84-2; benzamide, 55-21-0; formamide, 75-12-7; 2-pyrrolidinone, 616-45-5; urethane, 51-79-6; diphenylamine, 122-39-4; *N*-benzylisobutyramide, 4774-58-7; 3,4-dimethylbenzoic acid, 619-04-5; 3,4-dichlorobenzoic acid, 51-44-5; 3,5-dichlorobenzoic acid, 51-36-5; 4-chloro-3-nitrobenzoic acid, 96-99-1; 3,5-dinitrobenzoic acid, 99-34-3; 2,4-dinitrobenzoic acid, 610-30-0; 2,6-dihydroxybenzoic acid, 303-07-1; 3,5-dinitrophenol, 586-11-8; 3-(trifluoromethyl)-4-nitrophenol, 88-30-2; 2,6-*tert*-butyl-4-nitrophenol, 728-40-5; 2,4-dinitrophenol, 51-28-5; 2,6-dinitrophenol, 573-56-8; 4-chloro-2,6-dinitrophenol, 88-87-9; thiophenol, 108-98-5; oxalic acid, 144-62-7; malonic acid, 141-82-2; succinic acid, 110-15-6; glutaric acid, 110-94-1; adipic acid, 124-04-9; *o*-phthalic acid, 88-99-3.

Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase by 4-Amino-5-hexynoic Acid (γ-Ethynyl GABA)†

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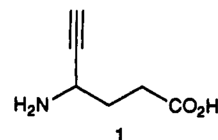
Abstract: γ-Aminobutyric acid (GABA) aminotransferase is a pyridoxal phosphate (PLP) dependent enzyme that catalyzes the degradation of γ-aminobutyric acid. The inactivation of GABA aminotransferase has been shown to be an important treatment for epilepsy. The mechanism of inactivation of GABA aminotransferase by γ-ethynyl GABA, a mechanism-based inactivator of GABA aminotransferase that shows anticonvulsant activity in animal models, is investigated in this paper. Although it appears that azaallylic isomerization (the normal catalytic pathway for substrates) of the PLP-bound inactivator occurs (pathway a, Scheme VII), little or no inactivation of the enzyme results from that isomerization. Essentially all of the inactivation is derived from a propargylic isomerization (pathway b) to the allenamine bound PLP adduct **10**, which undergoes nucleophilic attack at two different sites. It appears that an active site lysine residue reacts at the Schiff base to give the free enamine **18** (pathway c) or reacts at the allene to give the enzyme and cofactor bound enamine **12** (pathway d); possible attack by water (pathway e) would lead to metabolite **26**. The enamine **18** does not become attached to the PLP (Scheme III, pathway a), but a small amount (5–10%) may become attached to the enzyme at a site other than at lysine (**9**, Scheme III, pathway b). Adduct **9** also could be derived from azaallylic isomerization of the inactivator-PLP Schiff base followed by conjugate addition to the acetylene by an active site nucleophile other than a lysine residue (Scheme I). Mostly **18** is released into solution to give **27** (Scheme VII). Adduct **12** is believed to be a transient intermediate that partitions between conversion to metabolite **26** (Scheme VII, pathway f) and conversion to a more stable isomer (**13**, pathway g). Upon denaturation, adduct **13** partitions equally (Scheme VIII) between release of metabolite **26** and the formation of another covalent adduct (**17**). Isolation and identification of the amine and nonamine metabolites produced during processing of γ-ethynyl GABA showed that, on average, for every 13 molecules of γ-ethynyl GABA that are turned over, 1.2 undergoes transamination (pathway a, Scheme VII), 2.6 are metabolized to **27** (pathways b and c), 8.2 are converted to **26** (pathways b, d, and f and/or pathways b and e), and 1.0 becomes attached to the enzyme, almost all, as **13** (pathways b, d, and g), but possibly 5–10% as **9** (X ≠ Lys) as discussed above.

Epilepsy is a disease characterized by convulsive seizures that result from repeated and excessive electrical discharges in the neurons. In its many forms, epilepsy may affect as much as one percent of the world population.[1] Although the etiological mechanism leading to the electrical discharges in convulsive epilepsy is not yet understood, it is known that γ-aminobutyric acid (GABA) is a major neurochemical component in seizure inhibition. Indeed, convulsions occur when GABA levels fall below a certain threshold level in the brain,[2-6] and direct injection of GABA into the brain causes the convulsions to cease.[7]

The catabolism of GABA is catalyzed by the pyridoxal 5'-phosphate (PLP)-containing and α-ketoglutarate-dependent enzyme, GABA aminotransferase (EC 2.6.1.19), to yield succinic semialdehyde and L-glutamate. An important treatment of epileptic convulsions has focused on raising brain GABA levels by the irreversible inactivation of GABA aminotransferase. So far, this has been the most effective way to increase the presynaptic levels of GABA.[8]

γ-Ethynyl GABA (4-amino-5-hexynoic acid, **1**), one of the first rationally designed mechanism-based inactivators,[9] was shown to



be a potent inactivator of GABA aminotransferase.[10-12] In vivo, γ-ethynyl GABA causes a long-lasting decrease in mouse brain

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† Dedicated to Professor Robert H. Abeles, with admiration for his creative contributions to enzymology, on the occasion of his 65th birthday.

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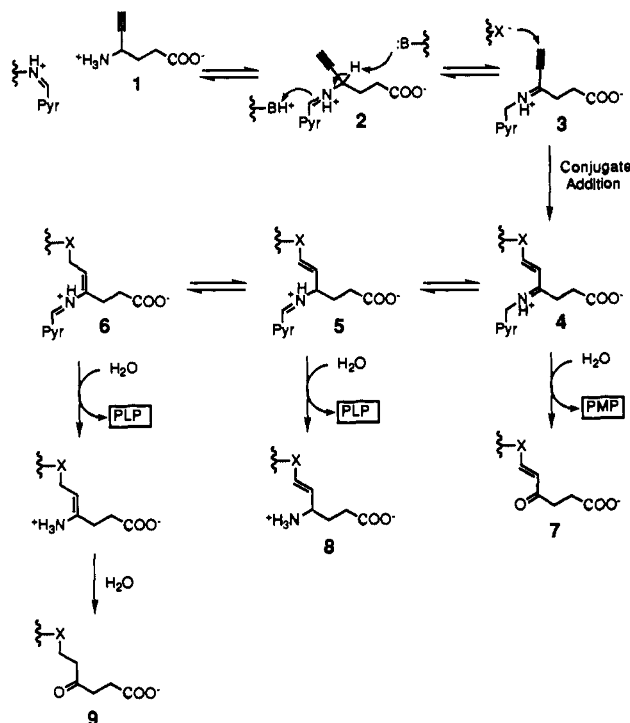
Scheme I. Potential Azaallylic Mechanism of Inactivation of GABA Aminotransferase by γ-Ethynyl GABA[a]



[a] The carbon–carbon double bonds shown are not meant to imply any specific stereochemistry. Pyr represents the pyridine ring of PLP.

GABA aminotransferase activity in a dose-dependent manner when administered peripherally and has been shown to increase synaptosomal GABA levels.[13] Studies with genetically epilepsy-prone gerbils have shown γ-ethynyl GABA to be a potent anticonvulsant agent.[14] The stereochemistry of γ-ethynyl GABA is important to its in vitro and in vivo activity. The (*R*)-isomer is a selective inactivator of GABA aminotransferase, but the (*S*)-isomer inactivates both brain GABA aminotransferase and the brain enzyme that biosynthesizes GABA from L-glutamate, namely, L-glutamic acid decarboxylase.[15] As a result of this effect by the (*S*)-enantiomer to decrease the production of GABA, the racemic mixture should not be as effective an anticonvulsant agent as is the (*R*)-enantiomer alone.[16]

Four mechanisms by which γ-ethynyl GABA could inactivate GABA aminotransferase are based on the well-accepted mechanism for pyridoxal 5′-phosphate (PLP)-dependent aminotransferases (see Scheme III in the following paper in this issue) and on earlier work with γ-ethynyl GABA.[10-12] One of these mechanisms (Scheme I) involves the formation of an imine between γ-ethynyl GABA and the cofactor (**2**) followed by normal azaallylic tautomerization to give **3**. This intermediate is a reactive Michael acceptor, and attack of an active-site nucleophile would give a covalently modified inactive enzyme, which could exist as one of (or an equilibrium mixture of) three tautomers (**4**–**6**); upon hydrolysis these would be converted into **7**–**9**, respectively.

Another possible mechanism (Scheme II) is one in which the enzyme, after formation of the imine between inactivator and cofactor (**2**), catalyzes a propargylic isomerization to the reactive conjugated allene **10**. Michael addition by an active-site nucleophile would give the covalently modified inactive enzyme as either **11**, **12**, or **13** or an equilibrium mixture of these.

A third possibility is a mechanism analogous to the enamine alkylation inactivation mechanism initially reported by Metzler and co-workers[17] for the inactivation of glutamate decarboxylase and aspartate aminotransferase by serine *O*-sulfate. That is, after propargylic tautomerization to the allene **10** a transimination with the active-site lysine would give the allenamine, **18** (Scheme III). Without leaving the active site, **18** could act as a nucleophile to add to the cofactor (pathway a), giving the covalently modified enzyme-bound cofactor (**19**) or the isomerized adduct (**20**). The allenamine (**18**) also could be protonated to give the electrophilic species **22** (pathway b), which could alkylate an active-site nucleophile and give the same adduct (**9**) as was obtained in Scheme I. The mechanism shown in Scheme III, pathway b, was suggested as the pathway for the formation of **9** (X = Cys) when γ-ethynyl GABA inactivates ornithine aminotransferase.[18]

Here we report our detailed studies directed at the elucidation of the mechanism of inactivation of GABA aminotransferase by γ-ethynyl GABA and the metabolism of γ-ethynyl GABA by GABA aminotransferase.

Table I. Binding Studies of [2-^{3}H]-γ-Ethynyl GABA with GABA Aminotransferase[a]

pH	denature with 6 M Urea?	temp, °C	equivalents bound
7.4	no	4	1.2 ± 0.19
7.4	no	25	1.0 ± 0.03
3.1	yes	4	0.7 ± 0.06
3.1	yes	25	0.6 ± 0.05
3.1	yes	50	0.5 ± 0.04
7.4	yes	4	1.3 ± 0.01
7.4	yes	25	0.9 ± 0.03
7.4	yes	50	0.5 ± 0.01
9.5	yes	4	1.1 ± 0.09
9.5	yes	25	0.6 ± 0.16
9.5	yes	50	0.6 ± 0.08

[a] Each result is the average of at least three experiments at each condition. See Experimental Section for details.

Results

Studies on the Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by γ-Ethynyl GABA. GABA aminotransferase that had been reconstituted with [^{3}H]PLP was inactivated with γ-ethynyl GABA and then denatured and precipitated with trichloroacetic acid. Greater than 95% of the radioactivity was released from the protein under these conditions. A control in which no γ-ethynyl GABA was present released 98% of its radioactivity under these conditions. Scintillation counting of the dialysate resulting from dialysis of the γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase showed no tritium as compared to a noninactivated control. Under these conditions, a level of 5% of the total tritium would have been detected.

The γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase was denatured with $NaDodSO_4$ at pH 2 to release the tritiated products for HPLC analysis. Under these conditions, HPLC analysis of the γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase showed that all of the radioactivity coeluted with PLP (Figure 1). A control containing no inactivator also released its radiolabel as 100% PLP. A control in which [^{3}H]PLP GABA aminotransferase was incubated with GABA in the absence of both γ-ethynyl GABA and α-ketoglutarate showed that the radioactivity was released as a 82:18 mixture of [^{3}H]PMP and [^{3}H]PLP.

Stability of the Bond Formed between Enzyme and Inactivator. When GABA aminotransferase was inactivated with [2-^{3}H]-γ-ethynyl GABA, there was between 1.0 and 1.2 equiv of radiolabel incorporated into the enzyme (Table I). When the inactivated enzyme was denatured with urea, however, some of the radiolabel

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Scheme II. Potential Allylic Mechanism of Inactivation of GABA Aminotransferase by γ-Ethynyl GABA[a]

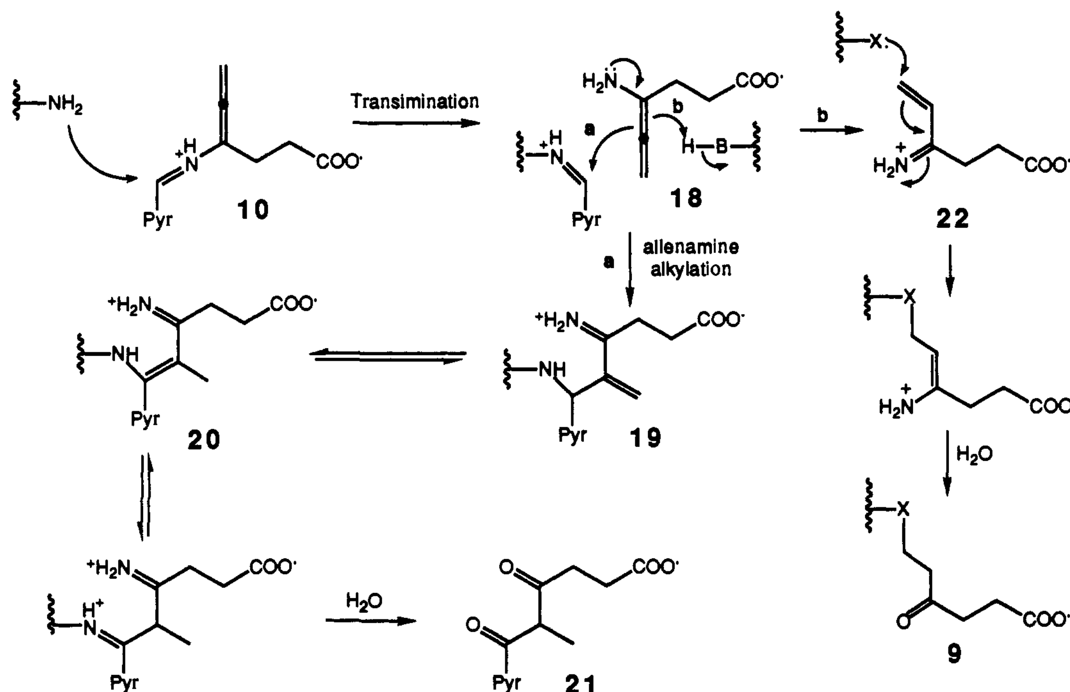
[a] The carbon–carbon double bonds shown are not meant to imply any specific stereochemistry. Pyr represents the pyridine ring of PLP.

was released from the enzyme as shown in Table I. When the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme was denatured and precipitated with trichloroacetic acid, 0.4 equiv of radiolabel remained bound to the enzyme. Incubation of the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme with $NaBH_4$ prior to denaturation and precipitation with trichloroacetic acid showed 0.8 equiv of radiolabel bound to the enzyme.

Identification of the Radiolabeled Products Released from [2-^{3}H]-γ-Ethynyl GABA-Inactivated GABA Aminotransferase upon Denaturation. The first experiment was designed to detect the formation of **27** (Scheme IV) after denaturation and precipitation of the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme with trichloroacetic acid followed by extraction of the supernatant with dichloromethane. No radioactivity was detected in the organic layer. A control experiment containing synthetically prepared **27** under the identical conditions showed it to be nearly completely extracted into the organic layer as determined by HPLC analysis. In a second experiment, HPLC of the $NaDodSO_4$ dialysate (Figure 2) showed no **27**, only amino acids.

The radiolabeled products released upon denaturation and precipitation of the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme were dansylated and identified by HPLC analysis. By this analysis, 71% of the radioactivity coeluted with dansylated **26**, 16% with dansylated γ-ethynyl GABA, and none with dansylated **24** (Figure 3). Direct paper chromatography analysis of the radiolabeled products released upon denaturation and precipitation of the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme showed that 82% of the radioactivity cochromatographed with **26**, 18% with

Scheme III. Potential Allenamine Mechanisms of Inactivation of GABA Aminotransferase by γ-Ethynyl GABA[a]



[a] The carbon–carbon double bonds shown are not meant to imply any specific stereochemistry. Pyr represents the pyridine ring of PLP.

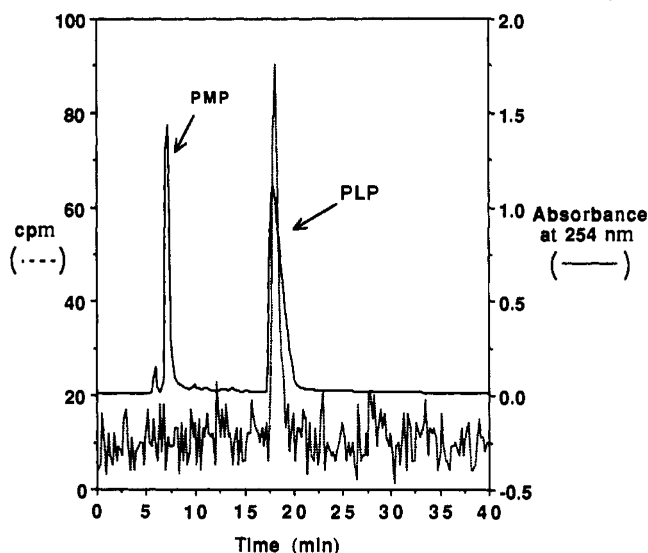


Figure 1. HPLC analysis of tritiated products released upon denaturation of γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase. [^{3}H]PLP GABA aminotransferase (1.32 nmol) in a total volume of 780 μL was inactivated with γ-ethynyl GABA as described in the Experimental Section. A control containing no inactivator was also prepared along with a control containing 4 mM GABA and neither inactivator nor α-ketoglutarate. The samples were dialyzed separately in the dark for 6 h at 25 °C against 500 mL of sodium phosphate buffer containing 0.25 mM β-mercaptoethanol at pH 7.0. The samples were titrated to pH 2 with HCl, and then solid $NaDodSO_4$ was added to make them 1% (w/v) in $NaDodSO_4$. A 470-μL aliquot was added with 30 μL of a standard mixture of PLP and PMP (1 mg each/mL) and analyzed by reversed-phase HPLC using an Alltech Econosil C18 10 μ 4.6 × 250 mm column. The solvent system used was 100 mM sodium phosphate pH 7.0. UV absorption was monitored at 254 nm. Radioactivity was measured with a Radiomatic Instruments Flo-One/Beta Model CR radioactivity detector using Packard Radiomatic Flo-Scint II scintillation cocktail. Under these HPLC conditions, PMP and PLP had retention times of 7 and 18 min, respectively.

γ-ethynyl GABA, and none with **24**.

[^{3}H]$NaBH_4$ Reduction of γ-Ethynyl GABA-Inactivated and Denatured GABA Aminotransferase. [^{3}H]$NaBH_4$ reduction of the γ-ethynyl GABA-inactivated and denatured enzyme resulted in tritium incorporation of 27.49 mCi/mmol (σ = 0.21, three experiments). [^{3}H]$NaBH_4$ reduction of a control containing denatured enzyme that had not been inactivated results in 26.54 mCi/mmol of tritium incorporation (σ = 0.53, three experiments).

As a control, a model reduction of a related ketone was carried out in order to account for any tritium isotope effect on reduction of the enzyme-bound adduct. [^{3}H]$NaBH_4$ reduction of 6-amino-4-oxohexanoic acid hydrochloride (**28**, Scheme V) was carried out. This reaction resulted in the incorporation of 1.90 mCi/mmol tritium into the lactone (**29**). If 1.90 mCi/mmol is set equal to 1.0 equiv of tritium, then the tritium incorporation into the γ-ethynyl GABA-inactivated enzyme is 14.47 ± 0.11 equiv (27.49/1.90) and the tritium incorporation into the uninactivated control is 13.97 ± 0.28 equiv (26.54/1.90). The difference suggests that an additional 0.5 equiv of tritium is incorporated into the inactivated enzyme that does not become incorporated into the active enzyme.

Acid and Base Stability of the [2-^{3}H]-γ-Ethynyl GABA-Inactivated and Denatured GABA Aminotransferase Adduct. Incubation of the [2-^{3}H]-γ-ethynyl GABA-inactivated and denatured enzyme at pH 1.8 (55 °C) left 0.32 equiv of radiolabel still bound to the enzyme. At pH 13 (76 °C) 0.38 equiv of radiolabel remained bound to the enzyme. A control which was neither heated nor treated with base retained 0.49 equiv of radiolabel.

Periodate Oxidation of 6-Amino-4-oxohexanoic Acid (28) and 5-Amino-4-oxopentanoic Acid (30). 6-Amino-4-oxohexanoic acid (**28**) and 5-amino-4-oxopentanoic acid (**30**) were oxidized with sodium periodate. HPLC analysis showed that both **28** and **30** produced a succinic acid peak comparable in size to that expected for stoichiometric conversion.

Periodate Oxidation of [2-^{3}H]-γ-Ethynyl GABA-Inactivated and Denatured GABA Aminotransferase. [2-^{3}H]-γ-Ethynyl GABA-inactivated GABA aminotransferase that had been denatured and dialyzed was oxidized with sodium periodate. Under these conditions, only 0.16 equiv of radiolabel remained bound to the enzyme. A control, which had not been oxidized, retained 0.57 equiv of radiolabel. HPLC analysis of the oxidized sample showed the only radiolabeled product to be succinic acid.

