

Evidence for a role of high K_m aldehyde reductase in the degradation of endogenous γ -hydroxybutyrate from rat brain

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γ -Hydroxybutyrate (GHB) is a putative neurotransmitter in brain. We have already demonstrated that it is transformed into γ -aminobutyrate (GABA) by rat brain slices incubated under physiological conditions. This conversion occurs via a GABA-transaminase reaction. Therefore, succinic semialdehyde, the oxidative derivative of GHB, appears to be the primary catabolite of GHB degradation. Apparently, the kinetic characteristics and pH optimum of GHB dehydrogenase (high K_m aldehyde reductase) in vitro do not favor a role for this enzyme in endogenous brain GHB oxidation. However, in the presence of glucuronate, glutamate, NADP and pyridoxal phosphate, pure GHB dehydrogenase, coupled to purified GABA-transaminase does produce GABA from GHB at an optimum pH close to the physiological value and with a low K_m for GHB.

γ -Hydroxybutyrate Catabolism Rat brain

1. INTRODUCTION

In brain, GHB fulfills the main criteria generally accepted for defining a neurotransmitter [1]. Its biosynthesis [2,3], transport [4], binding [5], release [6] and turnover rate [7] suggest that GHB plays an important role at the synaptic level. Recently we have described the catabolic pathway of this compound [8]. [3 H]GHB incubated in vitro under physiological conditions with rat brain slices produces [3 H] γ -aminobutyrate ([3 H]GABA). This transformation does not occur via succinate followed by the Krebs cycle, and it is strongly decreased by all the GABA-T inhibitors tested.

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Abbreviations: GABA-T, 4-aminobutyrate-2-oxoglutarate aminotransferase (EC 2.6.1.19); GHB, γ -hydroxybutyrate; GABA, γ -aminobutyrate; AET, aminoethylisothiuronium bromide, hydrobromide; SSA, succinic semialdehyde

Therefore, it seems likely that the first step of GHB catabolism is its oxidation to SSA via a GHB dehydrogenase, followed by the transamination of SSA to GABA via GABA-T.

An NADP-linked enzyme, able to interconvert GHB and SSA has been partially purified from hamster liver and brain [9]. This enzyme, which has an M_r of 38000 by SDS-PAGE, can reduce SSA with NADPH as cofactor at a pH optimum of 6.7. This reaction is inhibited by barbiturates, sodium valproate, phenylhydantoins and ethosuximide. D-Glucuronate appears to be a good substrate for this enzyme. Therefore, it seems that the GHB dehydrogenase purified by Kaufman et al. [9] is the high K_m aldehyde reductase isolated and purified by several authors (review [10]). This enzyme, designated as ALR₁ (EC 1.1.1.2 [11]) or succinic semialdehyde reductase (SSR₁) [2] can oxidize GHB to SSA. However, in view of the kinetic characteristics of the GHB dehydrogenation reaction, its role in the catabolism of the very

low endogenous concentration of GHB in brain may be questionable. We demonstrate in this work that purified GHB dehydrogenase (ALR₁ or SSR₁) and purified GABA-T, both from rat brain, added together are able to rapidly transform very small amounts of GHB into GABA.

2. MATERIALS AND METHODS

[2,3-³H]GHB potassium salt (spec. radioact. 40 Ci/mmol) was obtained from the CEA (France). Radiochemical purity was checked by thin layer chromatography in 3 different solvent systems as described [5].

2.1. Enzyme assays

For determination of GHB dehydrogenase activity, the initial rate of NADP reduction at 37°C was measured in phosphate buffer (300 mM, pH 8.0) containing 5 mM β -mercaptoethanol, 5 mM AET and suitable amounts of GHB and NADP (usually 10 mM and 50 μ M, respectively). The increase in NADPH fluorescence was measured at excitation 355 nm and emission 470 nm. In some cases, measurements of SSA production were carried out with cyclohexane 1,3-dione according to Beeler and Churchich [12].

