BIOSYNTHESIS OF Δ-AMINOLEVULINATE IN GREENING BARLEY LEAVES. IX. STRUCTURE OF THE SUBSTRATE, MODE OF GABACULINE INHIBITION, AND THE CATALYTIC MECHANISM OF GLUTAMATE 1-SEMIALDEHYDE AMINOTRANSFERASE

by

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Glutamic acid 1-semialdehyde hydrochloride was synthesized and purified. Its prior structural characterization was extended and confirmed by ^{1}H NMR spectroscopy and chemical analyses. In aqueous solution at pH 1 to 2 glutamic acid 1-semialdehyde exists in a stable hydrated form, but at pH 8.0 it has a half-life of 3 to 4 min. Spontaneous degradation of the material at pH 8.0 generated some undefined condensation products, but coincidentally a significant amount isomerized to 5-aminolevulinate. At pH 6.8 to 7.0, glutamate 1-semialdehyde is sufficiently stable to permit routine and reproducible assay for glutamate 1-semialdehyde aminotransferase activity. Only about 20% of the enzyme extracted from chloroplasts was sensitive to inactivation by gabaculine with no pretreatment. However, when the enzyme was exposed to 5-aminolevulinate, levulinate or 4,5-dioxovalerate in the absence of glutamate 1-semialdehyde, it was completely inactivated by gabaculine; 4,6-dioxoheptanoate had no effect on the enzyme. These results lead to the hypothesis that the aminotransferase exists in the chloroplast in a complex with pyridoxamine phosphate, which must be converted to the pyridoxal form before it can form a stable adduct with gabaculine. We propose that the enzyme catalyzes the conversion of glutamate 1-semialdehyde to 5-aminolevulinate via 4,5-diaminovalerate.

1. INTRODUCTION

5-Aminolevulinate, the biosynthetic precursor of porphyrins and chlorophylls, is synthesized from glutamate in plants and numerous bacterial species (7, 9, 13, 19, 21, 31, 32). In the first step of this pathway, glutamate is esterified to tRNA^{Glu} by glutamic acid-tRNA ligase, which also provides glutamyl-tRNA for protein synthesis (4, 14, 26). Glutamyl-tRNA then is reduced in an NADPH-requiring reaction. The

dehydrogenase that catalyzes this reaction is strongly inhibited by low concentrations of hemin (10, 31), which suggests that this step is the site of feedback control of porphyrin synthesis. The product of this second reaction is glutamate 1-semialdehyde (8, 15, 19). Finally, 5-aminolevulinate is formed from glutamate 1-semialdehyde by an isomerization that is catalyzed by an aminotransferase (12, 13, 30).

Glutamic acid 1-semialdehyde was previously synthesized chemically and characterized by IR spectroscopy and, as the diethylacetal derivative, by NMR and mass spectroscopy (8, 15). The chemically synthesized product is identical to the biosynthetic intermediate isolated from barley seedlings treated with gabaculine (15). However, because of its instability, doubt regarding the existence of glutamate 1-semialdehyde as an intermediate in the pathway to 5-aminolevulinate was expressed (11, 23). We have again purified and carefully reexamined the properties of the compound prepared in this laboratory that function as substrate for the enzymatic conversion to 5-aminolevulinate. In this report we provide further evidence that glutamic acid 1-semialdehyde is indeed the product of the chemical synthesis and is a precursor of 5-aminolevulinate. Glutamate 1-semialdehyde aminotransferase has been partially purified, and we describe improved assay conditions for the characterization of this enzyme. Furthermore, we have characterized the interaction of the aminotransferase with gabaculine, a powerful inhibitor of chlorophyll synthesis (6, 12, 15, 21).

2. MATERIALS AND METHODS
2.1. Chemicals
N-Carboxybenzoxy-L-glutamic acid 5-benzyl ester was obtained from Bachem AG, Switzerland,: 3-amino-2,3-dihydrobenzoic acid hydrochloride (gabaculine) was from Fluka AG, Switzerland; palladium powder, deuterium oxide, 99.8 atom % D, and 3-(trimethylsilyl) tetradeuteropropionate were from Aldrich-Chemie, W. Germany; phosphorus pentachloride and plastic-backed silica gel 60 TLC sheets were from Merck, W. Germany; 4,6-dioxohep-

tanoic acid was from Calbiochem, San Diego, CA; 5-aminolevulinic acid · HCl, 3-methyl-2-benzothiazolone hydrazone and Dowex 50W-X8 were from Sigma Chemical Co., St. Louis, MO; and 5-amino(4-^{14}C)levulinic acid · HCl was purchased from Amersham Denmark ApS. 4,5-Dioxovaleric acid was a generous gift from D. Dörnemann, Philipps-Universität, Marburg/Lahn, W. Germany.

2.2. Synthesis and purification of glutamic acid 1-semialdehyde
Glutamic acid 1-semialdehyde was synthesized as described previously (15), except that hydrogenation was allowed to proceed in ether for only 2 hr in a glass cylinder with a sintered glass base. H$_2$ gas, introduced from below the cylinder and dispersed by the sintered glass disc, maintained the palladium catalyst in suspension. Glutamic acid 1-semialdehyde was extracted with water, lyophilized and washed with ice-cold ether. Glutamic acid 1-semialdehyde · HCl constituted 1 to 2% and glutamic acid · HCl about 75% of this material by weight, while proline was among the other contaminants. When stored dry at -20 °C, the material showed no loss of substrate for glutamate 1-semialdehyde aminotransferase over a period of 12 months. Aqueous solutions (17 mg/ml) at pH 1 to 2 were stable for at least 5 months at 4 °C and were the source of substrate for most enzyme assays.

To purify glutamic acid 1-semialdehyde, the material was dissolved in water and applied to a column (1×30 cm) of Dowex 50W-X8 (Na$^+$-form). During application of the sample the pH of the eluant dropped to below 2.5. The column was then washed with Na citrate (0.2 M-Na$^+$), pH 3.6, until glutamate was completely removed from the column. Subsequently, glutamate 1-semialdehyde was eluted with Na citrate (0.2 M-Na$^+$), pH 4.2. Ninhydrin-reactive material was determined by spotting 5 µl aliquots of effluent fractions onto filter paper and spraying with ninhydrin solution. Glutamate 1-semialdehyde was detected in fractions by incubating an aliquot with partially purified glutamate 1-semialdehyde aminotransferase and measuring the

production of 5-aminolevulinate (see section 2.4).

Fractions containing glutamate 1-semialde-hyde were pooled, acidified to pH 2 with HCl and applied to a column (1×15 cm) of Dowex 50W-X8 (H+-form). The column was washed with 50 mM-HCl to eliminate citrate and then with 500 mM-HCl. Elution of glutamic acid 1-semialdehyde was detected by reaction with 3-methyl-2-benzothiazolone hydrazone (see section 2.6) and by enzymatic conversion to 5-aminolevulinate. Fractions containing active material were pooled and lyophilized.