Periodate Oxidation of $NaBH_4$-Reduced 6-Amino-4-oxohexanoic Acid (28) and 5-Amino-4-oxopentanoic Acid (30). Compounds **28** and **30** were reduced with sodium borohydride and then oxidized with sodium periodate. The production of succinic semialdehyde was analyzed by first adding 2,4-dinitrophenylhydrazine

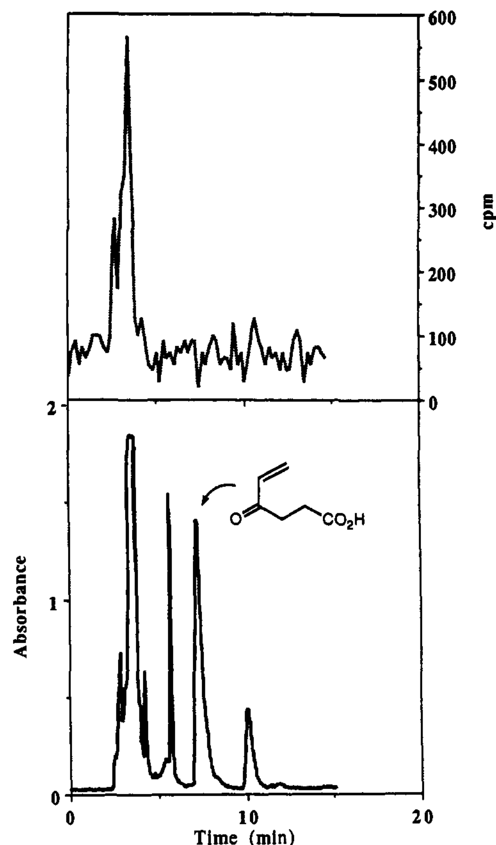


Figure 2. HPLC evidence against **27** as the product released by hydrolysis of the unstable adduct. GABA aminotransferase (6.6 nmol) in a total volume of 468 μL was inactivated with [2-^{3}H]-γ-ethynyl GABA as described in the Experimental Section. After dialysis against 100 mM sodium phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol for 7 h at 4 °C (changing dialysate once), it was microdialyzed against 5 mL of 25 mM sodium tetraborate, pH 9.5, containing 0.1% NaDodSO$_4$ for 24 h at 25 °C. A portion (500 μL) of the dialysate was spiked with **27** and was analyzed by HPLC as above. Radioactivity was measured with a Radiomatic Flo-Scint III scintillation cocktail (ratio of cocktail to HPLC eluate = 4:1).

reagent and then extracting with $CHCl_3$ so that any succinic semialdehyde would be isolated as its 2,4-dinitrophenylhydrazone. HPLC analysis of reduced and oxidized **30** showed a large peak at 10 min indicating the formation of an amount of succinic semialdehyde comparable in amount to that expected for stoichiometric conversion. Derivatization and HPLC analysis of reduced and oxidized **28** showed no peak at 10 min indicating that no succinic semialdehyde was produced. Controls of both of these oxidations in which the compounds were reduced with $NaBH_4$ but not oxidized with periodate were prepared, and HPLC analysis showed neither to have a peak at 10 min.

Periodate Oxidation of [2-^{3}H]-γ-Ethynyl GABA-Inactivated, Denatured, and $NaBH_4$-Reduced GABA Aminotransferase. [2-^{3}H]-γ-Ethynyl GABA-inactivated GABA aminotransferase that had been denatured, dialyzed, and reduced with $NaBH_4$ was oxidized with periodate. Under these conditions, only 0.06 equiv of radiolabel remained bound to the enzyme. A control that had not been oxidized retained 0.52 equiv of radiolabel. HPLC analysis of the oxidized sample showed the only radiolabeled product to be succinic semialdehyde (Figure 4).

Turnover Products Formed During [2-^{3}H]-γ-Ethynyl GABA Inactivation of GABA Aminotransferase. GABA aminotransferase that had been inactivated with γ-ethynyl GABA in the presence of [5-^{14}C]-α-ketoglutarate produced 1.2 equiv of [5-^{14}C]glutamate per enzyme molecule inactivated, indicating that 1.2 transamination events occurred per inactivation event.

The radiolabeled nonamine turnover products formed during [2-^{3}H]-γ-ethynyl GABA inactivation of GABA aminotransferase were separated from excess inactivator and any amine turnover products by ion exchange chromatography. It was found that 3.8 equiv of radiolabel, after subtracting a control containing no enzyme, did not remain bound to the ion-exchange column (nonamine radioactivity). HPLC analysis showed that 1.9 of these 3.8 equiv coeluted with **27**. Nearly all of the remaining radioactivity eluted in peaks at 4 and 8 min whose identities are unknown.

The radiolabeled excess inactivator and amine turnover products formed during [2-^{3}H]-γ-ethynyl GABA inactivation of GABA aminotransferase were isolated by cation-exchange chromatography. Dansylation and HPLC analysis showed, after subtracting out a control that contained no enzyme, only one radioactive product which coeluted with **26**. The amount of radioactivity in this peak corresponded to 8.2 equiv per inactivation event.

Changes in the Optical Spectrum of GABA Aminotransferase upon Inactivation with γ-Ethynyl GABA. When γ-ethynyl GABA was added to a solution of the enzyme in the presence of α-ketoglutarate, a rapid increase in the absorbance at 330 nm, as monitored by UV–vis spectrophotometry, was accompanied by a rapid decrease in the absorbance at 415 nm. Thereafter, the spectrum did not appreciably change during the course of the inactivation.

Discussion

If the inactivation of GABA aminotransferase by γ-ethynyl GABA proceeds through an enamine alkylation mechanism (Scheme III, pathway a), the cofactor in the inactivated enzyme would be irreversibly attached to both the enzyme and the inactivator. Since essentially all of the radiolabel is released from a denatured sample of γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase, an allenamine-alkylated enzyme could not have the structure **19** (tritium still would be at C-7 of PLP and would appear attached to the enzyme). However, tautomerization of **19** to **20** would result in an enamine adduct that does not contain any tritium. Adduct **20** also could be released eventually as the unusual PLP adduct **21** which would not contain tritium. In either case, however, the tritium would be released as tritiated water. Scintillation counting of the dialysate resulting from dialysis of the γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase showed no radioactivity released as compared to a noninactivated control. Under these conditions loss of tritiated water from the cofactor at a level of 5% of the total radiolabel would have been detected. This shows that the inactivation mechanism does not proceed through an allenamine alkylation mechanism. Recently, John and co-workers[18] isolated the peptides from a tryptic digestion of ornithine aminotransferase inactivated by γ-ethynyl GABA. These studies indicated that there are several different adducts produced during inactivation. The major radioactive adduct was a peptide that had both the inactivator and the cofactor attached to Lys-292, the lysine to which the PLP is attached in the native enzyme, and it was concluded that an enamine adduct similar to **19** was responsible. If that is the case, then there is at least one large difference in the inactivation of GABA aminotransferase and ornithine aminotransferase by γ-ethynyl GABA.

There are three other pathways, leading to seven other possible outcomes, that can be considered. Upon denaturation of five of these adducts (**5**, **6**, **12**, and **13** in Schemes I and II and from pathway b in Scheme III) the cofactor would be released as PLP. Denaturation of two of the adducts (**4** and **11**) results in the release of the cofactor as PMP. Since trichloroacetic acid interferes with the HPLC analysis of the released tritiated products, NaDodSO$_4$ at pH 2 was used to denature the protein and to release the tritiated products for HPLC analysis. Under these conditions, HPLC analysis of a control of [^{3}H]PLP-reconstituted GABA aminotransferase containing no inactivator showed that, as expected, 100% of its released radiolabel coeluted with PLP. Another control in which [^{3}H]PLP-reconstituted GABA aminotransferase was incubated with GABA in the absence of γ-ethynyl GABA

(18) De Biase, D.; Simmaco, M.; Barra, D.; Bossa, F.; Hewlins, M.; John, R. A. *Biochemistry* **1991**, *30*, 2239–2246.

or α-ketoglutarate showed the radioactivity released as an 82:18 mixture of [^{3}H]PMP and [^{3}H]PLP. Since GABA in this control would be expected to convert the cofactor completely into PMP, the 18% of [^{3}H]PLP present in this control may be derived either from inactive [^{3}H]PLP-reconstituted GABA aminotransferase formed during the reconstitution process[19] or may be due to the fact that in the absence of succinic semialdehyde dehydrogenase, the presence of GABA is not sufficient to convert the cofactor completely into the PMP form because of feedback inhibition of the enzyme by the product succinic semialdehyde.[20] HPLC analysis of γ-ethynyl GABA-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase that had been denatured with $NaDodSO_4$ showed all of its released radiolabel was in the form of PLP (Figure 1). Because only PLP is released, the possible enzyme adducts that would release PMP, namely **4** and **11**, can be discounted.

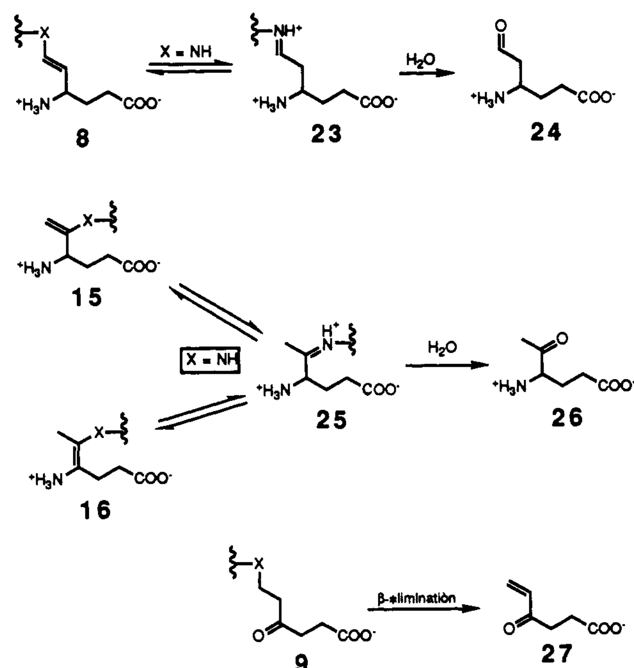
In order to differentiate the five possibilities associated with release of PLP after denaturation, the γ-ethynyl GABA was tritium-labeled, and the chemical properties of the adduct formed upon inactivation were investigated. When GABA aminotransferase was inactivated with [2-^{3}H]-γ-ethynyl GABA, about 1.0 equiv of radiolabel became incorporated into the enzyme (see Table I) resulting from covalent modification of the active site. When the inactivated enzyme was denatured with urea, however, some of the radiolabel was released from the enzyme. The amount released totaled no more than about 50% of the radiolabel, even after 24 h at 50 °C. When the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme was denatured and precipitated with trichloroacetic acid, again only about one-half (0.4 equiv) of radiolabel remained bound to the enzyme. These results suggest that there may be two or more different adducts formed upon inactivation of GABA aminotransferase by γ-ethynyl GABA: one adduct that is stable to denaturation, to pH 1, and to temperatures of 50 °C, and another adduct that is not stable to these conditions and, in fact, is released by denaturation of the enzyme at 25 °C at high or low pH or at 50 °C at neutral pH.

All of the possible adducts remaining could further degrade to become detached from the enzyme, especially if the active site nucleophile is the ε-amino group of a lysine residue. Since John and co-workers found that the major peptide isolated after inactivation of ornithine aminotransferase by γ-ethynyl GABA[18] and human liver GABA aminotransferase by γ-vinyl GABA[21] was a lysine adduct, it is reasonable that, at least, one of the adducts in the present study is a lysine adduct. If the active-site nucleophile is the ε-amino moiety of a lysine residue, then adducts **8**, **15**, and **16** would be enamines that could easily tautomerize to the corresponding imines and be cleaved from the enzyme by hydrolysis. As shown in Scheme IV, upon hydrolysis, **8** would release 4-amino-6-oxohexanoic acid (**24**) and **15** and **16** would release 4-amino-5-oxohexanoic acid (**26**). Another possible unstable adduct formed after denaturation is adduct **9** where the active site nucleophile would be a good leaving group such as in a cysteine, tyrosine, or histidine residue; β-elimination under denaturing conditions would release 4-oxo-5-hexenoic acid (**27**, Scheme IV).

