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An Overview of γ-Hydroxybutyrate Catabolism: The Role of the Cytosolic NADP$^+$-Dependent Oxidoreductase EC 1.1.1.19 and of a Mitochondrial Hydroxyacid-Oxoacid Transhydrogenase in the Initial, Rate-Limiting Step in This Pathway

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γ-Hydroxybutyrate (GHB) is a naturally occurring compound present in micromolar concentration in both brain (1,2) and in peripheral tissues (3). This endogenous compound is remarkable in that pharmacological doses of 200–500 mg/kg produce marked behavioral and electroencephalographic changes (4), a profound decrease in cerebral glucose utilization (5), an increase in striatal dopamine levels (6) and a decrease in body temperature (7). High doses of GHB have also been reported to protect neurons (8) and intestinal epithelium (9) against cell death resulting from experimental ischemia. Behavioral changes are not seen with doses of less than 30 mg/kg, but low doses stimulate the release of prolactin, growth hormone and cortisol (10,11), and doses of 5–10 mg/kg result in an increase in body temperature (12). These actions, and the discovery of high affinity binding sites for GHB in the central nervous system (13), suggest that GHB may have a biological function. Both the origin of endogenous GHB and its catabolism are, therefore, of considerable interest.

This review will cover the early work on the degradative pathway for GHB and the discovery of a dual pathway for the initial step in the oxidative catabolism of GHB. The factors which regulate the activity of the enzymes in these pathways, and as a consequence, regulate tissue levels of GHB are also discussed.

Walkenstein et al. (14) established that GHB is largely disposed of, *in vivo* by oxidation to CO_2 and water. These investigators could not find the ^{14}C label from GHB in succinate in urine, but they did find that the label could be trapped in hippuric acid in the urine of animals treated with sodium benzoate as might be expected if GHB were undergoing β-oxidation. They therefore proposed that GHB was metabolized by β-oxidation (14). Möhler et al. (15) and Doherty et al. (16), however, assayed citric acid cycle intermediates in the tissues of animals given [^{14}C]GHB and demonstrated that the carbon skeleton of GHB indeed does enter the citric acid cycle as succinate rather than as acetyl-CoA as would be expected if GHB were being oxidized through the β-oxidation pathway.

They proposed the following pathway:

1) GHB $\rightleftharpoons$ succinic semialdehyde
2) Succinic semialdehyde $\rightarrow$ succinate
3) Succinate $\rightarrow\rightarrow\rightarrow\rightarrow$ Co_2 + H_2O

The discovery of a metabolic disease in which GHB and succinic semialdehyde (SSA) are markedly elevated (17) in both blood and urine due to a block in SSA dehydrogenase (18) added evidence to support a degradative pathway in which GHB is oxidized to SSA, which in turn is oxidized to succinate.

At the time the pathway shown above was proposed, it was known that the enzymes of the citric acid cycle catalyzed the reactions in step (3) and that SSA

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Abbreviations used in this paper: GHB, γ-hydroxybutyrate; SSA, succinic semialdehyde; DTT, dithiothreitol.

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dehydrogenase (the enzyme missing in patients with GHB aciduria (18)) catalyzed the reaction in step (2). Step (1) is always depicted as an essential part of this scheme. However, at the time this pathway was proposed, an enzyme or enzymes which could catalyze this step had not been identified.

Isolation of a Cytosolic GHB Dehydrogenase. Two unusual oxidoreductases, one cytosolic (19) and the other mitochondrial (20), that catalyze the oxidation of GHB to SSA have now been isolated. The cytosolic enzyme, which will be referred to as GHB-dehydrogenase in this review, was first purified to homogeneity from hamster liver (19) and was found to be an $NADP^+$-dependent oxidoreductase. A study of the substrate specificity of purified GHB-dehydrogenase revealed that D-glucuronate and L-gulonate, the product of D-glucuronate reduction, were also good substrates (19). The physical characteristics, as well as the substrate and inhibitor specificity of this enzyme, indicate that the ability to catalyze the oxidation of GHB probably represents a previously unreported activity for the $NADP^+$-dependent oxidoreductase (EC 1.1.1.19) commonly known as D-glucuronte reductase (21). This enzyme may also be identical to the group of enzymes categorized in a 1985 review by Cromlish et al. (22) as "ALR-1", the high K_m aldehyde reductase or L-hexonate dehydrogenase.