The activity of the coupled enzyme reaction (GHB dehydrogenase plus GABA-T) was measured as follows: radioactive GHB ([2,3-³H]-GHB, 40 Ci/mmol) at a specific activity of 700 mCi/mmol was incubated in 500 μ l of 300 mM phosphate buffer (pH 8.0) containing 5 mM β -mercaptoethanol, 5 mM AET, 0.5 mM pyridoxal phosphate, 0.5 mM glutamate, 2 mM glucuronate, 50 μ M NADP and a suitable amount of purified GHB dehydrogenase (8 units) and purified GABA-T (10.3 units) (1 unit = 1 nmol SSA formed/min). These two levels of activities were chosen due to the similar specific activities of the two enzymes in homogenates of rat brain [13,14]. After incubation for 5 min at 37°C, the reaction was stopped by adding 50 μ l of 100% trichloroacetic acid. The [³H]GABA formed was separated from [³H]GHB by ion-exchange chromatography using Dowex 50 W X 8 (H⁺ form) resin packed into a Pasteur pipette (bed volume 3 $\times$ 0.5 cm). The column was rinsed 4 times with 1 ml distilled H₂O, and the amino acid fraction was eluted from the column with 1 M ammonium hydroxide and mixed with

5 ml Instagel (Packard Instruments) in a scintillation vial. Radioactivity was determined using a Beckman β counter at about 40% efficiency. Elution profiles of standards solution of [³H]GHB or [³H]GABA indicate clear cut separation.

2.2. Enzyme extraction and purification

2.2.1. High K_m ALR₁

High K_m ALR₁ which is identical to non-specific SSR₁ and glucuronate reductase [11] has been purified as described [2]. This enzyme is named GHB dehydrogenase in this work.

2.2.2. GABA-T

GABA-T from rat brain was partially purified (about 300-fold with regard to the initial homogenate). An acetone powder, prepared as described by Roberts and Frankel [15], was used as a source of enzyme. An acetone powder from 15 rat brains was homogenized in 100 ml of 2 mM phosphate buffer (pH 7.2) containing 5 mM β -mercaptoethanol, 5 mM AET and 0.5 mM pyridoxal phosphate. After rapid centrifugation at 30000 $\times g$ for 20 min, the supernatant was adsorbed onto a DEAE cellulose column. This chromatography and the following hydroxyapatite chromatography were carried out as described by Maitre et al. [16]. The GABA-T obtained from this last step has a specific activity of 0.31 μ mol/min per mg and was concentrated about 10-fold with an Amicon ultrafiltration system.

2.3. pH optima for the coupled GHB dehydrogenase and GABA-T reaction

300 mM phosphate buffer (pH 5.0–9.5) was used. All the substrates, including [³H]GHB (700 mCi/mmol) described for the measurement of the coupled reaction were added (final GHB concentration 30 μ M). GABA formation was measured as described above.

2.4. Apparent K_m for GHB when GABA-T is present in the medium

When GABA-T was present in the incubation medium (10.3 units/500 μ l) the apparent K_m for GHB was determined using [³H]GHB and isolating the [³H]GABA formed as described above. The incubation medium contained all the substrates indicated and GHB (0–60 μ M).

The product formed from [³H]GHB after the

conjugation of GHB dehydrogenase and GABA-T action was identified by dansylation according to Zanetta et al. [17]. After separation on Dowex 50 W X 8, two-dimensional thin-layer chromatography of the dansylated product was performed using pure dansylated amino acids as standards. Identification of radioactive spots on the chromatogram was carried out with a Berthold linear β -counter.

2.5. Substrate specificities

Various structural analogues of GHB (1 mM, final concentration) were substituted for GHB in the standard reaction medium at pH 8.0 and the relative initial reaction velocities were compared (incubation time 30 min at 37°C). Dehydrogenase activity was measured by monitoring NADPH

fluorescence. For the best substrate, K_m values were determined.

3. RESULTS

3.1. pH optimum

The pH optimum for GHB dehydrogenase is about 9.0 [9]. However, for the coupled reaction (GHB dehydrogenase plus GABA-T), the optimum pH gives a sharp peak at 8.0. At a pH 9.0, GABA formation was strongly reduced (fig.1).

3.2. Apparent K_m for transformation of GHB into GABA in the presence of GHB dehydrogenase and GABA-T

In the presence of 2 mM glucuronate, 0.5 mM glutamate, 50 μ M NADP and 0.5 mM pyridoxal phosphate, 30 μ M [3 H]GHB (280 Ci/mmol), at pH 8.0, is transformed into GABA. This reaction is linear for 5 min at 37°C (fig.2) when GHB dehydrogenase (final concentration 8 units/500 μ l phosphate buffer) and GABA-T (final concentration 10.3 units/500 μ l phosphate buffer) are used. GHB dehydrogenase or GABA-T incubated alone with the same substrates does not produce

