A Novel Reaction of the Coenzyme of Glutamate Decarboxylase with L-Serine *O*-Sulfate†

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ABSTRACT: Reaction of glutamate decarboxylase of *Escherichia coli* with L-serine *O*-sulfate causes inactivation of the enzyme and the appearance of an absorption band at 336 nm [Sukhareva, B. S., & Braunstein, A. E. (1971) *Mol. Biol. (Moscow)* *5*, 302]. The 336-nm absorbing material **1** is converted instantaneously at high pH to a low molecular weight yellow compound, **2** [Likos, J. J., & Metzler, D. E. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1545]. We have now characterized this compound by proton and ^{13}C NMR spectroscopy and by conversion to derivatives by dephosphorylation and by reduction. Compound **2** is a known compound which had been synthesized previously [Schnackerz, K. D., Ehrlich, J. H., Geisemann, W., & Reed, T. A. (1979) *Biochemistry 18*, 3557] by an aldol condensation between pyridoxal phosphate and pyruvate followed by dehydration. It is proposed that **1** is an adduct formed by attack of the β carbon of α-aminoacrylate on the "internal" Schiff base of pyridoxal phosphate with a lysine side chain from the protein. Aminoacrylate is generated from the quasi-substrate serine *O*-sulfate by enzyme-catalyzed β elimination of the sulfate group. Compound **2** is presumably released by attack of the hydroxyl ion on adduct **1**. Release is prevented by treatment with sodium borohydride or cyanoborohydride which is assumed to reduce the C=N or C=O linkage in **1** and thereby lower the acidity of the carbon-bound proton removed in the formation of **2**. Essentially the same set of reactions is proposed for another enzyme, aspartate aminotransferase, as is described in the following paper [Ueno, H., Likos, J. J., & Metzler, D. E. (1982) *Biochemistry* (following paper in this issue)]. These results suggest that the mechanisms of action of other "suicide inactivators" of pyridoxal phosphate dependent enzymes must be reevaluated.

Glutamate decarboxylase of *Escherichia coli* is a pyridoxal phosphate dependent enzyme whose properties have been described in considerable detail (Shukuya & Schwert, 1960; Strausbauch & Fisher, 1970a,b; Sukhareva & Tikhonenko, 1972; Mekhanik & Torchinsky, 1972; Mekhanik et al., 1972; Fonda, 1972a,b, 1975, 1976; O'Leary & Brummund, 1974). The hexameric enzyme has a subunit molecular weight of 50000. It contains one molecule of pyridoxal phosphate per subunit. As with other pyridoxal phosphate containing enzymes, the coenzyme exists as a Schiff base with a lysine side chain at low pH, the absorption maximum being at 420 nm. In a cooperative transition centered at pH 5.6 and involving simultaneous loss of four or more protons, the Schiff base is converted to another form, possibly a geminal diamine, with a peak at 330 nm.

Glutamate decarboxylase undergoes an interesting side reaction, a decarboxylative transamination with DL-α-methylglutamate. The products are pyridoxamine phosphate, which absorbs at 326 nm, CO_2, and levulinic acid (Huntley & Metzler, 1968). The same type of reaction has been shown by Sukhareva & Braunstein (1971) to occur very slowly with the normal substrate, L-glutamate, and to cause slow inactivation of the enzyme. These authors also observed that L-serine *O*-sulfate inactivates the enzyme. However, the absorption band of the inactive product is centered at 336 nm, not the same as that of free pyridoxamine phosphate. Indeed, as reported by Likos & Metzler (1976), the reaction is of a quite different nature than that with α-methylglutamate. In this report, we propose a structure that accounts for the 336-nm band and provide proof for the structure of a yellow compound released from the inactivated enzyme by sodium hydroxide. A preliminary report has been published (Likos et al., 1982).

Materials and Methods

All chemicals used were of reagent grade. L-Serine *O*-sulfate was synthesized according to the procedure of Tudball (1962). The purity and authenticity was checked by using high-voltage electrophoresis and NMR.