Initial evidence to support the existence of one or more of these possibilities as the adduct not stable to denaturation came from the reduction of [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme with $NaBH_4$, in which case 0.8 equiv of radiolabel remained bound to the enzyme upon denaturation and precipitation with trichloroacetic acid. The fact that most of the adduct previously not stable to denaturation could be stabilized by reduction with $NaBH_4$ suggests the presence of an imine bond (as in **23** and **25**) or a ketone (as in **9**). Reduction of any of these three adducts would produce an adduct stable to trichloroacetic acid denaturation.

The more definitive proof for the structure of the unstable adduct was obtained by identification of the radiolabeled product

Scheme IV. Hydrolysis of Possible Unstable Adducts[a]



[a] The carbon-carbon double bonds shown are not meant to imply any specific stereochemistry.

released (**24**, **26**, or **27**) from the inactivated enzyme upon denaturation. The first experiments carried out were designed to detect the formation of radiolabeled **27**. No radioactivity in the supernatant after denaturation and precipitation of the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme could be extracted with dichloromethane, whereas a control experiment containing synthetically prepared **27** under the identical conditions showed that it was possible to extract the observed amount of **27**. Furthermore, HPLC of the denatured enzyme showed that none of the keto acid **27** was present, only radioactive amino acids (Figure 2). Therefore, **6** (or **9**, Scheme I) is not the structure of the adduct that is unstable to denaturation, although the adduct that is stable to denaturation could still have this form, if the modified amino acid was not a sufficiently good leaving group to undergo β-elimination under these conditions.

The identity of the released product from the unstable adduct was determined by HPLC analysis of the dansyl derivative of the product. By this analysis, 71% of the radioactivity coeluted with dansyl **26**, 16% with dansyl γ-ethynyl GABA, and none with dansyl **24** (Figure 3). The radioactive peak coeluting with dansyl γ-ethynyl GABA becomes smaller the longer the sample is dialyzed before trichloroacetic acid treatment, suggesting that this peak comes from incomplete dialysis and not from the degradation of some covalent adduct upon trichloroacetic acid treatment. The small amount of radioactivity (13%) eluting in the void volume is probably due to amino acids that were not completely dansylated (i.e., the derivatization did not go to 100% completion). To verify this result and to avoid using any derivatization step, the released radiolabeled products were analyzed by paper chromatography. Using this method, it was found that 82% of the radioactivity cochromatographed with **26**, 18% with γ-ethynyl GABA, and none with **24**. This is the same ratio found using HPLC analysis of the dansyl derivatives, if the radioactivity eluting in the void volume is ignored. These results indicate that the unstable adduct is derived from either tautomer **15** or **16** (Scheme II), resulting from nucleophilic attack of an active-site lysine residue on the allene intermediate **10**. The existence of a lysine residue acting as the active-site nucleophile is indicated by the instability of this adduct to denaturing conditions and its stability after $NaBH_4$ treatment, which suggests that the adduct is in the form of an enamine after release of cofactor. The possibility that serine, cysteine, or tyrosine residues act as the nucleophile, which would produce either a vinyl

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(20) Alston, T. A.; Porter, D. J. T.; Wheeler, D. M. S.; Bright, H. J. *Biochem. Pharmacol.* **1982**, *31*, 4081.
(21) DeBiase, D.; Bolton, J. B.; Barra, D.; Bossa, F.; John, R. A. *Biochimie* **1989**, *71*, 491.

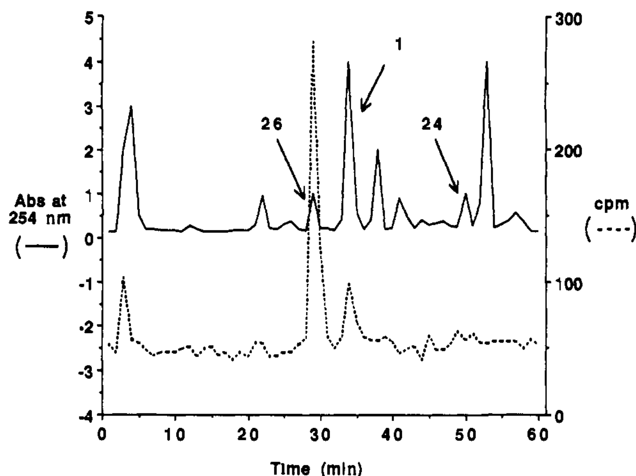
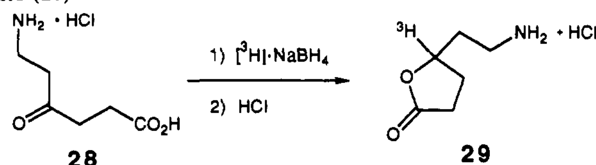


Figure 3. HPLC analysis of the dansylated derivative released upon denaturation of [2-^{3}H]-γ-ethynyl GABA-inactivated GABA aminotransferase. GABA aminotransferase (15.1 nmol) in a total volume of 1.18 mL was inactivated with [2-^{3}H]-γ-ethynyl GABA, dialyzed, precipitated with trichloroacetic acid, and centrifuged as described under trichloroacetic acid precipitation of GABA aminotransferase inactivated with [2-^{3}H]-γ-ethynyl GABA in the Experimental Section. A portion (3000 dpm) of the resulting supernatant was diluted 20-fold with water, added with a solution containing **24**, **26**, and γ-ethynyl GABA standards (1 mg each/mL, 15 μL), and applied to a Dowex 50 × 8-400 column (0.5 × 5 cm). The column was washed with 3 mL of water, and the radioactivity of the eluate from this application and washing was measured by liquid scintillation counting to be less than 10% of the radioactivity that was applied to the column. The column was then eluted with 10 mL of 1.5 M HCl, and this eluate was lyophilized and redissolved in 40 mM Li_2CO_3 (160 μL). A portion (80 μL) of this was then added to 100 μL of 20 mM dansyl chloride in acetonitrile and allowed to react for 1 h in the dark. This derivatized solution was then analyzed by reversed-phase HPLC using a Rainin Dynamax-60A 8 μM C18 4.6 × 250 mm column with a 0.6% HOAc, 0.03% triethylamine/methanol gradient (5–100% methanol over 60 min, flow rate 1.0 mL/min, detection of 254 nm). Fractions were collected manually every 1.0 min and analyzed for radioactivity by liquid scintillation counting. Under these HPLC conditions, the dansylated derivatives of **26**, γ-ethynyl GABA, and **24** had retention times of 29, 34, and 50 min, respectively.

ether, vinyl thioether, or phenyl vinyl ether, is excluded because all of these bonds would be expected to require more vigorous conditions for hydrolysis.[22] The lysine that is attached to PLP in the native enzyme and, presumably, acts as the active-site base in the azaallylic tautomerization during the normal catalytic mechanism would be ideally suited to act as the active-site nucleophile, as was observed for the inactivation of ornithine aminotransferase by γ-ethynyl GABA[18] and the inactivation of human liver GABA aminotransferase by γ-vinyl GABA.[21]

The possible adducts remaining after hydrolysis of the cofactor that may be stable to denaturation are **8**, **9** (Schemes I and III), **15**, and **17** (Scheme II); adducts **8** and **15** would be stable to denaturation only if the active-site nucleophile is some residue other than lysine (e.g., X = His, Ser, Cys, Tyr). To test the possibility of the denatured stable adduct having a structure such as **8** or **15**, the [2-^{3}H]-γ-ethynyl GABA-inactivated enzyme was subjected to conditions (pH 1.8, 55 °C) known to hydrolyze vinyl ethers, vinyl thioethers, and phenyl vinyl ethers.[22] Under these conditions, 0.32 equiv of radiolabel still remained bound to the enzyme. The fact that most of the denatured stable adduct remains bound under these conditions indicates that if it had the general structure of **8** or **15**, the active-site nucleophile could only be a histidine residue (i.e., X = imidazole), since *N*-vinylimidazoles are very stable and cannot be hydrolyzed in this manner. Aspartate aminotransferase is considered to be very similar to GABA aminotransferase in both mechanism and structure.[23] If it is assumed that both enzymes have similar active-site structures,

Scheme V. Sodium Borotritide Reduction of 6-Amino-4-oxohexanoic Acid (28)



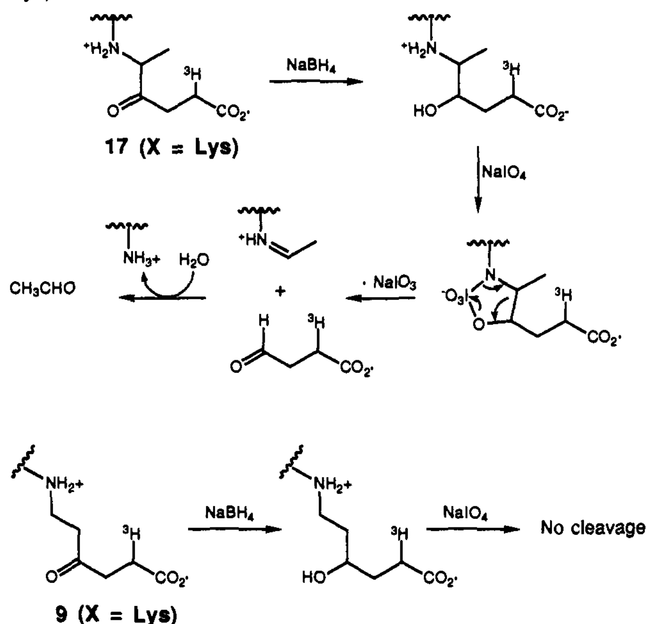
except for small variations that affect substrate specificity, then it would be unlikely that a structure such as **8** or **15** (X = histidine) results from inactivation and denaturation, since there is no histidine residue in the active site of aspartate aminotransferase (and presumably of GABA aminotransferase) that would be situated close enough to act as an active-site nucleophile.[24] A more likely candidate for the nucleophilic active-site residue is the lysine residue that has been implicated in the formation of the adduct that is unstable to denaturation (**15** or **16**).

The possibility of the denatured stable adduct having the general structure **9** (Schemes I and III) or **17** (Scheme II) was tested in several ways. Since both of these adducts are ketones, whereas **8** and **15** (X = His) are not, treatment of the stable adduct with [^{3}H]$NaBH_4$ should lead to incorporation of 0.5 equiv of tritium if **9** or **17** is the adduct, but not if **8** or **15** is the adduct. In order to model the reduction of the putative enzyme adducts so that the amount of tritium incorporation corresponding to 1 equiv of reduced ketone could be calculated, [^{3}H]$NaBH_4$ reduction of 6-amino-4-oxohexanoic acid hydrochloride (**28**, Scheme V) to the tritiated lactone (**29**) was carried out. Using this approach, tritium incorporation into the γ-ethynyl GABA-inactivated and denatured enzyme averaged 14.47 equiv (three experiments), whereas a noninactivated control averaged only 13.97 equiv. Since [^{3}H]-$NaBH_4$ in excess can reduce peptide bonds,[25] the high amount of nonspecific tritium incorporation in both the γ-ethynyl GABA-inactivated sample and the noninactivated control is expected. However, the consistency of these experiments suggests that this difference in tritium incorporation in the inactivated and control enzyme (0.5 equiv) is real. Under the conditions of this experiment, the adduct that is unstable to denaturation would have been removed prior to [^{3}H]$NaBH_4$ reduction and only the stable adduct, comprising 0.4–0.6 equiv, would remain. Since there is, on average, 0.5 equiv more incorporated into the γ-ethynyl GABA-inactivated and denatured enzyme than into the control, this provides initial support for the existence of a ketone such as **9** or **17** as the denatured stable adduct and provides evidence against a structure such as **8** or **15** (X = His).