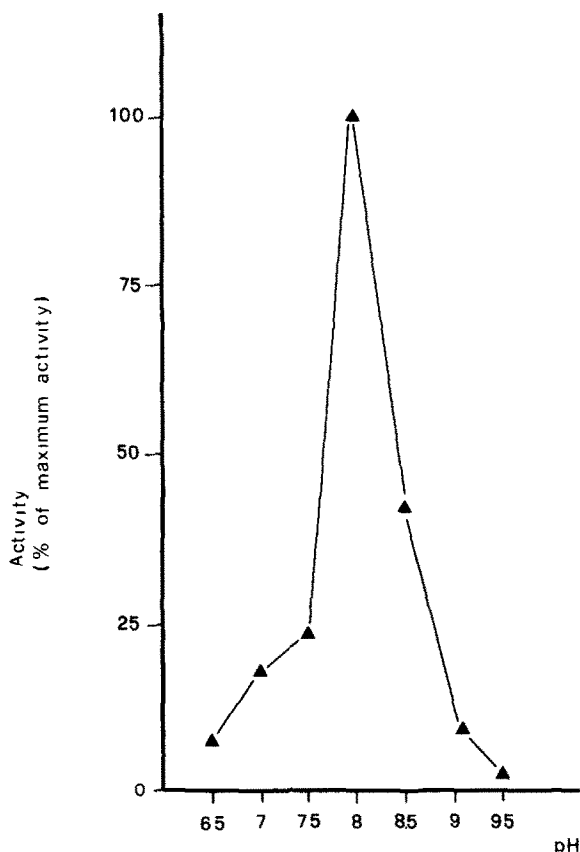


Fig.1. Determination of optimum pH for the production of GABA from [3 H]GHB in the presence of GHB dehydrogenase and purified GABA-T. Each point represents the mean of 2 experiments.

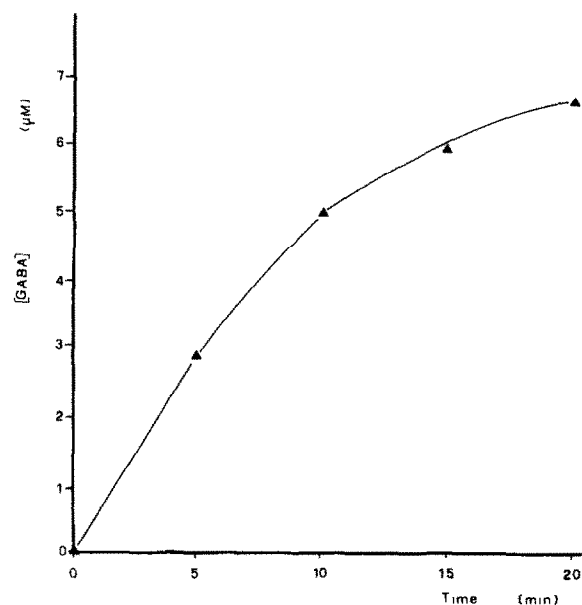


Fig.2. Time course of [3 H]GABA formation from [3 H]GHB in the presence of GHB dehydrogenase and purified GABA-T. Conditions are indicated in the text. Each point represents the mean of 2 determinations.