Spectra. Most of the nuclear magnetic resonance (NMR) spectra were obtained with a Bruker WM300-WB spectrometer, but JEOL-FX90Q and Bruker HX90 spectrometers were also used. Standard 5-mm sample tubes were used. Chemical shifts were recorded in parts per million (ppm) relative to an external standard present in a capillary tube. The standard for ^1H spectra was tetramethylsilane (Me_4Si) in $CDCl_3$ and that for ^{13}C spectra was dioxane. For the latter, 66.5 ppm was added to the observed shifts. Typically 16K or 32K words of computer memory were used for ^1H or ^{13}C measurements, giving a digital resolution of 0.49 or 1.22 Hz, respectively. To avoid an undesirably high HDO peak, we used 100% D_2O as the solvent which also provided the internal deuterium lock signal.

The value of pD was estimated by adding 0.41 to the reading on a Radiometer Model PHM64 pH meter. The pD was adjusted by addition of DCl or NaOD. High-resolution 5-mm sample tubes were used. A 30° flip angle (2.1-μs pulse) and 0.1-s delay time were used for most proton spectra which were measured with water saturation. For ^{13}C spectra, a 60° flip angle (15-μs pulse) and 0.2-s delay time were used. Broad band decoupled, gated decoupled or specific proton decoupled spectra were taken to assign the ^{13}C resonances.

Absorption spectra were measured with a Cary 1501 recording spectrophotometer interfaced through a Cary–Datex digital output to an IBM card punch. Spectra were corrected for base-line errors and small amounts of turbidity. The data were replotted automatically as molar absorptivity or absorbance vs. wave number (Johnson & Metzler, 1970; Metzler et al., 1973). The method of Nagano & Metzler (1967) was

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used to evaluate pK values, to plot spectrophotometric titration curves, and to calculate the spectra of the individual ionic forms of the compounds studied. The mass spectrum was obtained with a Finnegan 4000 mass spectrometer.

Enzyme. L-Glutamate decarboxylase (EC 4.1.1.15) was isolated from *E. coli* (ATCC 11246) as described by Fonda & DeGrella [1974; see also Yang & Metzler (1979)]. The final two steps make use of columns of DEAE-Sephadex A-50 and Sephacryl S-200, respectively. It was found that during the first of these steps, a protein "factor" running behind the glutamate decarboxylase was sometimes separated from the enzyme. It was necessary to add this factor back to the enzyme before a rapid reaction with serine sulfate could be observed (Ueno, 1982). Molar concentrations of the enzyme active sites were calculated from the height of the absorption band at 420 nm at pH 4.6 assuming a molar absorptivity of 10000 (Fonda, 1971). Enzyme activity was determined with a Gilson differential respirometer (Fonda, 1971).

Preparation of the Initial Product (**1**) *of Reaction of Serine Sulfate with Glutamate Decarboxylase.* In a typical preparation 52.5 mL of 6.1×10^{-5} M glutamate decarboxylase (160 mg of protein; 3.2 μmol) in 0.1 M potassium acetate buffer, pH 4.6, containing 10^{-4} M dithiothreitol was used. To this solution was added 62 mg of solid L-serine *O*-sulfate (to give a concentration of 5.3 mM) and, when necessary, 0.60 mL of a solution of the protein factor that had been separated from the enzyme. This contained 13 units of activity as defined by Ueno (1982). The reaction mixture was left at room temperature for about 6 h during which the absorbance at 420 nm dropped from 0.607 to 0.093. The modified enzyme (**1**) formed in this case was dialyzed overnight against three 2-L portion of redistilled water.

Preparation of Low molecular Weight Yellow Product **2**.[1] The pH of a solution of compound **1** was raised to 11.0 by addition of 0.1 N KOH. The resulting bright yellow compound (**2**) was separated from the protein by ultrafiltration through an Amicon PM10 membrane. The protein solution remaining (about 1–2 mL) was diluted with 3–5 mL of 0.05 N KOH and again filtered. This "washing" was then repeated 2 more times, and all the washings were combined with the initial ultrafiltrate. The resulting solution, which contained from 60 to 90% of the initially formed absorbance at 420 nm, was passed through a thoroughly washed 0.7×2 cm column of Dowex 50 (X8, 100–200 mesh) ion-exchange resin in the H^+ form. The column was eluted with 10 mL of water. The measured pH of the eluate was 5.0–5.5. The solution was concentrated under vacuum to the desired volume or was lyophilized.

An alternative method of preparation was to first raise the pH to 11 and to then add solid trichloroacetic acid to precipitate the protein. Over 90% of the chromophore remained in the supernatant.

