

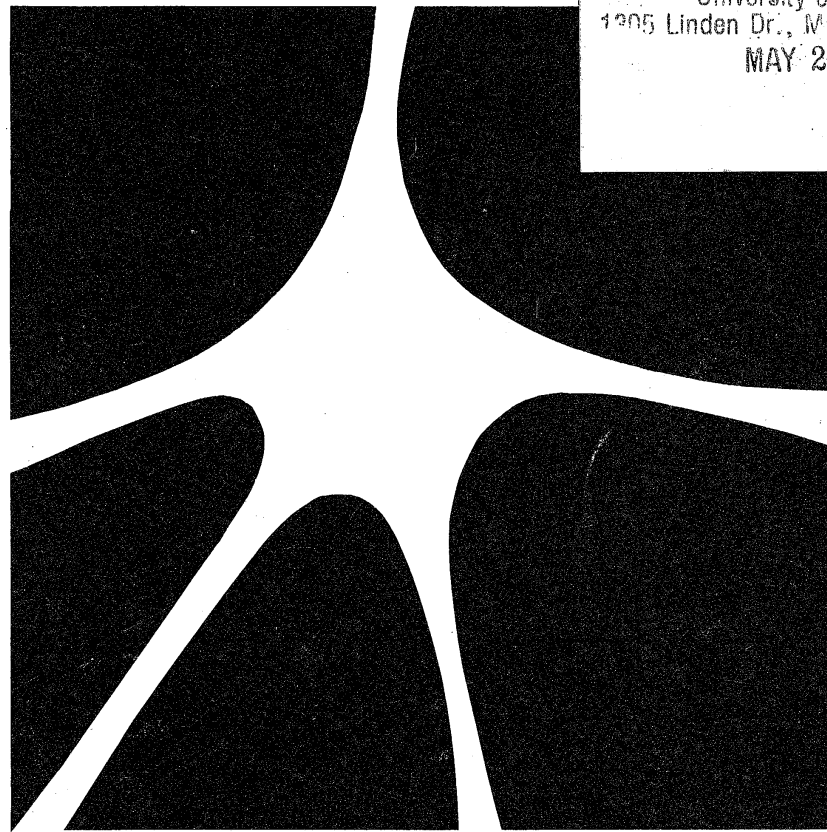
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JOURNAL OF NEUROCHEMISTRY

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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 CO₂ 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 \longleftrightarrow SSA
- (2) SSA \longrightarrow succinate
- (3) succinate $\rightarrow \rightarrow \rightarrow$ CO₂ + H₂O

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-

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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.). [¹⁴C]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 N₂ and assayed for GHB as described above.

Effects of D-glucuronate or L-gulonate on the $t_{1/2}$ of [¹⁴C]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 [¹⁴C]GHB (sp act 4.5 $\mu\text{Ci}/\mu\text{mol}$) 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/H₂O/NH₄OH, 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 H₂O. The fraction of total ¹⁴C in each plasma sample that migrated with authentic GHB was measured and used to determine the concentration of [¹⁴C]GHB found in the plasma sample in the presence of radioactive metabolites of GHB. The plasma [¹⁴C]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	$4.0 \times 10^{-3} M^a$ $4.8 \times 10^{-3} M^a$	$5.7 \times 10^{-5} M$
Salicylate	$1.2 \times 10^{-3} M$	$1.15 \times 10^{-4} M$
α -Ketoisocaproate	$1.7 \times 10^{-3} M$	$1.7 \times 10^{-4} M^b$
Phenyl acetate	$3.5 \times 10^{-3} M$	$5.0 \times 10^{-4} M^b$

^a The K_i of $4.8 \times 10^{-3} M$ for sodium valproate for SSA dehydrogenase is taken from Whittle and Turner (1978), and the value of $4.0 \times 10^{-3} M$ 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 $\cong 2.0$ 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 $\cong 2.0$ 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 \pm SEM, numbers of animals in parentheses.

^a $p < 0.05$.

^b $p < 0.01$.

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