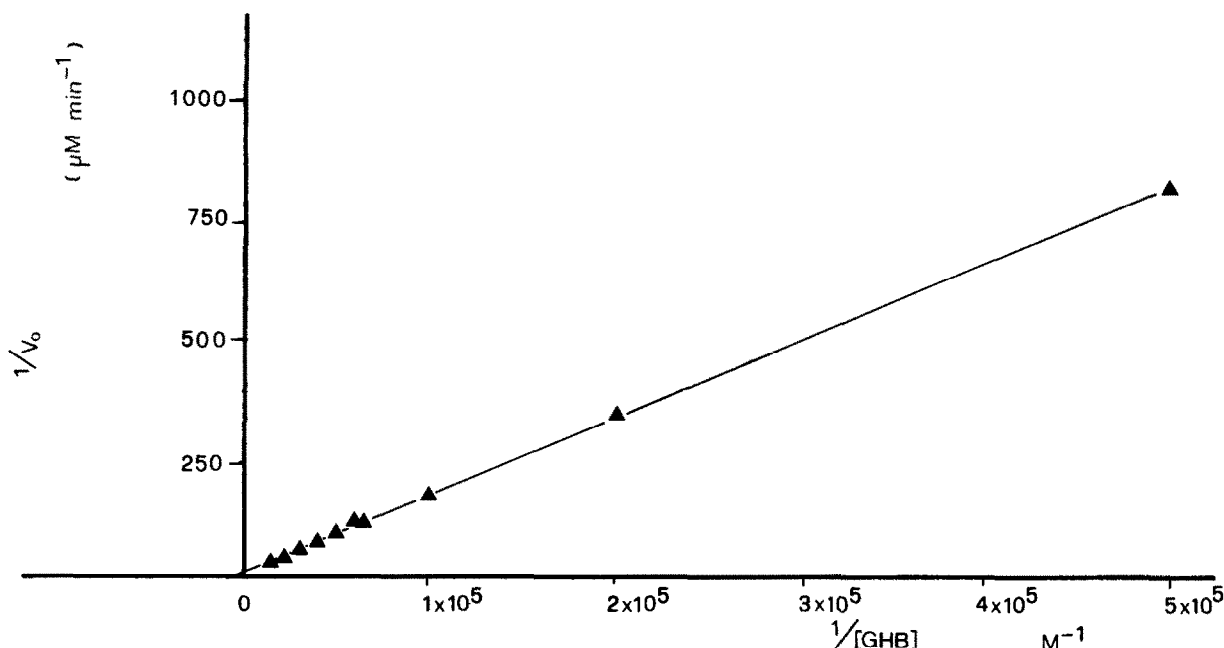


Fig.3. Apparent K_m value for GHB in the coupled enzyme reaction. Incubation conditions are given in the text. Ordinate: initial rate of [^3H]GABA formation. Each point represents the mean of 2 determinations.

[^3H]GABA. With GHB concentrations of 2–60 μM , and an incubation time of 5 min, an apparent K_m value for the coupled enzyme reaction was calculated to be $175 \pm 75 \mu\text{M}$ (fig.3). This value is about 15-fold lower than the K_m value for GHB in the presence of GHB dehydrogenase alone [9]. The K_m value determined for the coupled enzyme reaction is in good agreement with the K_m found for the multienzymatic system that transforms GHB into GABA in brain tissue slices [8]. For GHB dehydrogenase alone, the K_i for SSA ($14 \pm 2 \mu\text{M}$) was measured at pH 8.0 with SSA concentrations of 2–200 μM . The K_i for NADPH was $21 \pm 5 \mu\text{M}$ (not shown). Thus, the presence of glucuronate decreases the accumulation of NADPH and favors GHB dehydrogenation.

3.3. Substrate specificity

Table 1 shows the activities, as substrates of GHB dehydrogenase, of a series of compounds structurally related to GHB. In particular, α , β or γ substituted GHB analogues (compounds 1–17), *trans*- γ -hydroxycrotonic acid (*trans*-HCA), which may be considered as extended or semi-extended GHB analogues, and γ -substituted derivatives

(compounds 24–36) were tested, and their activities were compared to that of GHB. GHB is probably recognized by the enzymatic system in an extended or semi-extended conformation, as is shown by the high activity of *trans*-HCA. On the other hand, introduction of substituents generally led to a decrease, or complete loss of activity; only slight modifications (introduction of a methyl group) are tolerated in the α or β position (compounds 1,3,15,24).

4. DISCUSSION

Interest in studying GHB dehydrogenase is stimulated by the fact that GHB possesses several interesting neuroactive properties [18]. In many respects, it can be considered as a neurotransmitter in brain [1]. Thus, the identification of the catabolic route for GHB in brain appears to be of importance.

We have recently demonstrated that when GHB (in micromolar concentrations) is incubated under physiological conditions with rat brain slices, it is transformed rapidly into [^3H]GABA [8]. This catabolism occurs via GABA-T activity since it is