2.3. Preparation of glutamate 1-semialdehyde aminotransferase

Most experiments were performed with enzyme partially purified by passing chloroplast stroma through columns of Sephacryl S-300, Cibacron blue-Sepharose, Procion red-agarose and chlorophyllin-Sepharose as described previously (14, 30). Because this preparation contained porphobilinogen synthase and porphobilinogen deaminase, assays for the formation of 5-aminolevulinate included 10 mM-levulinate to block utilization of the product. Some experiments were repeated with enzyme that had been purified further with a column (2×80 cm) of Sephacryl S-200, which resolved the aminotransferase from the other two enzymes.

2.4. Assay of glutamate 1-semialdehyde aminotransferase

Each assay mixture (1.0 ml) contained 0.1 M-Na 2-(N-morpholino)ethanesulfonate-0.1 M-Na phosphate, pH 6.8, 10 mM-levulinate, partially purified enzyme (100 to 500 μg protein) and 10 to 50 μM-glutamate 1-semialdehyde. The substrate was added last, and the mixtures were then incubated for various times at 28 °C. Reactions were terminated by adding 25 μl of 70% perchloric acid, and the precipitated protein was removed by centrifugation. The supernatants were transferred into graduated tubes, 200 μl of 1 M-Na acetate, pH 4.8, were added to each sample and the pH was adjusted to 4.6±0.1 with NaOH. To each tube, 100 μl of

acetylacetone were added before heating for 20 min in a boiling water bath to produce the 5-aminolevulinate pyrrole. The tubes were then cooled to room temperature and the volume of liquid per tube adjusted to 1.5 ml with water before mixing with 1.5 ml of modified Ehrlich's reagent (20). Twenty minutes later absorption spectra were recorded from 450 to 650 nm. An assay without protein and substrate served as a reference for spectrophotometry. An extinction coefficient at 553 nm of 60 l · mmole^{-1} for a 1-cm light path was determined with 5-aminolevulinic acid · HCl and used for calculation of amounts of this product formed in the assays. At pH 4.6 the nonenzymatic conversion of glutamate 1-semialdehyde to 5-aminolevulinate during pyrrole formation is negligible, since zero-time controls gave virtually no Ehrlich's reaction. The previously reported positive reaction for glutamate 1-semialdehyde (12, 15) was largely due to nonenzymatic production of 5-aminolevulinate prior to and during pyrrole formation at pH 6.9.

For the determination of enzymatic formation of 5-aminolevulinate, the amount of product formed in control assays without enzyme was subtracted from that formed in assays with enzyme. Glutamate 1-semialdehyde concentrations in stock solutions were estimated indirectly from the amount of 5-aminolevulinate produced by enzymatic catalysis with 500 μg or more of crude enzyme protein in reactions that went to completion in under 10 min at pH 6.8 at 28 °C.

Formation of uroporphyrin from glutamate 1-semialdehyde or 5-aminolevulinate took place in 0.1 M-Tricine-NaOH, pH 8.2 without levulinate. After reactions were terminated with perchloric acid, samples were exposed to near-UV light to enhance oxidation of uroporphyrinogen to the fluorescent uroporphyrin.

2.5. Proton NMR spectra

Spectra were obtained at 300 MHz on a Bruker WM 300 wide bore spectrometer. Spectra were recorded at ambient temperature (ca. 20 °C) with a 60° pulse angle (10 μsec), a 6-sec recycle time, and a spectral width of ± 1500 Hz

from the carrier with quadrature phase detection. Samples were dissolved in 99.8 atom % deuterium oxide immediately prior to data acquisition. For solutions of glutamic acid 1-semialdehyde, the pD (uncorrected meter reading) was 1.84. Chemical shifts are reported relative to external 3-(trimethylsilyl)tetradeuteropropionate. The spectra (16 K data points) were processed with a 0.2 Hz line-broadening function.

2.6. Assay of aldehyde function

The assay was adapted from the procedure described in (25). Twenty µl of solutions of glutamate 1-semialdehyde were mixed with 100 µl of 0.4% 3-methyl-2-benzothiazolone hydrazone. After 30 min at room temperature, 250 µl of 0.2% $FeCl_3$, freshly diluted from a 20% stock solution, were added. Color was allowed to develop for 30 min before the sample was diluted with 500 µl of acetone and the absorbance measured at 650 nm. Absorbance was linear with concentration of glutamate 1-semialdehyde up to 1.8.

2.7. Other methods

Thin layer chromatography was performed on silica gel 60 sheets with n-butanol-acetic acid-water (60:20:20, v/v/v) as solvent. Protein was estimated by the Lowry procedure (18). Amino acid analysis was performed with a standard single-column analyzer and a lithium citrate buffer system (Pierce Chemical Co., Rockford, IL).

3. RESULTS

3.1. Properties of glutamic acid 1-semialdehyde

Elution of glutamic acid 1-semialdehyde from Dowex 50W-X8 columns was detected by its reaction with the aldehyde-specific reagent 3-methyl-2-benzothiazolone hydrazone (25), reaction with ninhydrin, and enzymatic conversion to 5-aminolevulinate. Purified glutamic acid 1-semialdehyde migrated as a single, ninhydrin-positive component during thin-layer
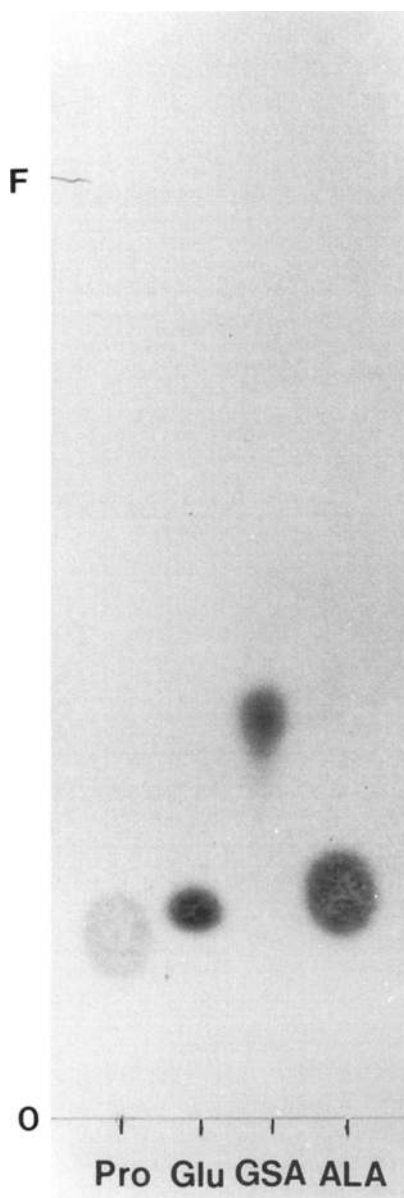