More direct evidence for the structure of the stable adduct was obtained. As discussed above, a lysine residue is thought to act as the active-site nucleophile, and, therefore, the two resulting possible denatured stable adducts, **9** (X = NH, Scheme I) and **17** (X = NH, Scheme II), would be β- or α-amino keto acids, respectively. In an effort to differentiate between these two possibilities, the chemical stabilities of compounds of these general structures were determined. As was described for the stable adduct associated with inactivation of this enzyme by γ-vinyl GABA,[26] conditions (7 M urea, pH 13.3, 76 °C) that produce complete β-elimination with the model compound 4-oxo-6-piperidinohexanoic acid (a model for **9**) but which cause only slight degradation of the model compound 4-oxo-5-piperidinohexanoic acid (a model for **17**) were used on the radiolabeled enzyme that had been denatured to release the unstable adduct. Since 78% of the stable adduct remained bound to the enzyme under these conditions as compared to a control that was not heated with base, these results are consistent with **17** as the stable adduct, since this adduct would be expected to be only slightly degraded under these conditions. It is not clear if the small amount of radioactivity released in this experiment is the result of the expected small

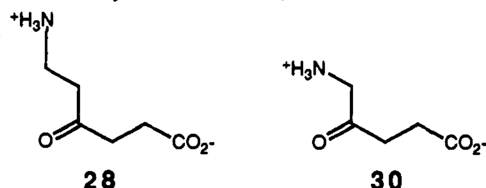
(22) Washtien, W.; Abeles, R. A. *Biochemistry* **1977**, *16*, 2485.
(23) Dunathan, H. C.; Voet, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3888.
(24) Kirsch, J. F.; Eichele, G.; Ford, G. C.; Vincent, M. G.; Jasonius, J. N. *J. Mol. Biol.* **1984**, *174*, 497.
(25) Jensen, M. D. Doctoral dissertation, Cornell University, 1973.
(26) Nanavati, S. M.; Silverman, R. B., following paper in this issue.

Scheme VI. Sodium Borohydride Reduction and Sodium Periodate Oxidation of Potential Stable Adducts **17** (X = Lys) and **9** (X = Lyx)



amount of degradation observed in the model study for the α-piperidino analogue or that there is a small amount of an adduct such as **9** where the active-site nucleophile is, for example, a cysteine or serine residue (as is the case in Schemes I or III), since the resulting β-thioalkoxy- or β-alkoxyketone would be more susceptible to elimination than is a β-amino ketone.

In an effort to get even more direct evidence for the existence of **17** (X = NH) as the denatured stable adduct, the [2-^{3}H]-γ-ethynyl GABA-inactivated, denatured, and dialyzed enzyme was subjected to sodium borohydride reduction followed by oxidative cleavage using sodium periodate, as described in the following paper in this issue[26] and briefly below. The enzyme molecule itself should remain intact due to the fact that the amide linkage of proteins is not susceptible to periodate oxidation.[27] A great amount of work has been done on the periodate cleavage of amino alcohols,[28] with the conclusion that both primary and secondary amino alcohols are only cleaved when the nitrogen and oxygen are on adjacent carbons.[29-31] Therefore, 1,3-amino alcohols are immune to periodate cleavage. With this in mind, the stable adduct could be reduced with sodium borohydride (Scheme VI), leading either to a 1,2-amino alcohol (if **17**, X = NH is the adduct) or a 1,3-amino alcohol (if **9**, X = NH is the adduct); periodate oxidation could, then, differentiate these two possibilities. In this case, the radioactive oxidation product would be succinic semialdehyde, since the tritium in the γ-ethynyl GABA is at the 2-position. In fact, $NaBH_4$ reduction of the α-amino keto acid model compound, **30**, followed by periodate oxidation produced succinic semialdehyde, whereas the β-amino keto acid model, **28**,



did not produce any succinic semialdehyde under identical conditions. When the [2-^{3}H]-γ-ethynyl GABA-inactivated, denatured, and $NaBH_4$-reduced enzyme was treated with periodate, 88% of the radiolabeled denatured stable adduct was released from the enzyme, and HPLC analysis showed that the released radiolabeled product was succinic semialdehyde (Figure 4). This provides direct evidence that nearly all of the denatured stable adduct is an α-substituted keto acid of general structure **17** (X = NH) which, like the adduct that is not stable to denaturation (**15** or **16**), comes ultimately from attack of an active-site nucleophile onto an allene intermediate, **10** (Scheme II). Regarding the active-site residue that is alkylated to give **17**, the possibility that a serine, threonine, cysteine, aspartate, or glutamate residue is modified can be ruled out due to the fact that the 1,2-alkoxy alcohols, 1,2-thioalkoxy alcohol, and 1,2-acyl alcohols that would result after $NaBH_4$ reduction of the denatured enzyme are known to be immune to periodate cleavage.[29-32] This, again, provides support for the denatured stable adduct being a modified lysine residue, since an α-lysyl keto acid is the only form of **17** that would be expected to yield succinic semialdehyde upon $NaBH_4$ reduction and periodate oxidation. The small amount of radiolabel (0.06 equiv) remaining on the denatured and reduced enzyme after periodate oxidation may be due to the presence, after denaturation, of a small amount of some other adduct such as **9** (X is not Lys; Scheme I or Scheme III), which, when reduced with $NaBH_4$ and

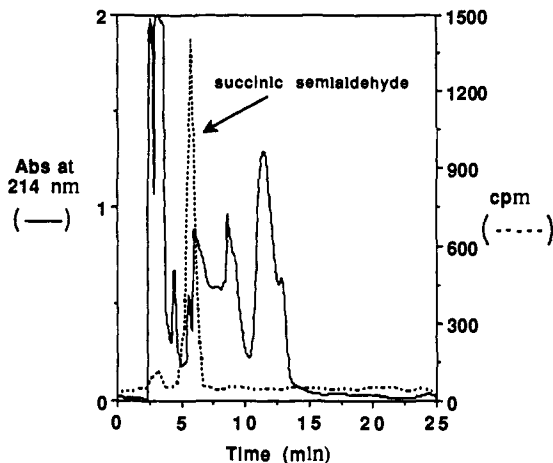
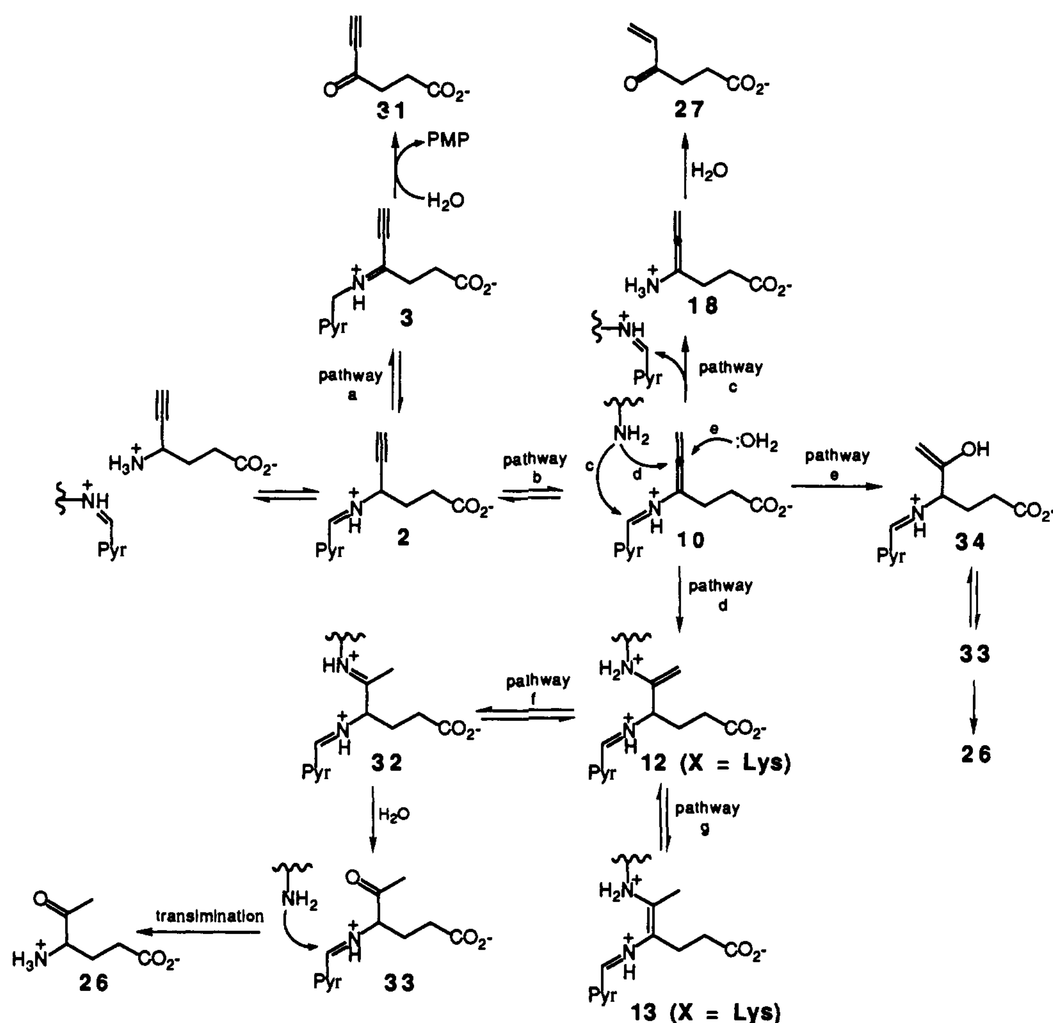


Figure 4. HPLC analysis of the product released by sodium borohydride reduction and sodium periodate oxidation of the stable adduct. GABA aminotransferase (18.6 nmol) in a total volume of 880 μL was inactivated with [2-^{3}H]-γ-ethynyl GABA as described under binding studies of [2-^{3}H]-γ-ethynyl GABA and GABA aminotransferase in the Experimental Section. The sample was then dialyzed at 25 °C for 8 h (changing the dialysate once) against 4 L of 6 M urea in 100 mM sodium phosphate buffer containing 1 mM β-mercaptoethanol at pH 7.4. To this sample was added 100-μL aliquots of a 190 mM solution of $NaBH_4$ in 150 mM NaOH at 0, 2, 4, and 6 h while incubating at 25 °C. After an additional 11 h, the sample was acidified with acetic acid and dialyzed at 25 °C for 4 h against 1 L of 6 M urea in 50 mM citric acid at pH 3.2 and then against 1 L of 6 M urea in 100 mM sodium phosphate buffer at pH 7.4 for 4 h at 25 °C. A portion (400 μL) of this [^{3}H]-γ-ethynyl GABA-inactivated, denatured, and $NaBH_4$-reduced enzyme was made 100 mM in sodium periodate and allowed to incubate at 25 °C for 28 h. A control sample was also prepared by incubating 400 μL of this [^{3}H]-γ-ethynyl GABA-inactivated, denatured, and $NaBH_4$-reduced enzyme at 25 °C for 28 h. Both the oxidized and control samples were then analyzed by reversed-phase HPLC by combining a 100-μL aliquot with 10 μL of 1 M succinic acid and 10 μL of 15% (w/w) succinic semialdehyde and injecting this onto an Alltech Econosil C18 10 μ 4.6 × 250 mm column (solvent, 0.1% aqueous trifluoroacetic acid; flow rate, 1.0 mL/min; detection, 214 nm). Fractions were collected every 0.5 min and analyzed for radioactivity by liquid scintillation counting. Under these HPLC conditions, succinic semialdehyde and succinic acid had retention times of 6 and 8 min, respectively. The remaining portions of the oxidized and control samples were then separately dialyzed against 1 L of 6 M urea in 100 mM sodium phosphate buffer at pH 7.4. The protein concentrations were assayed and then the radioactivity contents determined by liquid scintillation counting.