Compound **2** was sometimes purified further by high-voltage electrophoresis. Sheets (23×57 cm) of Whatman 3MM paper were used for electrophoresis with 0.1 M acetate buffer, pH 4.7, as the electrolyte. Electrophoresis was conducted for 20 min at 2000 V. Under these conditions **2** had the greatest electrophoretic mobility and moved 6.5 cm. The sample could be located by spraying a test strip of the electropherogram with Gibbs' reagent or by its distinct orange fluorescence when illuminated by ultraviolet light (Mineralight UVS 11, Ultraviolet Products Inc., San Gabriel, CA). Compound **2** was eluted from the dried electropherogram with water and applied to a 0.7×1 cm column of Dowex 50 (H^+ form, 100–200 mesh). It was again eluted with water and lyophilized.

Synthesis of Compound **2**. The spectral properties of compound **2** appeared to be similar (see Results) to those of a compound prepared by aldol condensation of pyridoxal phosphate and pyruvate followed by dehydration (Schnackerz et al., 1979). This reference compound was pepared and purified as described by Schnackerz et al. Fractions from the Dowex 50 (X8) column containing the product were identified by adding a small aliquot of each fraction to 3-mL portions of 0.02 M NaOH and measuring the absorbance spectrum. Those fractions with an absorption maximum at 425 nm were pooled and concentrated. The product was crystallized from a water–ethanol mixture.

Preparation of **3** *by Dephosphorylation of* **2**. A solution of **2** at pH 11 as obtained by ultrafiltration of the inactivated and base-treated enzyme was adjusted to pH 8.1 with 0.05 N HCl. Approximately 0.03 mg of alkaline phosphatase from *E. coli* (code BAPF, Worthington Biochemicals) was then added. The dephosphorylation reaction was followed by the decrease in absorbance at 406 nm. After approximately 13 h at room temperature, the spectrum did not change further. The solution was lyophilized. The residue was dissolved in 0.6 mL of water and was passed through a 1.5×68 cm column of Bio-Gel P2 with water as the eluant. Compound **3** emerged shortly after the void volume in a sharp band. An alternative purification procedure was used for some samples. The lyophilized residue was dissolved in 0.3 mL of water and was subjected to high-voltage electrophoresis in 0.1 M acetic acid adjusted to pH 4.7 with pyridine. Electrophoresis was carried out for 20 min at 2000 V. Under these conditions the major product (as determined qualitatively by the intensity of the Gibbs test) migrated 2.5 cm toward the anode. After the electropherogram was dried, the material (**3**) was eluted with water and was lyophilized.

Synthesis of **3**. The dephosphorylated analogue of **2** was synthesized from pyridoxal hydrochloride and sodium pyruvate as described by Schnackerz et al. (1979). Fractions from a Dowex 2 (X8) column containing the desired product were identified as described above for **2**. The 425-nm-absorbing fractions were combined and dried by rotary evaporation. Compound **3** was crystallized from water.

Preparation of **5** *by the Reduction of Isolated* **3**. The pH of a solution of **3** was lowered to 7.0 with 0.1 N HCl. Solid sodium cyanoborohydride was then added. The reduction reaction was followed by the decrease in the absorbance at 406 nm. The reaction solution was left overnight at room temperature and was then acidified by the addition of 50 μL of 6 N HCl. The product was partially purified by gel filtration. The yield was about 55%.

Synthetic **5**. Crystalline **5** was prepared by dissolving 150 mg (0.63 mmol) of synthetic **3** in 40 mL of 4.4 M acetic acid. Solid $NaBH_4$ (360 mg, 9.5 mmol) was added in small increments ($\sim$10 mg per addition). The rate of addition was sufficiently slow to allow for complete cessation of H_2 production before the next addition of solid $NaBH_4$. The extent of reaction was determined by diluting an aliquot of the reaction mixture with 0.1 M NaOH and measuring the absorbance spectrum. Lack of absorption at 425 nm was indicative of complete reduction. The indicated large excess of $NaBH_4$ was required for complete reduction. The reaction mixture

[1] Abbreviations: compound **2**, 4-[3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxo-3-butenoic acid; compound **3**, 4-[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]-2-oxo-3-butenoic acid; compound **4**, 4-[3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxobutyric acid; compound **5**, 3-(1,3-dihydro-7-hydroxy-6-methyl-1-furo[3,4-*c*]pyridinyl)-2-oxopropionic acid; compound **6**, 4-[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]butyric acid.

was taken to dryness, redissolved in H_2O, and applied to a 1 × 30 cm ion-exchange column (AG 1, X8, 200–400 mesh, formate form). The reaction products were eluted with 0.5 L of formic acid with a 0–2 M linear gradient. The major Gibbs' positive material eluted between 94 and 122 mL. These fractions were combined and taken to dryness. After being dried down from water several times, the solid was crystallized from a water–ethanol mixture.