Figure 1. Migration of glutamic acid 1-semialdehyde (GSA) relative to proline (Pro), glutamic acid (Glu) and 5-aminolevulinic acid (ALA) during silica gel thin-layer chromatography. O = origin; F = solvent front.

chromatography on silica gel with an $R_f = 0.43$ (Figure 1). A single ninhydrin-positive component, with a retention time similar to that of isoleucine, was detected with an amino acid
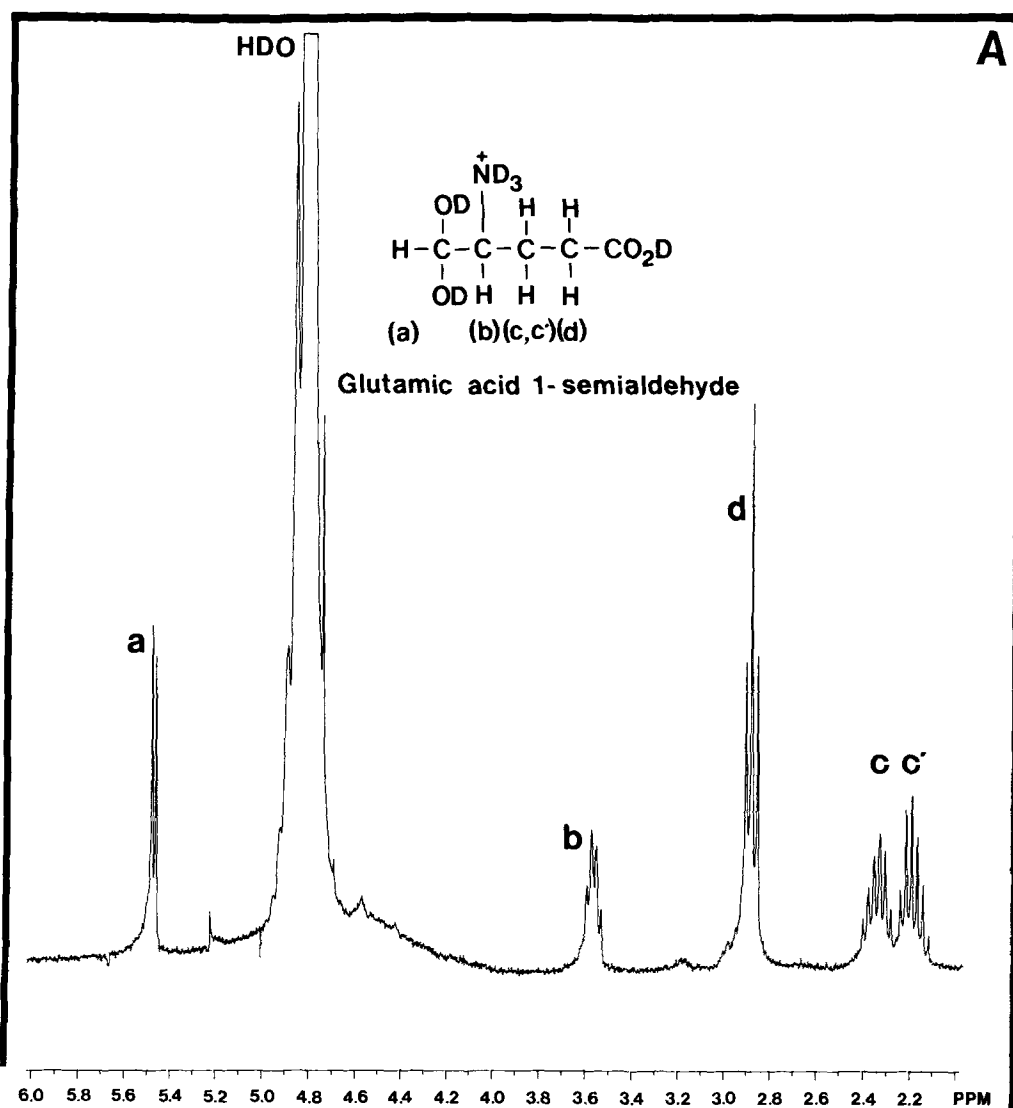
Figure 2A. Proton NMR spectrum of glutamic acid 1-semialdehyde in D_2O.

analyzer. Glutamic acid 1-semialdehyde was well resolved from 5-aminolevulinic acid, which migrated more slowly on thin-layer silica gel sheets and in the amino acid analyzer. After elution with HCl from a Dowex 50W-X8 column (see section 2.2), the only contaminant detected in the sample was NaCl, which was introduced during chromatography.

