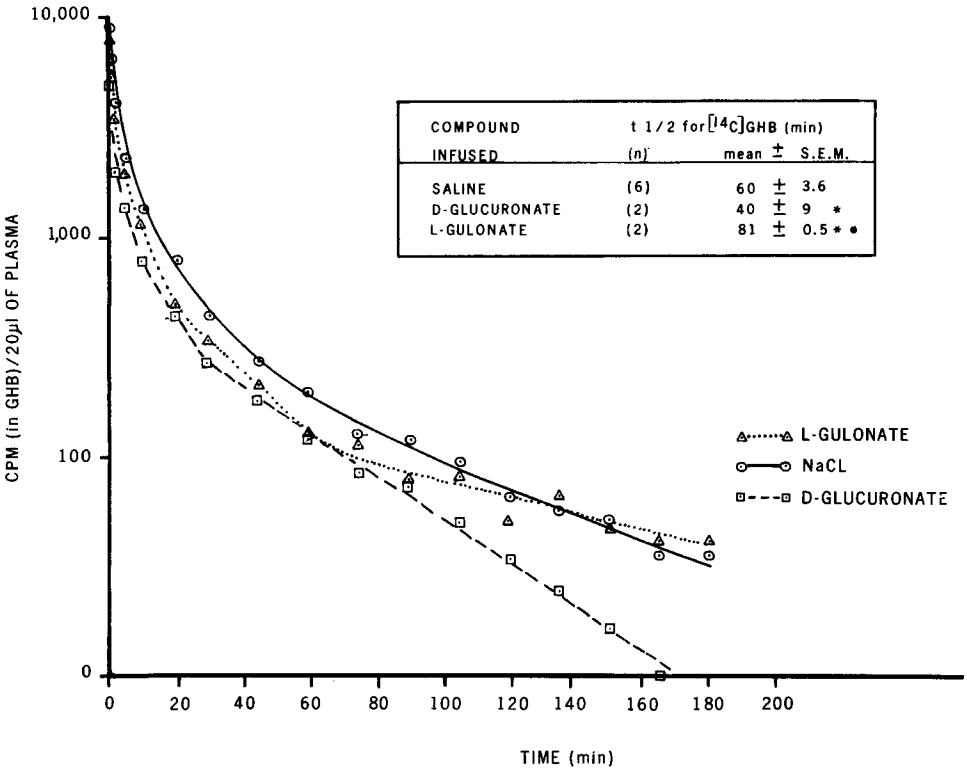

FIG. 1. Animals that received D-glucuronate received a bolus containing 240 mg followed by an infusion of 320 mg/h for 3 h. Animals receiving L-gulonate received a bolus of 333 mg and an infusion of 556 mg/h for 3 h. The $t_{1/2}$ for the disappearance of [^{14}C]GHB from plasma was determined as described under Materials and Methods. *$p < 0.05$; **$p < 0.025$.

DISCUSSION

This investigation was designed to determine whether GHB dehydrogenase, an enzyme that catalyzes the oxidation of GHB to SSA in vitro, plays a significant role in the disposition of GHB in vivo. We have also examined the possibility that the information obtained in this study might provide a reasonable explanation for the significant increases in GHB levels in brain brought about by the acute administration of sodium valproate and other drugs used in the treatment of petit mal epilepsy (Snead et al., 1980). This would be of interest since investigations of the inhibition of aminobutyrate aminotransferase (EC 2.6.1.19), SSA dehydrogenase (EC 1.2.1.16), as well as an NADPH-dependent aldehyde reductase (EC 1.1.1.2) by sodium valproate (Godin et al., 1969; Harvey et al., 1975; Sawaya et al., 1975; Whittle and Turner, 1978), have failed to produce a satisfactory explanation for the elevated GHB levels following an acute dose of sodium valproate.

Marked inhibition of SSA dehydrogenase might be expected to lead to increased tissue levels of SSA and thereby to increased synthesis of GHB. Indeed, in patients with GHB aciduria, a genetic disease in which this dehydrogenase is low or missing (Gibson et al., 1983), exactly such increases do occur. Little or no inhibition of SSA dehydrogenase would be expected to result from relatively high doses (100 mg/kg) of sodium valproate since the K_i of sodium valproate for SSA dehydrogenase has been reported to be 4.0×10^{-3} M by Maitre et al., (1976) or 4.8×10^{-3} M by Whittle and Turner (1978). At tissue concentrations of 0.1 mM–1.0 mM, the highest concentrations likely to be found in clinical use (Sawaya et al., 1975), inhibition would vary from a negligible amount at 0.1 mM to 12–15% inhibition at 1.0 mM.

In vitro studies of GHB dehydrogenase (Kaufman et al., 1979) have provided the first clue to the metabolic basis for the increased level of GHB in brain following acute administration of sodium valproate (Snead et al., 1980). The activity of SSA dehydrogenase in the brain of the adult rat (Pitts and Quick, 1967) is approximately 1,000 times greater than that of GHB dehydrogenase (Kaufman et al., 1979). Therefore, in the sequence of steps leading to the formation of succinate from GHB, GHB dehydrogenase would catalyze the rate-limiting step. If GHB dehydrogenase is the first enzyme in one of the quantitatively significant pathways for the degradation of GHB, then administration of inhibitors of this enzyme might lead to increased tissue levels of GHB. Phenyl acetate, α-ketoisocaproate, valproate, and salicylate, all potent inhibitors of GHB dehydrogenase with K_i values in the range of 10^{-4}–10^{-5} M, were selected to test this hypothesis. Indeed, administration

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