Preparation of **4** *and* **6** *from* **2**. Compound **2** was reduced with sodium borohydride at pH 8, the reaction being followed by the decrease in the absorbance at 420 nm. The solution was then acidified with 50 μL of 6 N HCl to destroy the excess borohydride. Partial purification was accomplished by gel filtration on a Bio-Gel P2 column eluted with water. The pH of a solution of **4** was adjusted to 8–9, and ~0.03 mg of *E. coli* alkaline phosphatase was added. The dephosphorylation reaction was run overnight at room temperature, and then the solution was lyophilized.

Alkaline Hydrolysis of **2**. To 200 μL of a solution containing 18 nmol of **2** in a 1.1 × 10 cm Pyrex test tube was added 20 μL of 1 M KOH. The pH was 12.5–13.0. The test tube was sealed with a torch and was heated at 100 °C for 4 h. Portions of the solution as well as a sample of unheated **2** were assayed for pyridoxal phosphate as follows: the apo form of the cytoplasmic isoenzyme of aspartate aminotransferase of pig hearts was prepared (Yang & Metzler, 1979) at a concentration of 6.4 mg/mL (1.4×10^{-4} M) in 0.02 M triethanolamine hydrochloride–NaOH buffer, pH 8.3. To a series of 50-μL portions, each containing 7 nmol of the enzyme, were added amounts of pyridoxal phosphate ranging from 0.05 to 3 equiv per equiv of apoenzyme. After 10 min at room temperature, 10 mL of water was added to each tube. The diluted samples were then assayed for aspartate aminotransferase activity as described by Furbish et al. [1969; see also Yang & Metzler (1979)].

Results

Serine *O*-sulfate (12.5 mM) and glutamate decarboxylase (4.1×10^{-5} M) that had been purified through the step of chromatography on DEAE-Sephadex A-50 were incubated in 0.05 M sodium acetate buffer, pH 4.6, 25 °C. Six-microliter aliquots were taken at timed intervals and were assayed for decarboxylase activity (the dilution into the 3-mL assay mixture containing glutamate quenched the inactivation reaction immediately). Activity decayed very rapidly at first but more slowly later. Half of the activity was lost in the first 5 min. Less than 10% remained after 90 min, and the enzyme was totally inactivated in 4 h. Although the reaction mixture normally contained 10^{-4} M dithiothreitol, no difference was seen when it was omitted. Preliminary kinetic studies have been reported by Likos (1977).

Protein Factor. Several batches of enzyme reacted just as described in the preceding paragraph, but later we obtained a presumably more highly purified enzyme that failed to react with serine sulfate. At this point we began to add back other fractions from the DEAE-Sephadex A-50 column and discovered that a fraction running behind the glutamate decarboxylase enhanced dramatically the rate of reaction with serine sulfate. While the kinetics of the inactivation reaction are complex, the rate was determined largely by the concentration of the factor present in this fraction. An assay for the factor has been devised, and some purification has been achieved (Ueno, 1982). It appears to be a protein of molecular weigfht about 30000. The partially purified factor alone has no apparent effect on serine sulfate as judged by electrophoresis and has little effect on the enzymatic activity of glutamate decarboxylase acting on its normal substrate. The course of the inactivation reaction was unaffected by preincubation of the protein factor with the serine sulfate for 10 min.