(27) Sklarz, B. *Quarterly Rev.* **1967**, *21*, 3.
(28) Maro, L.; Molnar-Perl, I.; Schissel, E.; Szerdahelyi, V. *J. Chem. Soc., Perkin Trans. II* **1980**, 39.
(29) March, J. *Advanced Organic Chemistry*, 3rd ed.; John Wiley & Sons: New York, 1985; p 1063.
(30) Perlin, A. S. In *Oxidation*; Augustine, R. L., Ed.; Marcel Dekker: New York, 1969; Vol. 1, pp 189–212.
(31) Jackson, E. L. *Org. React.* **1944**, *2*, 341.
(32) Fatiadi, A. J. In *Synthetic Reagents*; Pizey, J. S., Ed.; Halsted Press: New York, 1981; Vol. 4, p 227.

Scheme VII. Proposed Pathways for the Metabolism of γ-Ethynyl GABA and Inactivation of GABA Aminotransferase[a]



[a] Pyr represents the pyridine ring of PLP.

treated with periodate, would not be oxidatively cleaved from the enzyme.[29-32]

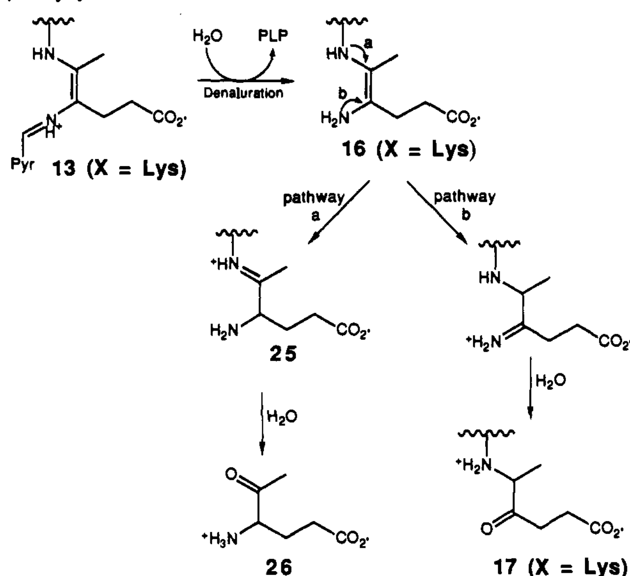
In order to complete the metabolic picture of γ-ethynyl GABA during processing by GABA aminotransferase, turnover products of this compound were investigated. Transamination of γ-ethynyl GABA to 4-oxo-5-hexynoic acid (**31**, Scheme VII, pathway a) as a released turnover product would produce PMP which, in the presence of [5-^{14}C]-α-ketoglutarate, would be converted back into PLP with concomitant formation of [5-^{14}C]glutamate. The fact that 1.2 equiv of [5-^{14}C]glutamate are produced per enzyme molecule inactivated would suggest that **31** is formed 1.2 times per inactivation event. Furthermore, inactivation of GABA aminotransferase with [2-^{3}H]-γ-ethynyl GABA produced 3.8 equiv of radiolabeled nonamine turnover products per inactivation event. Of these, at least 1.9 equiv are due to the formation of 4-oxo-5-hexenoic acid (**27**, Scheme VII, pathways b and c) as determined by HPLC analysis. Allylic isomerization of **2** to the allene **10** (pathway b) is followed by transimination with the active-site lysine to yield the allenamine **18** (pathway c), which is released into solution and hydrolyzed to **27**. Of the other 1.9 equiv of radiolabeled nonamine turnover products, 1.2 equiv are, presumably, due to the formation of **31** from the normal azaallylic tautomerization of **2** to **3**; the remaining 0.7 equiv of unassignable nonamine turnover products may be due to the degradation of **27** by either polymerization or Michael addition of water or β-mercaptoethanol (**27** had variable stability under the conditions of the experiment). The fact that **18** appears to be formed but does not inactivate the enzyme by allenamine alkylation of the cofactor (Scheme III, pathway a) suggests that **18** cannot obtain the correct orientation for alkylation, presumably because of steric hindrance in the active site. In the case of the inactivation of ornithine aminotransferase by γ-ethynyl GABA,[18] the enamine adduct (**19**, Scheme III) was proposed as the major inactivation species.

Since reactive intermediate **3** is presumably formed but does not lead to enzyme inactivation (Scheme I), either the enzyme does not have an active-site nucleophile correctly positioned for conjugate addition into **3** or the covalently bound adduct, **4**, **5**, or **6** is not stable enough to cause irreversible inactivation. In fact, **27** could arise from a spontaneous β-elimination from **9**, if X were a good enough leaving group.

Only one radioactive amine-containing metabolite (8.2 equiv per inactivation event) was generated during [2-^{3}H]-γ-ethynyl GABA inactivation of GABA aminotransferase, namely, **26** (Scheme VII), the product of denaturation of the unstable adduct. The fact that **26** was produced both from enzymatic turnover and from the denatured unstable inactivation adduct can be rationalized by the mechanism shown in Scheme VII (pathways b and e or pathways b, d, and f). Attack at **10** by water could lead to **26** (pathways b and e); alternatively, conjugate addition of the lysine residue to the allene **10** produces **12** (X = Lys), which could isomerize to **32** then hydrolyze, and undergo transimination to **26** (pathways b, d, and f). Since tautomerization of **12** to **13** should be favored over tautomerization of **12** to **32** (**13** should be more stable than **32**), but the formation of **26** occurs about eight times more often than inactivation, then this suggests that **26** is formed more likely by pathways b and e than by pathways b, d, and f.

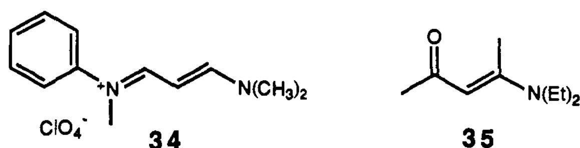
Addition of the active site lysine to the allene **10** should produce initially adduct **12**, which, as indicated above can lead to me-

Scheme VIII. Proposed Pathways for the Conversion of **16** (X = Lys) to the Unstable and Stable Adducts Upon Denaturation of γ-Ethynyl GABA-Inactivated GABA Aminotransferase[a]



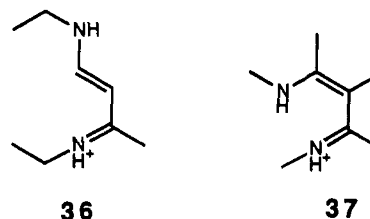
[a] Pyr represents the pyridine ring of PLP.

tabolite **26**. We believe that **12** partitions mostly to the more stable tautomer **13** (pathway g) and that **13** is the major inactivated form of the enzyme that results when γ-ethynyl GABA inactivates GABA aminotransferase. This is analogous to the conclusions of Schwab et al.[33] on the inactivation of β-hydroxydecanoyl thioester dehydrase by 3-decynoyl-*N*-acetylcysteamine. Following propargylic isomerization to the allenic thioester, an active site histidine is believed to add to the allene to give the β,γ-unsaturated enamine (equivalent to **12** in our case), which slowly isomerizes through the iminium ion to the stabilized α,β-unsaturated enamine (equivalent to **13** in our case). The rate of this isomerization in the case of 3-decynoyl-*N*-acetylcysteamine is slow because the intermediate iminium ion that would form during isomerization when histidine is the nucleophile is much higher in energy than that which would form when lysine is the nucleophile. In fact, when a model reaction of an allenic thioester was carried out with histidine methyl ester, the amino group rather than the imidazole group reacted with the allene to form initially the β,γ-unsaturated thioester which underwent rapid isomerization to the α,β-unsaturated thioester.[34] The carbon–carbon double bond of **13** is in conjugation with the cofactor and, therefore, is much more stable than the carbon–carbon double bond of **12** which is not conjugated. Conjugated enamines, such as **34** and **35**, are known to be relatively stable in aqueous solutions,[35,36] and there is precedence for the existence of stable conjugated enamines as adducts resulting from enzyme inactivation. For instance, estradiol dehydrogenase is inactivated by 3-hydroxy-14,15-secoestra-1,3,5-(10)-trien-15-yn-17-one to form a stable conjugated enamine adduct irreversibly, as determined by NMR studies.[37]



We believe that the stable and unstable adducts are *both* formed from **13** upon denaturation (Scheme VIII). When the enzyme is denatured, the adduct is completely accessible to water, and the imine bond to the cofactor would be hydrolyzed to release PLP and produce **16** (X = Lys). The resulting unstable en-1,2-diamine adduct would be expected to collapse in two ways with roughly equal probability. Pathway a leads to the release of **26** observed after denaturation of γ-ethynyl GABA-inactivated GABA aminotransferase and was suggested to be the result of the decomposition of the unstable adduct. Pathway b leads to the stable adduct (**17**, X = Lys).

In an effort to further verify the existence of a conjugated enamine adduct (**13**, X = Lys) as the inactivated enzyme adduct, optical spectra were recorded during inactivation. It was hoped that an absorption would appear at a unique wavelength indicative of a conjugated enamine adduct. Conjugated enamines **34** and **35** are known to have absorption maxima at 316 and 327 nm, respectively,[35,36] and model enamines **36** and **37** also have absorption maxima in this region (308 and 322 nm, respectively).[38,39]



However, PMP strongly absorbs at 330 nm, so an absorption of a conjugated enamine adduct would be obscured by the strong absorption of PMP in this region. Indeed, the absorbance at 330 nm rises immediately when γ-ethynyl GABA is added to the enzyme and stays at this level as the inactivation progresses, with no rise in absorption seen at any other wavelength.

Conclusions

These studies indicate that the inactivation of GABA aminotransferase by γ-ethynyl GABA proceeds completely via a propargylic isomerization of the Schiff base of γ-ethynyl GABA and PLP (Scheme VII, pathway b) to intermediate **10**. Almost, if not, all of the inactivation results from nucleophilic attack of what appears to be a lysine residue at the incipient conjugated allene of **10** to give **13** (pathways d and g). A few percent of the inactivation may arise from transimination of the PLP–allenamine adduct **10** to give the allenamine **18**, which eventually alkylates an active-site nucleophile other than lysine (possibly cysteine) to give **9** (Scheme III, pathway b). Azaallylic isomerization of the Schiff base of γ-ethynyl GABA and PLP to give **3** (Scheme VII, pathway a) apparently occurs and a small percentage of the time may undergo nucleophilic attack by a residue other than a lysine to give **9** (Scheme I).