Table 1
Substrate specificities

Substrate	Relative activities	K_m (mM)
GHB	100	10 ± 3
1 α -Methyl GHB	73	
2 α -Phenyl GHB	33	
3 β -Methyl GHB	167	2.5 ± 2
4 β -Ethyl GHB	100	
5 β - <i>n</i> -Propyl GHB	nd	
6 β -Isopropyl GHB	nd	
7 β -Benzyl GHB	27	
8 β -Phenyl GHB	7	
9 γ -Methyl GHB	nd	
10 γ -Phenyl GHB	nd	
11 γ -(<i>p</i> -Chloro)phenyl GHB	13	
12 γ -(<i>p</i> -Methoxy)phenyl GHB	11	
13 γ -(<i>p</i> -Methyl)phenyl GHB	22	
14 γ -(<i>p</i> -Fluoro)phenyl GHB	26	
15 α,β -Dimethyl GHB	613	0.7 ± 0.1
16 Cyclohexanol-2-acetic acid	nd	
17 Hydroxymethyl-2-benzoate	nd	
18 α -Hydroxybutyrate	17	
19 β -Hydroxybutyrate	13	
20 δ -Hydroxyvalerate	20	
21 β -Hydroxypropane sulfonate	33	
22 γ -Hydroxybutane sulfonate	20	
23 <i>trans</i> -HCA	140	5 ± 2
24 β -Methyl <i>trans</i> -HCA	96	
25 β -Phenyl <i>trans</i> -HCA	10	
26 γ -Methyl <i>trans</i> -HCA	40	
27 γ -Cyclohexyl <i>trans</i> -HCA	13	
28 γ -Phenyl <i>trans</i> -HCA	nd	
29 γ -(<i>p</i> -Nitro)phenyl <i>trans</i> HCA	nd	
30 γ -(<i>p</i> -Fluoro)phenyl <i>trans</i> -HCA	20	
31 γ -(<i>p</i> -Chloro)phenyl <i>trans</i> -HCA	27	
32 γ -(<i>p</i> -Methyl)phenyl <i>trans</i> -HCA	24	
33 γ -(<i>p</i> -Trifluoromethyl)phenyl <i>trans</i> -HCA	26	
34 γ -(<i>o</i> -Chloro)phenyl <i>trans</i> -HCA	nd	
35 β -Methyl- γ -phenyl <i>trans</i> -HCA	nd	
36 Cyclohexanol-2-yliden acetic acid	60	

nd: not detected under our assay conditions. Each point represents the mean of 3 determinations. K_m values (for GHB dehydrogenase alone – see section 2) were determined for the best substrates

strongly reduced by GABA-T inhibitors. Moreover, transformation of glutamate into 2-ketoglutarate paralleled the synthesis of

[^3H]GABA [8]. It is therefore of interest to show that in vitro, the kinetic characteristics of pure GHB dehydrogenase coupled to purified GABA-T are consistent with the rapid transformation of micromolar amounts of GHB present in vivo in rat brain [19]. The enzyme named in the present work GHB dehydrogenase appears to be identical to the high K_m ALR₁ which has been implicated in D-glucuronate and *myo*-inositol metabolism [10]. The reduction of D-glucuronate can also be coupled with γ -hydroxybutyrate oxidation. We have demonstrated that in the presence of glucuronate, glutamate, NADP and pyridoxal phosphate, the apparent K_m of GHB for the coupled enzyme reaction ($K_m = 175 \mu\text{M}$) is very much closer to the value required to metabolize endogenous brain GHB. Moreover, in this case, the optimum pH of the polyenzymatic reaction (pH 8.0) is more consistent with the physiological conditions than the optimum pH of GHB dehydrogenase acting alone (pH 9.0). In vitro, in the conditions described, GHB at concentrations close to those existing in vivo [19] is rapidly converted to GABA despite the relatively high K_m found for the coupled reaction in vitro. It seems likely that in vivo the subcellular concentrations of GHB are higher than those found in whole brain tissue [19]. This synthesis of GABA has been confirmed by analysis after dansylation of the tritiated compound produced by the polyenzymatic reaction. This transformation of GHB into GABA in vitro, confirmed by experiments on brain slices [8] is in accordance with most previous results (review [8]) although Möhler et al. [20] working with [^{14}C]GHB administered in vivo did not observe the formation of [^{14}C]GABA.

Snead et al. [21] have reported that acute administration of ethosuximide and sodium valproate significantly increase the GHB level in brain. In view of the demonstrated inhibition of these compounds on GHB dehydrogenase, the mechanism of this increase in GHB can be explained by an inhibition of its degradation.

Therefore, it seems likely that inhibition of the degradation of GHB and an increase of this substance in brain might have anticonvulsant properties. This result is paradoxical, considering that injection of GHB to animals produces a modification in the electroencephalographic pattern (EEG) which resembles that of petit mal

epilepsy [21]. This modification in the EEG profile is antagonized by ethosuximide, trimethadione and sodium valproate [21]. To explain this apparent paradoxical phenomenon, we postulate that inhibition of GHB dehydrogenase leads to a decrease in GABA formation from GHB. This GABA pool might be involved in the negative feedback regulation of a GABAergic synapse or of a GHBergic synapse.

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