3.2. Structure of glutamate 1-semialdehyde

The ^1H NMR spectrum for glutamic acid 1-semialdehyde in D_2O is shown in Figure 2A. Since the concentration of the compound in the sample was not known, for interpretation of the integrated intensities for this spectrum the resonance for the methylene protons on carbon 4 (δ 2.89) were assigned a value corresponding to
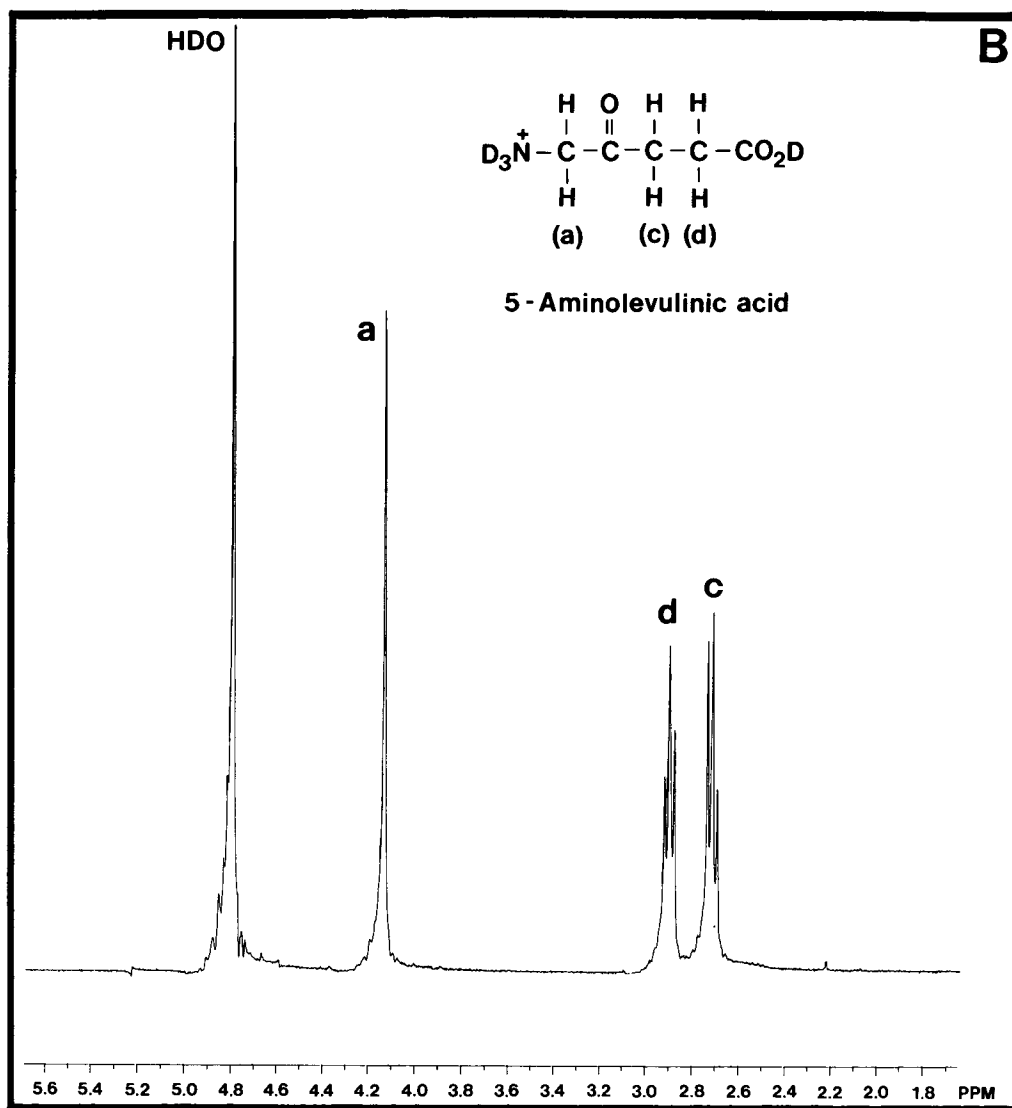
Figure 2B. Proton NMR spectrum of 5-aminolevulinic acid in D_2O.

2 protons. On the basis of this assumption the following assignments can be made: δ 2.18 (1 H, m, ‾OOC-CH_2-C$\underline{H}$H-), δ 2.35 (1 H, m, ‾OOC-CH_2-C$\underline{H}$H-), δ 2.89 (2 H, t, J = 7.6 Hz, ‾OOC-C$\underline{H}_2$-), δ 3.57 (1 H, m, > C$\underline{H}$ND$_3^+$), and δ 5.48 (1 H, d, J = 4.9 Hz, -C$\underline{H}$(OD)$_2$). The doublet at δ 5.48 corresponded to the resonance of the proton in hydrated amino aldehydes (1, 17). The region between 8 and 10 ppm did not exhibit any detectable resonance attributable to the proton

of a free aldehyde. The ^{1}H NMR spectra of 5-aminolevulinic acid (Figure 2B) and glutamic acid (Figure 2C) were distinctly different from that of glutamic acid 1-semialdehyde. Integration of the intensities of the resonances in the spectrum of glutamic acid 1-semialdehyde gave the expected value of one proton for the doublet at δ 5.48 (Table I). Two separate batches of glutamic acid 1-semialdehyde gave identical spectra. Furthermore, the other non-exchange-
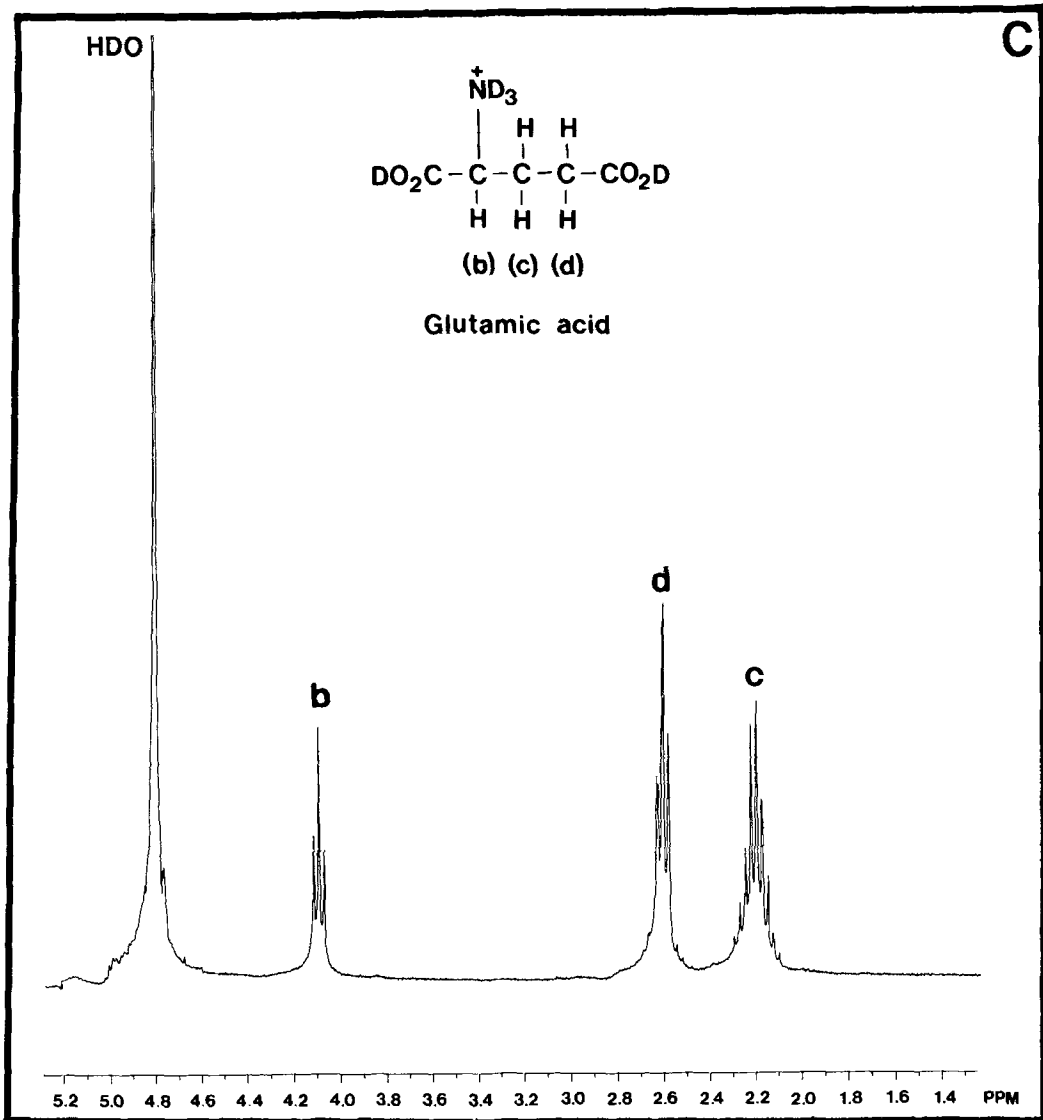
Figure 2C. Proton NMR spectrum of glutamic acid in D₂O.

able protons in the semialdehyde, glutamic acid and 5-aminolevulinic acid also gave values close to those expected.