Although the oxidation of GHB catalyzed by this GHB-dehydrogenase proceeds at an easily measurable rate when assayed in vitro under optimal conditions, the very low activity found in the in vitro system under conditions simulating those in the cytosol raises the question of how, or indeed whether, this enzyme catalyzes the oxidation of GHB in vivo. An answer to this question may have been found when it was discovered that GHB dehydrogenase could catalyze the reduction of D-glucuronate coupled to the oxidation of GHB (23) as shown below:

$$\text{GHB} + \text{NADP}^+ \rightleftharpoons \text{SSA} + \text{NADPH} + \text{H}^+$$

$$\text{D-glucuronate} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{L-gulonte} + \text{NADP}^+$$

The overall or "coupled" reaction is:

$$\text{GHB} + \text{D-glucuronate} \rightleftharpoons \text{SSA} + \text{L-gulonate}$$

When the kinetic constants for the coupled system were determined, it was found that they were more favorable to oxidation of GHB under conditions present in cytosol of most tissues than were those for the uncoupled system.

The time course of the coupled reaction (Figure 1) in which both products, SSA and NADPH, were measured, shows that in the presence of an adequate concentration of D-glucuronate, only a very small net amount of NADPH is formed even though SSA formation is proceeding rapidly. This is in contrast to the control reaction mixture without glucuronate in which NADPH and SSA are formed in stoichiometric amounts (19). These results indicate that NADPH is being used for the reduction of D-glucuronate at the same rate at which it is being produced by the oxidation of GHB. This would account for the low steady state level of NADPH seen in Figure 1. The effect of increasing concentrations of D-glucuronate on the rate of oxidation of GHB to SSA in the presence of limiting amounts of $NADP^+$ and inhibitory amounts of NADPH is shown in Figure 2. Under these conditions, 2 mM D-glucuronate increased SSA formation 8-fold.

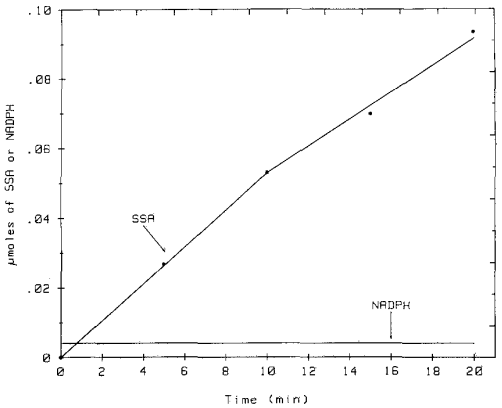

Fig. 1. Time course of succinic semialdehyde (SSA) and NADPH formation in the presence of D-glucuronate (23). The reaction mixture contains 10 mM GHB, 1.0 mM D-glucuronate, 0.1 mM $NADP^+$, 80 mM phosphate buffer, pH 7.6, enzyme and sufficient water to bring the volume to 1.0 ml. SSA and NADPH were determined as previously described (19).

Other important changes in the kinetic constants for this reaction occur in the presence of D-glucuronate (Table I). The K_m (4.5×10^{-4}M) for the coupled reaction is five-fold lower and the V_{max} (1.52 μmol/min/mg protein) is 1.8 times higher than in the uncoupled reaction. The effects of coupling and changes in pH on the rate of degradation of GHB are, however, more accurately described by changes in k (the first order rate constant for the reaction) than they are by changes in V_{max}. The concentration of GHB in the tissues is much lower than K_m and under these conditions the quantity V_{max}/K_m provides a good approximation of k (24). In the coupled reaction, the rate constant for GHB degradation, V_{max}/K_m, is increased 9-fold as compared to the 1.8-fold increase in V_{max} at saturating concentrations of GHB and $NADP^+$ (Table I). The effect of D-glucuronate on the rate of the reaction is much greater at the very low sub-

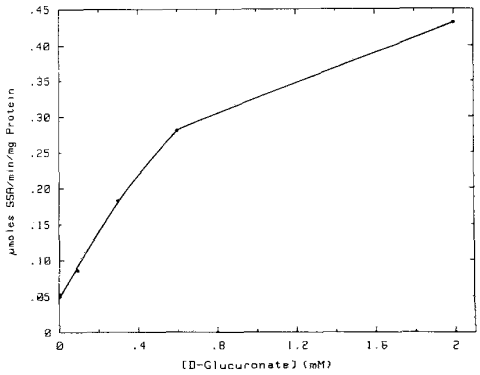

Fig. 2. The effect of D-glucuronate concentration on the rate of conversion of GHB to succinic semialdehyde (SSA) in a reaction mix containing NADPH and a low concentration of NADP+ (23). Reaction mixture: 10 mM GHB, 0.01 mM NADP+, 0.02 mM NADPH, 80 mM phosphate, pH 7.6, enzyme, D-glucuronate as indicated and water to 1 ml. The assay for SSA was carried out as previously described (19).