On average, for every 13 molecules of γ-ethynyl GABA that are turned over, 1.2 undergo transamination (Scheme VII, pathway a), 2.6 are metabolized to **27** (pathways b and c), 8.2 are converted to **26** (pathways b and e or b, d, and f), and 1.0 becomes attached to the enzyme, almost all as **13** (pathways b, d, and g) but possibly 5–10% as **9** (X $\neq$ Lys; Scheme I or Scheme III, pathway b).

Experimental Section

Analytical Methods. Optical spectra and GABA aminotransferase assays were recorded on either a Perkin-Elmer Lambda 1 or Beckman DU-40 UV–vis spectrophotometer. NMR spectra were recorded on a Varian XL-400 400-MHz spectrometer. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (TMS) as the internal standard in $CDCl_3$ or from 3-(trimethylsilyl)propanesulfonic acid, sodium salt (DSS) in D_2O. An Orion Research Model 601 pH meter with a general combination electrode was used for pH measurements. Mass spectral analyses were performed by the Analytical Services Laboratory at the Department of Chemistry, Northwestern University. Thin-layer chromatography was performed using Whatman

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PE SIL/UV silica gel plates with UV indicator. Flash column chromatography utilized silica gel 60 (230–400 mesh ASTM) fro Merck. Elemental analyses were performed by G.D. Searle & Co., Skokie, IL. Radioactivity was measured by liquid scintillation counting using a Beckman LS-3133T counter and Research Products International 3a70b scintillation cocktail. [^{14}C]Toluene (4×10^5 dpm/mL) and [^{3}H]toluene (2.19×10^6 dpm/mL, corrected for first-order decay) from New England Nuclear were used as internal standards. Radiopurity of radioactive compounds was assessed by cutting TLC plates (Merck Kieselgel 60 plates without fluorescent indicator) into strips and counting each strip with scintillation cocktail in the scintillation counter. HPLC was performed using Beckman 110B pumps in series with a Beckman 160 absorbance detector. Amines were visualized on TLC plates by spraying with a solution of ninhydrin (300 mg) with pyridine (2 mL) in acetone (100 mL) and then heating. Aldehydes and ketones were visualized on TLC plates by spraying with a standard 2,4-dinitrophenylhydrazine reagent.[40]

Reagents. The syntheses of [2-^{3}H]-4-amino-5-hexynoic acid ([2-^{3}H]-γ-ethynyl GABA, 2.25 mCi/mmol),[11] 4-amino-5-oxohexanoic acid hydrochloride (**26**),[41] 6-amino-4-oxohexanoic acid hydrochloride (**28**),[42,43] 5-ethoxy-2-pyrrolidone,[44] and 4-oxo-5-hexenoic acid (**27**)[45] were previously reported. Allyltrimethylsilane, 5-amino-4-oxopentanoic acid (i.e., δ-aminolevulinic acid), dansyl chloride, sodium periodate, and titanium tetrachloride were purchased from Aldrich. Phosphoric acid, potassium phosphate, and sodium phosphate were purchased from Mallinckrodt. γ-Aminobutyric acid, citric acid, α-ketoglutarate, β-mercaptoethanol, NADP, potassium pyrophosphate, sodium borohydride, and sodium tetraborate were purchased from Sigma. (D,L)-γ-Ethynyl GABA was a generous gift of Merrell Dow Research Institute. [5-^{14}C]-α-Ketoglutarate (24.2 mCi/mmol) was obtained from Amersham. Ultrapure urea was purchased from ICN Biomedicals. [^{3}H]Sodium borohydride (200 mCi/mmol) was obtained from Research Products International. $NaDodSO_4$ was purchased from Bio-Rad Laboratories. HPLC-grade acetonitrile, methanol, and water were obtained from Mallinckrodt and were filtered and degassed prior to use.

5-Allyl-2-pyrrolidone. To a stirred solution of 5-ethoxy-2-pyrrolidone[42] (5.9 g, 46 mmol) in dichloromethane (65 mL) was added titanium tetrachloride (3.0 mL, 27 mmol) dropwise under nitrogen. The cloudy yellow mixture was allowed to stir for 5 min at room temperature, and then allyltrimethylsilane (9.2 mL, 58 mmol) was added in one portion. The solution was allowed to stir an additional 10 min before being quenched with the dropwise addition of water (50 mL). The aqueous layer was separated and extracted with chloroform (3×150 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was removed in vacuo to give a colorless oil. Silica gel flash chromatography (chloroform/methanol 9:1) yielded the desired product as a colorless oil (4.1 g, 72%): ^{1}H NMR ($CDCl_3$) δ 1.80–2.30 (m, 6 H), 3.67 (m, 1 H), 5.05 (m, 2 H), 5.70 (m, 1 H), 7.50 (br s, 1 H); high-resolution electron impact mass spectrometry (m/z + H) for $C_7H_{11}NO$ = 126.0927 (+6.3 ppm). Anal. Calcd for $C_7H_{11}NO$: C, 67.17; H, 8.86; N, 11.19; Found: C, 67.00; H, 8.73; N, 11.39.

4-Amino-5-heptenoic Acid, Lithium Salt. 5-Allyl-2-pyrrolidone (3.9 g, 31 mmol) was refluxed in a solution of LiOH (1.0 g, 42 mmol) in 40 mL of water for a period of 3 h. After allowing to cool, the solution was neutralized with concentrated HCl and applied to a Dowex 50×8-400 column (2.5×25 cm) and washed with water. The column was then eluted with 2 M NH_4OH, and the ninhydrin-positive fractions were combined and lyophilized leaving a colorless solid (0.3 g, 6%). TLC (silica gel, 3:1:1 *n*-butanol/acetic acid/water) showed a single ninhydrin-positive spot at R_f 0.75: ^{1}H NMR (D_2O) δ 1.75 (m, 2 H), 2.10–2.30 (m, 4 H), 3.74 (m, 1 H), 5.03–5.20 (m, 2 H), 5.73 (m, 1 H); high-resolution fast atom bombardment spectrometry (m/z – Li) for $C_7H_{12}NO_2Li$ = 142.0834 (–25.6 ppm). Anal. Calcd for $C_7H_{12}NO_2Li$: C, 56.38; H, 8.11; N, 9.39. Found: C, 56.49; H, 8.19; N, 9.28.

4-Amino-6-oxohexanoic Acid·HCl (24). Ozonized oxygen was passed for 20 min through a solution of 4-amino-5-heptenoic acid, lithium salt (200 mg, 1.3 mmol) in 1.5 M HCl (3 mL) cooled in an ice bath. The solution was then diluted with 60 mL of ice–water and applied to a Dowex 50×8-400 column (1×10 cm) that had been prewashed sequentially with 1 M HCl and water. After washing the column with water, it was eluted with 1 M HCl. The 2,4-dinitrophenylhydrazine-positive fractions were combined and lyophilized, yielding a slightly yellow solid (86 mg, 35%). TLC (silica gel, 3:1:1 *n*-butanol/acetic acid/water) showed a single ninhydrin-positive spot at R_f 0.8 that was also 2,4-dinitrophenylhydrazine-positive. ^{1}H NMR, ^{13}C NMR, and mass spectral analysis indicated the presence of both the desired product, **24**, and its dimer (ratio ca. 4:1 by ^{1}H NMR): ^{1}H NMR (D_2O) δ 1.70–2.07 (m, H-C(3) and H-C(5) of **24** and dimer), 2.42 (t, H-C(2) of **24** and dimer), 3.34 (m, H-C(4) of dimer), 3.49 (m, H-C(4) of **24**), 5.14 (t, H-C(6) of dimer), 5.28 (t, H-C(6) of **24**); ^{13}C NMR (D_2O) δ 29.9 (C(2) of **24** and dimer), 31.9 (C(3) of **24** and dimer), 38.0 (C(5) of **24** and dimer), 49.6 (C(4) of **24**), 50.7 (C(4) of dimer), 90.9 C(6) of dimer), 101.4 (C(6) of **24**), 178.9 (C(1) of **24** and dimer); low-resolution fast atom bombardment spectrometry (m/z – Cl) for **14** ($C_6H_{12}NO_3Cl$) = 146, (m/z) for **15** ($C_{12}H_{18}N_2O_4$) = 254. Anal. Calcd for $C_6H_{12}NO_3Cl$: C, 39.68; H, 6.66; N, 7.71. Found: C, 38.18; H, 6.92; N, 7.70.

Enzymes and Assays. Pig brain GABA aminotransferase was purified to homogeneity using the published procedure.[46] Succinic semialdehyde dehydrogenase was isolated from GABAse (Boehringer Mannheim Biochemicals) using the method of Jeffery et al.[47] Protein concentrations were determined using Pierce Coomassie protein assay reagent No. 23200 using bovine serum albumin for standard curves. All buffers and enzyme solutions were prepared with deionized distilled water. Unless noted, GABA aminotransferase activity was assayed using a modification of the coupled assay of Scott and Jakoby.[48] The assay solution had final concentrations of 10 mM GABA, 1 mM NADP, 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and excess succinic semialdehyde dehydrogenase in 50 mM potassium pyrophosphate buffer at pH 8.5. With this assay, the change in absorbance at 340 nm, corresponding to the formation of NADPH from NADP, at 25 °C, is proportional to the GABA aminotransferase activity.

Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by γ-Ethynyl GABA. GABA aminotransferase reconstituted with [7-^{3}H]-pyridoxal 5′-phosphate ([^{3}H]-PLP) was prepared using the procedure of Silverman and Invergo.[49] This [^{3}H]-PLP GABA aminotransferase (1.64 nmol, 20 mCi/mmol) in 100 mM potassium phosphate buffer containing 0.5 mM α-ketoglutarate, 0.4 mM β-mercaptoethanol, and 2 mM γ-ethynyl GABA at pH 7.4 was incubated at 25 °C while protected from light. A control was run that was identical except that there was no γ-ethynyl GABA present. After 3 h, the control had 104% of its original enzyme activity, whereas the sample containing γ-ethynyl GABA was completely inactive. The two samples were then each dialyzed separately in the dark against 500 mL of water for 3 h at 25 °C. The radioactivity in 1-mL aliquots of the dialysates was determined by liquid scintillation counting. The enzyme samples were made 10% (w/w) in trichloroacetic acid by the addition of solid trichloroacetic acid. After centrifugation (9000 g) for 5 min, the supernatants were separated, and the pellets were washed three times (100 μL each) with 10% (w/w) trichloroacetic acid. The remaining pellets were dissolved in 500 μL of 2 M KOH, and the radioactivity content was determined by liquid scintillation counting.

Binding Studies of [2-^{3}H]-γ-Ethynyl GABA with GABA Aminotransferase. In a typical experiment, GABA aminotransferase (5.9 nmol) was incubated with [2-^{3}H]-γ-ethynyl GABA (2 mM) in 100 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM α-ketoglutarate and 1 mM β-mercaptoethanol at 25 °C. A control containing no inactivator also was prepared. The sample containing [2-^{3}H]-γ-ethynyl GABA had no remaining activity after 10 h, whereas the control had 92% of its original activity. The inactivated sample was dialyzed against 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol for 3 h at 4 °C to remove excess inactivator. It was then dialyzed at either 4, 25, or 50 °C for 24 h (changing the dialysate three times) against either 100 mM potassium phosphate, 1 mM β-mercaptoethanol, pH 7.4; 6 M urea in 100 mM potassium phosphate, 1 mM β-mercaptoethanol, pH 7.4; 6 M urea in 100 mM citric acid, 1 mM β-mercaptoethanol, pH 3.1; or 6 M urea in 50 mM sodium tetraborate, 1 mM β-mercaptoethanol, pH 9.5. The protein concentrations were assayed, and then the radioactivity contents were determined by liquid scintillation counting.