3.3. Stability of glutamate 1-semialdehyde

Glutamic acid 1-semialdehyde in acid solutions (pH 1-2) is relatively stable, as reported previously (15). When the pH is raised, however, decay of the compound occurs at an increasingly rapid rate. As Figure 3 illustrates, at pH 8.0 the aldehyde function as determined with 3-methyl-2-benzothiazolone hydrazone (see 2.6) decayed to 35-40% of the original amount and then remained constant. Proton NMR analysis of such a degraded sample detected a resonance of an aldehydic proton at δ 8.5, but no amino group was available in this material for reaction

Table I. Integration values from proton NMR spectra.

Compound	Proton designation[§]	ppm		Integral
A. Glutamic acid	a	5.48	(d)	0.89
1-semialdehyde	b	3.57	(m)	0.98
	c	2.35	(m)	0.90
	c'	2.18	(m)	0.95
	d	2.89	(t)	2.00[+]
B. 5-Aminolevulinic	a	4.15	(s)	1.6
acid	c	2.71	(t)	1.7
	d	2.90	(t)	2.00[+]
C. Glutamic acid	b	4.16	(t)	0.90
	c	2.20	(m)	2.00
	d	2.65	(t)	2.00[+]

[§] Letter designations for protons are as indicated in Figures 2A, 2B, 2C.

[+] The integration values for the methylene protons next to the carboxyl group (C-4 in glutamic acid 1-semialdehyde) were arbitrarily set to 2.0.
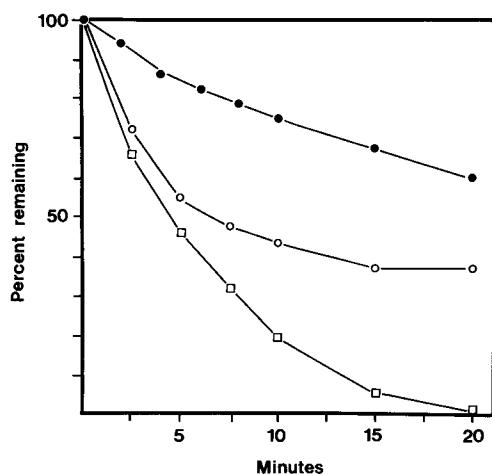

Figure 3. Loss of glutamate 1-semialdehyde at 28 °C determined chemically at pH 6.8 (●) and at pH 8.0 (○) and determined enzymically at pH 8.0 (□). Using an initial concentration of 50 μM-glutamate 1-semialdehyde relative aldehyde contents were determined chemically as given in 2.6 or enzymically as given in 2.4.

during decay suggested that condensation products were formed.

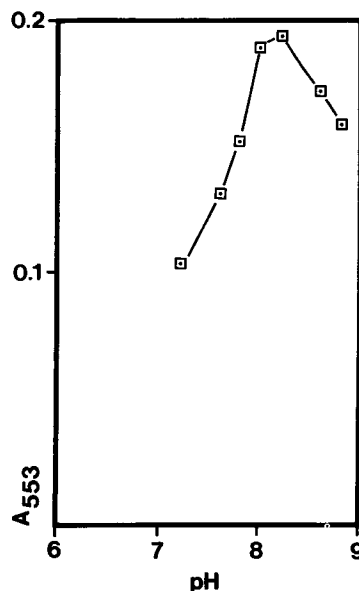Figure 3 also shows that the amount of glutamate 1-semialdehyde detected by enzymatic

Figure 4. The pH dependence of the nonenzymatic formation of 5-aminolevulinate from glutamate 1-semialdehyde. Portions of a fresh solution of 25 μM-glutamate 1-semialdehyde in 0.1 M-Na phosphate, pH 7.2, were adjusted to the indicated pH with Na_3PO_4 and incubated at room temperature for 75 min.

with ninhydrin. The $t_{1/2}$ for the decay at pH 8.0 to this plateau was 3 min. A short half-life for glutamate 1-semialdehyde after exposure to pH 8.0 was also found using an amino acid analyzer. An increase in absorbance of a solution of glutamate 1-semialdehyde at 345 and 274 nm

production of 5-aminolevulinate decayed with a $t_{1/2}$ of about 4 min at pH 8.0. Loss of substrate as measured using enzyme was complete in 20 min, even though residual aldehyde as determined chemically remained.

The rate of loss of glutamate 1-semialdehyde, as assayed chemically for aldehyde content, was much slower at pH 6.8 (Figure 3). About 15 min was required at pH 6.8 to achieve the same loss as in 3 min at pH 8.0.

3.4. Nonenzymatic conversion of glutamate 1-semialdehyde to 5-aminolevulinate

A surprisingly high rate of nonenzymatic production of 5-aminolevulinate occurred in solutions of glutamate 1-semialdehyde during incubation at pH 8. Figure 4 shows the strong pH dependence of this reaction between 7.2 and 8.2. The fall in the curve above pH 8.2 may be due in part to instability of 5-aminolevulinate in alkaline solutions. Because spontaneous formation of 5-aminolevulinate interferes with studies of the enzymatic reaction, we examined several parameters more closely. As shown by the time-course in Figure 5, the reaction was not complete at pH 7.2 after 1 hr. In another experiment, even after 2 hr the reaction produced only 62%

as much product as at pH 8.2. After 2 hr at pH 7.2, 50 nmol and 25 nmol of glutamate 1-semialdehyde produced 6 nmoles and 2.2 nmoles of 5-aminolevulinate, respectively.

This apparent lack of linearity of product formed with initial concentration of glutamate 1-semialdehyde was examined by studying the concentration dependence of the reaction over a wider range. Figure 6 shows that at all concentrations tested, the reaction was more rapid at pH 8.2 than at pH 6.8. At both pH values the reaction was not strictly linear with concentration of glutamate 1-semialdehyde, particularly at the lower concentrations.