strate concentrations found *in vivo* than in the standard in vitro assay where V_{max} conditions are used. In the coupled reaction the K_m for NADP+ is decreased from 2×10^{-5} to 1.4×10^{-6}M and the inhibition produced by NADPH ($K_i = 7 \times 10^{-6}$M) has been eliminated (Figure 3). GHB can now be oxidized in the presence of an otherwise extremely inhibitory concentration of NADPH. All of the kinetic constants for the coupled reaction are closer to the tissue concentration range shown in Table I (25) than are those for the uncoupled reaction.

The rate of GHB oxidation is pH-dependent. Earlier work had shown that the pH optimum for the cytosolic oxido-reductase under V_{max} conditions was 9.0 (19), but at a more physiological pH (7.0–7.2) the enzyme was only half as active. The pH optimum was dependent on GHB concentration in both the coupled and uncoupled reactions and in both cases was above 8.0 when saturating concentrations of substrate were used (26). As the concentration of GHB decreases toward levels found in vivo the pH optimum for the coupled reaction shifts toward pH 7.0 (26). A plot of V_{max}/K_m against pH (Figure 4) shows that, at substrate concentrations near those found in vivo, the pH optimum approaches the intracellular pH, i.e. 7.5 for the uncoupled reaction and 7.0 or lower for the coupled reaction (26). Vayer et al. (27) subsequently reported a pH optimum of 8.0 under different conditions from those described above. In their system the oxidation of GHB catalyzed by the cytosolic oxidoreductase was coupled to both the reduction of D-glucuronate and the transamination of SSA to form GABA.

GHB dehydrogenase is inhibited by a number of products of intermediary metabolism (Table II) which includes the ketone bodies, α-ketoglutarate and branched α-ketoacids derived from amino acid degradation as well as degradation products of phenylalanine (26). As has been found with certain aldehyde reductases (28,29), anticonvulsants such as barbiturates, diphenylhydantoin and valproate are good inhibitors of GHB dehydrogenase (30). In addition, GHB dehydrogenase is inhibited by salicylates (30).

GHB dehydrogenase like lysozyme, ribonuclease and a number of other proteins (26), may contain disulfide bridges which are essential for its activity. It is inhibited by compounds such as β-mercaptoethanol and dithiothreitol (DTT), which can reduce disulfide bonds (26). DTT has the most pronounced effect; addition of 2.5 mM DTT produces an 85% inhibition of the activity

Table I. The Effect of D-Glucuronate on the Kinetic Constants for γ-Hydroxybutyrate (GHB), NADP+, and NADPH

		Kinetic Constants		Tissue concentration
		Uncoupled assay	Coupled assay	(range between brain, liver, kidney, muscle)[a]
GHB	$K_{m_{app}}$	2.3×10^{-3} M	4.5×10^{-4} M[b]	$0.1–5 \times 10^{-5}$ M
NADP+	$K_{m_{app}}$	2×10^{-5} M	1.4×10^{-6} M[c]	$2 - 11 \times 10^{-6}$ M
NADPH	K_i	7×10^{-6} M	No inhibition	$1 - 30 \times 10^{-5}$ M
GHB	V_{max}[d]	0.83	1.52[b]	
V_{max}/K_{mGHB}	(k)[e]	0.36	3.38	

[a]The tissue concentrations of GHB are from reference (3). The molar concentration of NADP+ and NADPH in the various tissues were calculated from data taken from reference (25).
[b]1 mM D-glucuronate.
[c]2 mM D-glucuronate.
[d]μmol/min/mg protein.
[e]first order rate constant when $[S] << K_m$
Data in this table are from reference (23)

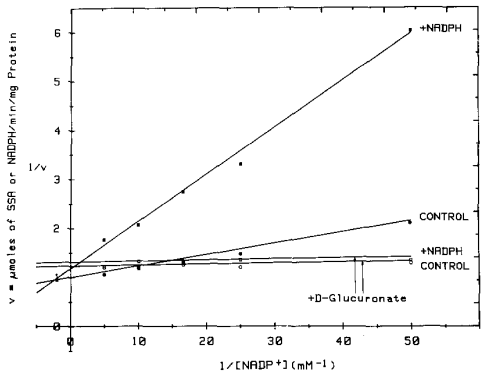

Fig. 3. The effect of D-glucuronate on the kinetics of the oxidation of GHB with $NADP^+$ as the variable substrate (23). In the absence of D-glucuronate, NADPH formation was measured; in the presence of D-glucuronate, succinic semialdehyde (SSA) formation was measured. ● = control, ■ = control with 0.02 mM NADPH, ○ = control with 2.0 mM D-glucuronate, □ = control with 0.02 mM NADPH and 2.0 mM D-glucuronate.