Trichloroacetic Acid Precipitation of GABA Aminotransferase Inactivated with [2-^{3}H]-γ-Ethynyl GABA. GABA aminotransferase (4.81 nmol) in a total volume of 640 μL was inactivated with [2-^{3}H]-γ-ethynyl GABA as described above. After being dialyzed against 100 mM sodium

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phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol for 16 h (changing dialysate three times) at 4 °C, it was made 10% (w/w) in trichloroacetic acid by the addition of solid trichloroacetic acid. After centrifugation (9000 g) for 5 min, the supernatant was separated from the pellet, the pellet was resuspended in 400 μL of 10% (w/w) trichloroacetic acid and recentrifuged, and the supernatant was removed. The pellet was dissolved in 500 μL of 2 M KOH and its radioactivity content, along with the radioactivity content of the combined supernatants, was measured by liquid scintillation counting. Another enzyme sample was prepared in an identical manner except that before adding the trichloroacetic acid, 100 μL of a 176 mM $NaBH_4$ solution in 100 mM NaOH was added at 0, 2, 4, and 6 h. After an additional 13 h, the trichloroacetic acid was then added, centrifuged, and counted.

Monitoring for Radiolabeled 4-Oxo-5-hexenoic Acid (27) Released from [2-^{3}H]-γ-Ethynyl GABA-Inactivated GABA Aminotransferase upon Denaturation. GABA aminotransferase (23.5 nmol) in a total volume of 640 μL was inactivated with [2-^{3}H]-γ-ethynyl GABA, dialyzed, precipitated with trichloroacetic acid, and centrifuged as described above. The supernatant was then removed and washed with dichloromethane (3 × 1 mL). The organic layer was dried over $MgSO_4$, concentrated to dryness under a stream of nitrogen, and redissolved in 100 mM sodium phosphate pH 7.4 buffer (100 μL), and the radioactivity content was measured by liquid scintillation counting. A control was prepared by dissolving **27** (10 mg) in 100 mM sodium phosphate pH 7.4 buffer (1 mL), making it 10% (w/w) in trichloroacetic acid, and extracting with dichloromethane (3 × 1 mL). The organic layer was dried over $MgSO_4$, concentrated to dryness under a stream of nitrogen, and then redissolved in 100 mM sodium phosphate pH 7.4 buffer (300 μL). The presence of **27** in this extracted control sample was verified by HPLC analysis using a Rainin Microsorb 5 μm C8 4.6 × 250 mm column (solvent: 90% 100 mM sodium phosphate pH 6.0, 10% methanol; flow rate: 1.0 mL/min; detection: 254 nm). Under these HPLC conditions, **27** had a retention time of 7.3 min.

Paper Chromatography of the Radiolabeled Products Released from [2-^{3}H]-γ-Ethynyl GABA-Inactivated GABA Aminotransferase upon Denaturation. A portion (1900 dpm) of the same supernatant from that prepared in Figure 3 was added with a solution of **24**, **26**, and γ-ethynyl GABA (1 mg each/mL, 20 μL) and applied to one end of a 3 × 25 cm piece of Whatman 3 MM filter paper. The amino acids were separated using descending paper chromatography (3:1:1 *n*-butanol/acetic acid/water). The positions of the three standards were determined by spraying with ninhydrin, and then the chromatogram was cut into 20 10-mm sections and subjected to liquid scintillation counting after first soaking each section in 1 mL of water for 48 h in the scintillation vials.

[^{3}H]$NaBH_4$ Reduction of γ-Ethynyl GABA-Inactivated and Denaturated GABA Aminotransferase. GABA aminotransferase (7.76 nmol) in 690 μL of 100 mM sodium phosphate, pH 7.4, containing 1 mM β-mercaptoethanol, was inactivated with γ-ethynyl GABA as described above. A control containing no inactivator also was prepared. The samples were each dialyzed separately for 4.5 h at 25 °C against 2 L of 6 M urea in 100 mM sodium phosphate buffer containing 1 mM β-mercaptoethanol at pH 7.4. To these samples were then added 100-μL aliquots of a 190 mM solution of [^{3}H]$NaBH_4$ (5 mCi/mmol) in 150 mM NaOH at 0, 2, 4, and 6 h while being incubated at 25 °C. After an additional 13 h, the samples were acidified with 2 M HCl and then dialyzed at 25 °C for 20 h (changing dialysate three times) against 6 M urea in 50 mM citric acid, pH 3.2, containing 1 mM β-mercaptoethanol. The protein concentrations were assayed, and then the radioactivity contents were determined by liquid scintillation counting.

[^{3}H]$NaBH_4$ Reduction of 6-Amino-4-oxohexanoic Acid. Using the method of Kirk et al.,[50] 100 mg of 6-amino-4-oxohexanoic acid hydrochloride (**28**) was reduced using a 190 mM solution of [^{3}H]$NaBH_4$ (5 mCi/mmol) in 150 mM NaOH. The radiopurity of the resulting 4-[^{3}H]-6-aminohexanoic acid 1,4-lactone hydrochloride (**29**) was shown to be greater than 99% by TLC (silica gel, 3:1:1 *n*-butanol/acetic acid/water). The amount of tritium incorporation was determined by liquid scintillation counting.

Acid and Base Stability of the [2-^{3}H]-γ-Ethynyl GABA-Inactivated and Denatured GABA Aminotransferase Adduct. GABA aminotransferase (6.4 nmol) was inactivated with [2-^{3}H]-γ-ethynyl GABA as above and dialyzed against 100 mM sodium phosphate buffer, pH 7.4, for 24 h (changing dialysate three times) at 25 °C and then against 6 M urea in 100 mM phosphoric acid, pH 1.8, at 55 °C for 6 h. The protein concentration was assayed, and then the radioactivity content was determined by liquid scintillation counting.

GABA aminotransferase (9.3 nmol) was inactivated with [2-^{3}H]-γ-ethynyl GABA as above and dialyzed against 6 M urea in 100 mM sodium phosphate, 1 mM β-mercaptoethanol, pH 7.4 for 16 h at 25 °C (changing the dialysate once). The labeled enzyme was then dialyzed at 76 °C for 10 h against 7 M urea which had been adjusted to pH 13 with KOH. A control was prepared in the same manner except that the final dialysis was done at 25 °C using 6 M urea in 100 mM sodium phosphate, 1 mM β-mercaptoethanol, pH 7.4. The protein concentrations were assayed, and then the radioactivity contents were determined by liquid scintillation counting.

Periodate Oxidation of $NaBH_4$-Reduced 6-Amino-4-oxohexanoic Acid (28) and 5-Amino-4-oxopentanoic Acid (30). To solutions containing 50 mg of either **28** or **30** in 2 mL of 6 M urea in 0.1 M NaOH was added 1.5 mL of a solution of 0.12 M $NaBH_4$, 0.1 M NaOH in 6 M urea. After allowing to react at 25 °C for 4 h, the samples were acidified with acetic acid, and the pH was adjusted to 7.0 with solid NaOH. A solution of 100 mM $NaIO_4$ in 6 M urea (500 μL) was added to 500 μL of each reduced sample. Controls containing no $NaIO_4$ were also prepared by adding 500 μL of 6 M urea to 500 μL of each reduced sample. After incubating at 25 °C for 19 h, 100 μL of each sample was added to 50 μL of 2,4-dinitrophenylhydrazine reagent and was allowed to react for 30 min. This was then extracted with 2 × 1 mL $CHCl_3$, evaporated under a stream of nitrogen and redissolved in 110 μL of 50% aqueous CH_3CN. Reversed-phase HPLC analyses of these derivatized samples were performed by injecting 25 μL of a sample onto an Alltech Econosil C18 10 μ 4.6 × 250 mm column with a water/CH_3CN gradient (isocratic at 25% CH_3CN for first 10 min, then 25–100% CH_3CN over 20 min; flow rate 1.0 mL/min; detection at 254 nm). Under these HPLC conditions, 2,4-dinitrophenylhydrazine and the 2,4-dinitrophenylhydrazone of succinic semialdehyde eluted at 8 and 10 min, respectively.

Extent of Transamination of γ-Ethynyl GABA during Inactivation. GABA aminotransferase (1.6 nmol) in 200 μL of 90 mM potassium phosphate buffer containing 1 mM β-mercaptoethanol, 2 mM γ-ethynyl GABA, and 5 mM [5-^{14}C]-α-ketoglutarate (1.45 mCi/mmol) at pH 7.4 was incubated at 25 °C until no enzyme activity remained (15 h). A control containing no inactivator also was run. Each sample was quenched with 25 μL of 20% trichloroacetic acid, and the [5-^{14}C]-glutamate formed was isolated and counted as previously reported.[51]

Non-Amine Turnover Products Formed during [2-^{3}H]-γ-Ethynyl GABA Inactivation of GABA Aminotransferase. GABA aminotransferase (4.7 nmol) in 520 μL of 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol, 9 mM [^{3}H]-γ-ethynyl GABA, and 0.5 mM α-ketoglutarate was incubated at 25 °C until no enzyme activity remained (9 h). A control containing no enzyme was also prepared. Each sample was applied to a Dowex 50 × 8-400 column (0.5 × 5 cm), and then the column was rinsed with 5.5 mL of water. The eluate from this application and wash was lyophilized and redissolved in 1.25 mL of 9:1 sodium phosphate (pH 6)/methanol. The radioactivity content of a 960-μL aliquot was then measured by liquid scintillation counting. A portion (250 μL) of this solution of non-amine turnover products was added with 50 μL of 75 mM **27** and analyzed by HPLC as described under **Monitoring for Radiolabeled 4-Oxo-5-hexenoic Acid (27) Released from [2-^{3}H]-γ-Ethynyl GABA-Inactivated GABA Aminotransferase upon Denaturation.** Fractions were collected every 1.0 min and analyzed for radioactivity by liquid scintillation counting.

Amine Turnover Products Formed during [2-^{3}H]-γ-Ethynyl GABA Inactivation of GABA Aminotransferase. GABA aminotransferase (4.7 nmol) in 520 μL of 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol, 2 mM [2-^{3}H]-γ-ethynyl GABA, and 0.5 mM α-ketoglutarate was incubated at 25 °C until no enzyme activity remained (15 h). A control containing no enzyme also was prepared. Each sample was then microdialyzed against 14 mL of water for 6 h at 25 °C. The microdialysate was then applied to a Dowex 50 × 8-400 column (0.5 × 5 cm). The column was washed with 5 mL of water and then eluted with 10 mL of 1.5 M HCl. The HCl eluate was lyophilized and redissolved in 40 mM Li_2CO_3 (150 μL). A portion (100 μL) of this solution was added to a solution containing **24**, **26**, and γ-ethynyl GABA standards (1 mg each/mL, 15 μL). A solution of 20 mM dansyl chloride in acetonitrile (100 μL) was then added and allowed to react for 1 h in the dark. This solution was then analyzed by reversed-phase HPLC for radioactivity content as described in Figure 3.

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