That 5-aminolevulinate was indeed made in this nonenzymatic reaction was verified by co-elution of the product from a Dowex 50W-X8 (H^+-form) column with 5-amino(4-^{14}C)levulinate, by co-chromatography with 5-aminolevulinate on silica gel 60 sheets, and by conversion of the product to uroporphyrin as described in 2.4. Reduction of glutamate 1-semialdehyde with $NaBH_4$ destroyed its ability to be converted
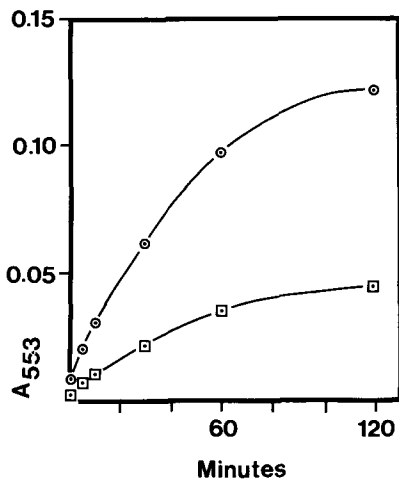

Figure 5. Time-course at 28 °C for nonenzymatic formation of 5-aminolevulinate at pH 7.2 with initial concentrations of glutamate 1-semialdehyde of 25 μM (□) and 50 μM (○).
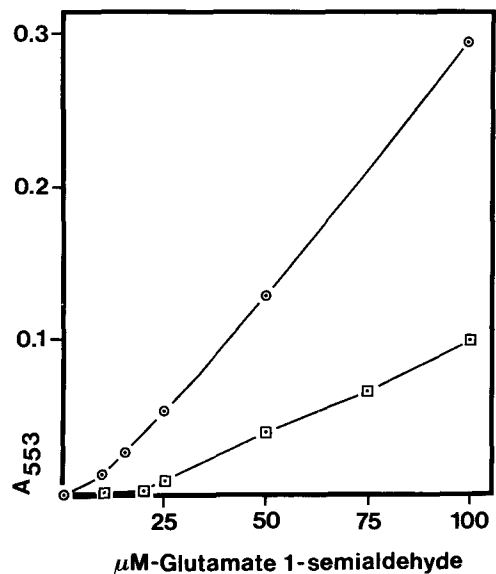

Figure 6. Nonenzymatic formation of 5-aminolevulinate in 10 min at 28 °C as a function of the initial concentration of glutamate 1-semialdehyde in 0.1 M-Na 2-(N-morpholino)ethanesulfonate-0.1 M-Na phosphate, pH 6.8 (□), and in 0.1 M-Tricine-NaOH, pH 8.2 (○).

to 5-aminolevulinate. The reduced product, presumably the amino alcohol, migrated more slowly on silica gel thin-layer sheets, with an R_f = 0.25-0.30.

3.5. Development of an assay for glutamate 1-semialdehyde aminotransferase

Because of the increased stability of glutamate 1-semialdehyde and the much slower rate of nonenzymatic conversion to 5-aminolevulinate below pH 7.2 than at higher pH, where earlier work (12, 13, 15) was performed, pH 6.8 buffer was chosen for assays of glutamate 1-semialdehyde aminotransferase. The enzyme was shown earlier to retain significant activity in this pH range (13). In most experiments unpurified rather than purified glutamic acid 1-semialdehyde was dissolved and used as substrate, because the former was more stable in the solid form and provided more consistent results. Also, the larger amounts of the unpurified material available allowed more experimentation with a single batch and thus direct comparisons within a larger set of data. Samples of purified

Figure 8. Time course of enzymatic synthesis of 5-aminolevulinate from glutamate 1-semialdehyde at pH 6.8 with initial concentrations of glutamate 1-semi-aldehyde of 25 μM (□), 40 μM (△) and 50 μM (○). The assays contained 250 μg protein in 1 ml assay mixtures and incubations were carried out at 28 °C.

glutamic acid 1-semialdehyde in which no contaminating compound other than NaCl was detected (see sections 2.2 and 3.1) showed the same characteristics in enzymatic assays as unpurified material.

The typical reaction mixture contained 50 μM-glutamate 1-semialdehyde, 250 μg of protein, and 10 mM-levulinate in 1.0 ml of 0.1 M-Na 2-(N-morpholino)ethanesulfonate-0.1 M-Na phosphate, pH 6.8. Under these conditions the enzyme catalysed formation of 5-aminolevulinate from glutamate 1-semialdehyde was nearly an order of magnitude faster than the spontaneous conversion of substrate to the product (Figure 7). Addition of glycerol, dithiothreitol or $MgCl_2$ to the assay, which were included in earlier work on this enzyme (12, 13, 15), had no effect on the activity. Glutamate or proline added to an assay with purified glutamate 1-semialdehyde did not affect activity. The reaction was allowed to proceed for various periods of time up to an hour. Figure 8 shows that the rate of enzymatic synthesis of 5-aminolevulinate was constant for nearly 40 min under these conditions but ceased abruptly after this time since the substrate was essentially exhausted. With 25 μM-substrate, the reaction stopped after about 20 min when a bit more than
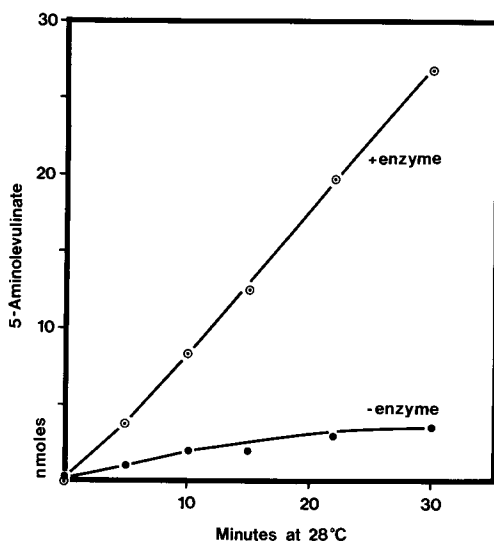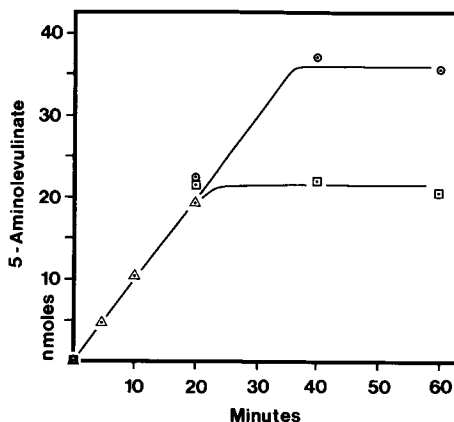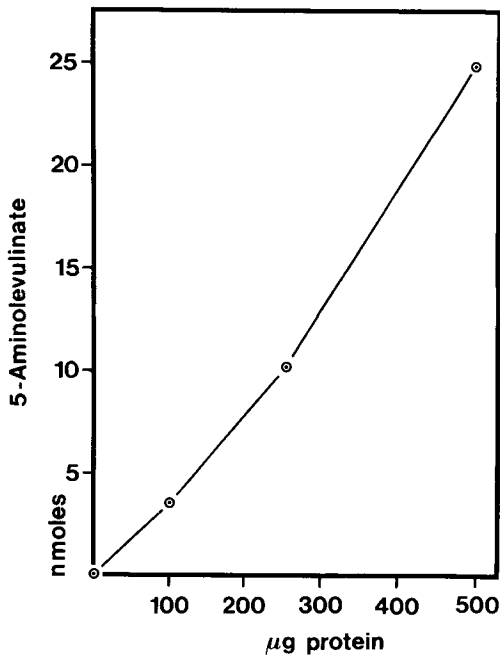