Table II. Kinetic Constants for the Inhibition of GHB-Dehydrogenase (26,30)

Inhibitor	K_i (mM)	
	Uncoupled reaction	Coupled reaction
DL-β-Hydroxybutyrate	3.9	4.4
Acetoacetate	3.0	
α-Ketoglutarate	1.1	0.6
ρ-Hydroxyphenylpyruvate	1.0	
Phenylacetate	0.5	0.4
Pyruvate	7.0	
α-Ketoisovalerate	0.33	0.2
α-Ketoisocaproate	0.17	
DL-α-keto-β-methyl *n*-Valerate	0.06[a]	
Valproate	0.057	
Salicylate	0.115	

Assays for the coupled reaction and the uncoupled reaction were carried out as described (26).
[a]It should be noted that the K_i has only been reported for the DL-mixture.

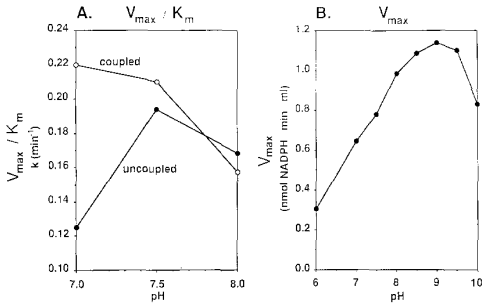

Fig. 4. The effect of pH on the ratio of V_{max}/K_m and on V_{max} for the oxidation of GHB by GHB dehydrogenase. The data for Figure 4A were taken from reference (26). The data in Figure 4B are from reference (19). V_{max}/K_m = the first order rate constant, k, when [S] << K_m.

of the purified enzyme and 2.0 mM DTT inhibits 84% of the activity found in rat liver cytosol. The inhibition produced by DTT can be partially reversed by the addition of H_2O_2 or completely reversed by oxidized glutathione. Preincubation of GHB dehydrogenase with 2.5 mM DTT before it is added to the reaction mixture (and thereby diluted 50 fold) does not inhibit the enzyme. Under these conditions, the sulfhydryl groups are probably reoxidized by molecular oxygen. Little or no stimulatory effect is seen when compounds such as H_2O_2, oxidized glutathione and cystamine, all of which can oxidize sulfhydryl groups, are added to enzyme that has not been *previously* inhibited by sulfhydryl reducing agents.

When the activity of GHB dehydrogenase is being measured, the inclusion of reducing compounds such as β-mercaptoethanol or DTT in the assay mixture as has been reported by some laboratories (27) could lead to incorrect results.

Identification of a Mitochondrial Transhydrogenase Which Catalyzes the α-Ketoglutarate-Dependent Oxidation of GHB. The discovery of GHB dehydrogenase made it possible to complete a sequence of reactions leading from GHB to CO_2 and H_2O. Development of a polyclonal antibody to the GHB dehydrogenase then made it possible to determine whether or not there were additional enzymes in the cytosol or in other subcellular fractions that could catalyze the initial, and probably rate-limiting, step in the oxidation of GHB to CO_2 (31). The antibody, which inhibited ≈ 95% of the ability of the cytosolic fraction to oxidize GHB to SSA, failed to inhibit approximately 60% of this activity in kidney homogenate (Figure 5). These results suggested that there was at least one additional enzyme which catalyzed the conversion of GHB to SSA. The mitochondrial fraction, which had previously been shown to lack GHB dehydrogenase activity (19), could oxidize [^{14}C]GHB to $^{14}CO_2$ and could catalyze the pyridine nucleotide-independent oxidation of GHB to SSA (31). The mitochondrial enzyme was subsequently isolated and shown to be a hydroxyacid-oxoacid transhydrogenase which catalyzed the following reaction (20):

GHB + α-ketoglutarate → SSA + α-hydroxyglutarate.