Figure 7. Formation of 5-aminolevulinate from substrate in the presence and absence of glutamate 1-semialdehyde aminotransferase. Incubations were carried out at 28 °C either with or without 250 μg of enzyme protein in 1 ml typical reaction mixtures as described in 3.5.

Figure 9. Effect of enzyme concentration on conversion of glutamate 1-semialdehyde (40 μM) to 5-aminolevulinate at pH 6.8. Assays were incubated for 10 min at 28 °C.
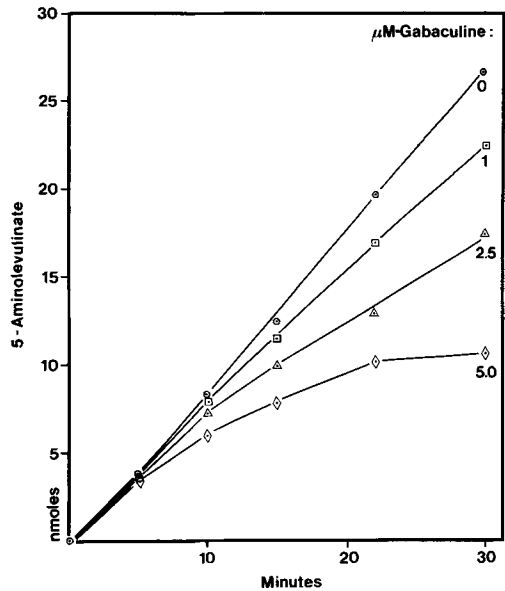

Figure 10. Progressive inactivation of glutamate 1-semialdehyde aminotransferase with increasing time in the presence of gabaculine at pH 6.8. The assays contained 50 μM-glutamate 1-semialdehyde, 10 mM-levulinate and 250 μg protein.

half as much product had been formed enzymatically. The rates of the enzymic reactions with initial concentrations of 25, 40 and 50 μM-glutamate 1-semialdehyde were the same, and no diminution in the rate (ca. 1 nmole 5-aminolevulinate formed per minute) was observed.

The relation of the rate of the enzymatic reaction with respect to the amount of protein added to the assay mixtures is shown in Figure 9.

3.6. Characteristics of gabaculine inhibition of glutamate 1-semialdehyde aminotransferase

The assay system described above was used to determine the effect of gabaculine on the aminotransferase. Figure 10 shows that in the presence of substrate the onset of inhibition by gabaculine was slow and concentration dependent. At gabaculine concentrations of 1 and 2.5 μM, inhibition remained incomplete up to 30 min, whereas full inhibition was established by 5 μM-gabaculine after about 20 min of incubation.

When aminotransferase was first preincubated with gabaculine at pH 6.8 in the presence of 10 mM-levulinate and then substrate added, the enzyme was an order of magnitude more sensitive to gabaculine than when glutamate 1-semialdehyde was presented together with gabaculine and levulinate (Figure 11). Only 0.25 μM-gabaculine was sufficient to produce 50% inhibition in 15 min in the absence of substrate, whereas 3 to 4 μM-gabaculine was required to produce 50% inhibition in 15 min in the presence of 50 μM-substrate.

Sensitivity of glutamate 1-semialdehyde aminotransferase to gabaculine was dependent upon the presence of a keto compound, such as levulinate, which apparently served as an amino group acceptor. To determine whether gabaculine was interacting with the keto compound or with the enzyme, aminotransferase was incubated with keto compounds, which then were

Table II. Treatment of glutamate 1-semialdehyde aminotransferase with keto-compounds induces sensitivity to gabaculine. Enzyme in 1.0 ml was preincubated with the various compounds at pH 6.8 for 15 min at 28 °C and then passed through a column (1×8 cm) of Sephadex G-50 to remove the compound. An aliquot of this enzyme was assayed directly for activity, whereas another aliquot was incubated with gabaculine (10 μM) at pH 6.8 for 15 min at 28 °C. The enzyme was again passed through the gel filtration column to remove free gabaculine and then assayed for activity using the typical reaction mixture given in section 3.5. Protein was measured in each case and results are expressed as percent of control specific activity. The MES-phosphate buffer described (section 2.4) was used throughout.

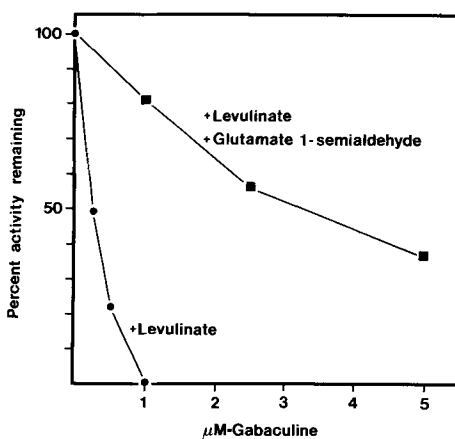| Preincubation	Gabaculine treatment	Percent enzyme activity remaining
None	–	100
	+	80 ± 5
5-Aminolevulinate (5 mM)	–	97
	+	4
Levulinate (10 mM)	–	93
	+	0
4,5-Dioxovalerate (1 mM)	–	81
	+	0
4,6-Dioxoheptanoate (1 mM)	–	84
	+	88
Pyruvate (10 mM)	+	68

Figure 11. Protection by substrate against inactivation of glutamate 1-semialdehyde aminotransferase by gabaculine in the presence of levulinate. The enzyme (250 μg protein) was preincubated at pH 6.8 with levulinate (10 mM) (●) and gabaculine (0.025, 0.5 or 1.0 μM) for 15 min at 28 °C in the absence of substrate. Then substrate (50 μm) was added. Reactions were stopped after a further 15 min at 28 °C. The points show relative enzyme activities, as assayed after preincubation. Enzyme incubated together with substrate, gabaculine and levulinate without preincubation (■). Relative enzyme activities remaining after 15 min at 28 °C were estimated from the slopes, taken between 15 and 22 min, of the curves in Figure 10.

separated from the enzyme by gel filtration. The eluted enzyme fraction was then incubated with gabaculine and again subjected to gel filtration to remove gabaculine. As listed in Table II, when the enzyme preparation was incubated first in buffer and then with gabaculine, $80 \pm 5\%$ of the activity was recovered. However, complete inactivation resulted when the enzyme was preincubated with levulinate or 4,5-dioxovalerate before exposure to gabaculine. Preincubation with 5-aminolevulinate caused nearly all the enzyme to become sensitive to gabaculine, but the extent of inactivation was not affected by 4,6-dioxoheptanoate and only slightly, if at all, by pyruvate.

4. DISCUSSION

The present results establish that glutamate 1-semialdehyde is a substrate for synthesis of 5-aminolevulinate. Proton NMR spectroscopy showed that at low pH the aldehyde is nearly quantitatively in the hydrated form. Similarly, an aldehydic proton was not detected in the proton NMR spectrum of 4,5-dioxovaleric acid, a compound proposed as another precursor of 5-aminolevulinate (5, 16). Evidence was pre-
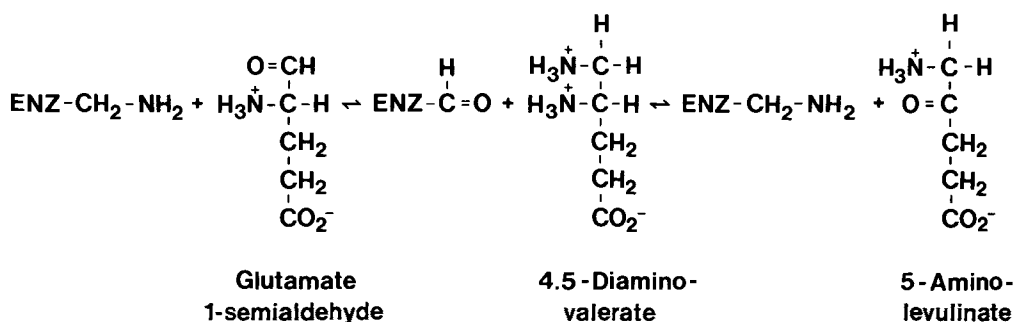
Figure 12. Proposed mechanism for the conversion of glutamate 1-semialdehyde to 5-aminolevulinate by the aminotransferase. The enzyme is represented as pyridoxamine ($-CH_2-NH_2$) or pyridoxal ($-CHO$) forms.

sented that the aldehyde group in 4,5-dioxovaleric acid also exists in the hydrated form (5). In this regard, these compounds are similar to other aldehydes and amino aldehydes (1, 17). The separation of the doublet at δ 5.48 from the amino-carbon proton at δ 3.57 in the NMR spectrum of glutamic acid 1-semialdehyde was the same as that reported for leucinal, an analogous compound (1). Since the activity of leucinal and other amino aldehydes as protease inhibitors requires the free aldehyde (1, 17), it would be interesting to determine whether hydration affects the substrate properties of glutamate 1-semialdehyde.

The apparent high efficiency of the aminotransferase has revealed the need for a more sensitive assay method to examine the enzymatic parameters of this protein. The enzyme was saturated with concentrations of glutamate 1-semialdehyde in the assay below 25 μM, and no diminution in the rate of conversion to 5-aminolevulinate was detected until the reaction had essentially reached completion. Direct determination of the K_M value of the aminotransferase for glutamate 1-semialdehyde, therefore, was not possible with the current assay methods.

A double-reciprocal plot of the data represented in Figure 11 suggested that glutamate 1-semialdehyde and gabaculine competed for the same site on the enzyme. Because kinetic parameters of the enzyme could not be determined directly, approximate dissociation constants were calculated by considering gabaculine as "substrate" and glutamate 1-semialdehyde a competitive "inhibitor" of the inactivation reaction. Appar-

ent dissociation constants for gabaculine and glutamate 1-semialdehyde were estimated as 0.5 and 3 μM, respectively. These values, obtained indirectly, should be considered as preliminary estimates for these parameters and must be confirmed by a detailed analysis of the properties of the enzyme. It is obvious that the enzyme is adapted to convert a highly reactive and unstable substrate to 5-aminolevulinate, which is required in large amounts for chlorophyll synthesis during chloroplast development. The K_M for glutamate:4,5-dioxovalerate transaminase from Chlorella, which catalyzes an alternate reaction for formation of 5-aminolevulinate, is 0.48 mM for glutamate and 2 mM for 4,5-dioxovalerate (22). The K_M for 4,5-dioxovalerate for transaminases from Clostridium (2) and radish (28) were reported as 0.26 and 0.71 mM, respectively, which are several orders of magnitude greater than that estimated for glutamate 1-semialdehyde with the aminotransferase.

Glutamate 1-semialdehyde aminotransferase may be among the most sensitive enzymes, including 4-aminobutyrate transaminase (24, 29), to inactivation by gabaculine. Inhibition of the former enzyme by aminooxyacetate (13) and by gabaculine suggest that it is a vitamin B_6-containing protein. SOPER and MANNING (29) demonstrated that only the pyridoxal phosphate form of transaminases is sensitive to gabaculine. Likewise, sensitivity of glutamate 1-semialdehyde aminotransferase to gabaculine required incubation in the presence of an amino acceptor. The enzyme apparently is extracted

from the chloroplast largely in the gabaculine-insensitive, pyridoxamine form. The pyridoxal form of the enzyme can be generated by incubating the enzyme with compounds such as levulinate and 4,5-dioxovalerate. Interestingly, these compounds have been used as inhibitors of the next enzyme in the porphyrin biosynthetic pathway, porphobilinogen synthase (3, 27), which uses 5-aminolevulinate as substrate. Positive identification of pyridoxamine phosphate as the cofactor in the aminotransferase is a task for the future.

We propose that the mechanism of the reaction catalyzed by glutamate 1-semialdehyde aminotransferase follows the scheme shown in Figure 12. For the enzyme to form 5-aminolevulinate, it must first donate the pyridoxamine amino group to glutamate 1-semialdehyde to form the intermediate 4,5-diaminovalerate. Subsequent transfer of the 4-amino group to the enzyme then liberates 5-aminolevulinate and regenerates the amino form of the enzyme. We expect that the enzyme should catalyze a reversible reaction, although our results suggest that glutamate 1-semialdehyde is converted essentially quantitatively to 5-aminolevulinate.

In conclusion, we have defined the properties of glutamate 1-semialdehyde aminotransferase with respect to structure of the substrate, suitable conditions for assaying the activity of the enzyme, and the interaction of the enzyme with gabaculine. The basis is now available to begin a detailed study of the kinetic and mechanistic properties of the aminotransferase.

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