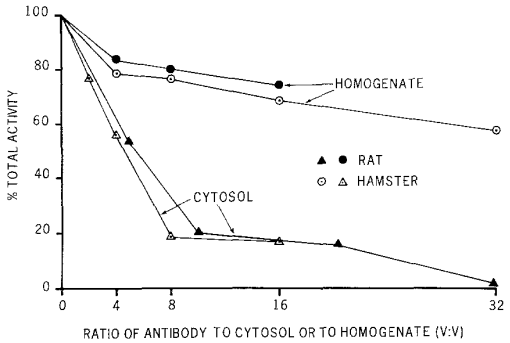

Fig. 5. Titration of GHB dehydrogenase activity in cytosol and homogenate with the antibody to GHB dehydrogenase (31). The conversion of [1-^{14}C]GHB to $^{14}CO_2$ was used as a measure of activity in the experiments in which homogenate was used. When cytosol was used, the conversion of GHB to SSA was measured spectrophotometrically. The cytosol and homogenate used in this experiment were derived from the same amount of kidney. Antibody was added to the reaction mixture in the amounts indicated.

The oxidation of GHB by the mitochondrial enzyme was found to be completely dependent on the presence of α-ketoglutarate. The reaction was reversible. The mitochondrial enzyme also catalyzed the conversion:
α-hydroxyglutarate + SSA → α-ketoglutarate + GHB
with the two products being formed in stoichiometric amounts. The substrate specificity is shown in Table III and the kinetic constants for the principal substrates in Table IV (20). The assumption that this enzyme is a transhydrogenase was confirmed when it was shown by gas chromatographic-mass spectroscopy that a deuterium on the hydroxyl-bearing carbon of one of the optical isomers of DL-γ-deutero-γ-hydroxybutyrate was transferred to the ketone-bearing carbon of α-ketoglutarate. This hydroxyacid-oxoacid transhydrogenase which has been found in the soluble fraction of mitochondria from liver, kidney and brain has been partially purified (20).

Comparison of the Cytosolic and Mitochondrial Enzymes which Oxidize GHB to SSA. With the discovery of the mitochondrial transhydrogenase it became apparent that there was a dual pathway for the initial step in the oxidative pathway for GHB. Both cytosolic GHB dehydrogenase and the mitochondrial enzyme are oxidoreductases, but of remarkably different types. The cytosolic enzyme is an NADP$^+$-dependent dehydrogenase, whereas the mitochondrial enzyme is a pyridine nucleotide-independent, α-ketoglutarate-dependent transhydrogenase. These two enzymes, despite some striking differences, have one property in common: the activity of each of these enzymes is regulated by coupling the oxidation of GHB, the hydroxyacid, to the simultaneous reduction of an oxoacid. Though GHB dehydrogenase can function in the uncoupled state, conditions in the cytosol of most tissues (Table I) would not be favorable to the uncoupled reaction. The mitochondrial enzyme, on the other hand, has an absolute requirement for an oxoacid, which suggests that the metabolism of GHB by the mitochondrial enzyme would depend on the steady state levels of citric acid cycle intermediates and of α-ketoglutarate in particular. The relative activities (V_{max} and v) of these two enzymes in brain and kidney are shown in Table V. The activities of the two enzymes were measured under V_{max} conditions; the rate of GHB oxidation (v) at average tissue GHB concentrations could then be calculated (Table V). How well these values correspond to measurements of GHB catabolism in vivo can be determined by examination of the half-life of GHB in brain. Doherty et al. (16) and Möhler et al. (15) have both reported a half-life ($t_{1/2}$) for labeled GHB in brain of 5 min. If one assumes that the rate of disposal of GHB follows first order kinetics, then from the fol-

Table III. The Relative Rates of the Transhydrogenase Reaction With Other Hydroxyacids and Oxoacids (20)

Hydroxyacid (with SSA as co-substrate)	Relative Rate	*Oxoacid* (with GHB as co-substrate)	Relative Rate
D-α-Hydroxyglutarate	100	α-Ketoglutarate	100
L-α-Hydroxyglutarate	0	α-Ketoadipate	24
DL-β-Hydroxybutyrate	43	Oxalacetate	18.5
L-β-Hydroxybutyrate	45	Pyruvate	8.0
D-β-Hydroxybutyrate	0	α-Ketobutyrate	4.3
DL-α-Hydroxybutyrate	0	Acetoacetate	0
D-Lactate	0	β-Ketoglutarate	0.5
L-Lactate	1.2	β-Ketoadipate	0
D-Malate	0		
L-Malate	0		

The data in this table are derived from reference (20)

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