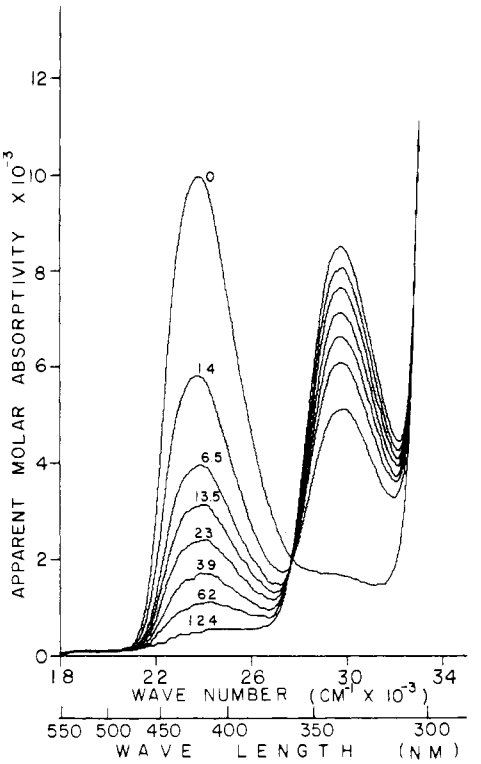


FIGURE 1: Reaction of glutamate decarboxylase with L-serine *O*-sulfate. The curve marked 0 is 4.05×10^{-5} M holoenzyme. The numbers by the other curves give the times in minutes after addition of 12.5 mM serine sulfate. The pH was 4.6, with 0.05 M sodium acetate buffer, 10 °C.

Compound **1**. Inactivation of glutamate decarboxylase by serine sulfate was accompanied by the characteristic spectral change shown in Figure 1. The inactive product, designated **1**, has a well-defined absorption maximum at 336 nm whose position, shape, and intensity are characteristic of a substituted 3-hydroxypyridine compound in a dipolar ionic form.

Dialysis of the inactivated enzyme (compound **1**) against 0.05 M acetate buffer, pH 4.6, for 24 h did not alter the spectrum. Under the assumption that the molar absorptivity at 282 nm is the same as that of the holoenzyme (88000 M^{-1} cm^{-1}, based on monomer; Fonda, 1971), the molar absorptivity of the chromophore was estimated as 8.2×10^3 M^{-1} cm^{-1} at 336 nm. Analysis of the supernatant fluid after the inactivated enzyme had been precipitated with trichloroacetic acid indicated that the chromophore precipitated with the protein. By contrast, reaction of the enzyme with α-methylglutamate gave a product (pyridoxamine phosphate) that absorbs at 325 nm and which is easily separated from the protein.

Dialysis of **1** overnight against 8 M urea in 0.05 M acetate buffer, pH 4.5, led to a hypsochromic shift of about 8 nm and a 50% decrease in absorbance at the 336-nm peak (relative to the 280-nm peak height). This change did not appear to involve any loss of coenzyme. When **1** was denatured with 10 M urea and the protein was precipitated with trichloroacetic acid, no 3-hydroxypyridine compound was released. By comparison, precipitation of the native enzyme with trichloroacetic acid releases about 35% of the bound pyridoxal phosphate.

Formation of Yellow Compound **2**. When the pH of a solution of **1** was raised to 11, an intense yellow color developed, and the peak moved to 420 nm. While compound **1** appears to be tightly bound to the protein, the yellow product

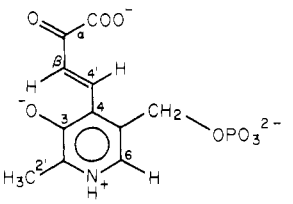
2 formed at high pH can be removed from the protein readily by ultrafiltration. Suspension of trichloroacetate-precipitated **1** at pH 11 also resulted in the immediate formation of **2**.

The bright yellow color of **2** appears almost instantaneously when a solution of **1** is raised to a pH above 9. However, **2** cannot be separated readily from the protein by ultrafiltration unless the pH is raised to about 11. Similarly if trichloroacetic acid is added to a solution of **1** that has been raised to pH 11, the protein is precipitated, but **2** is left in solution. However, if the pH is raised only to 9, no **2** is found in the supernatant after addition of trichloroacetic acid.

The position of the absorption band of **1** was not altered by the addition of an excess of sodium borohydride or sodium cyanoborohydride at pH 4.5. However, if the pH of the borohydride-treated solution was then raised to 11, no compound **2** was formed. Compound **2** was partially purified as described under Materials and Methods.

Both β-chloroalanine and β-phosphoserine also react with glutamate decarboxylase to yield inactive enzyme absorbing at 336 nm. Addition of base to the product from either of these amino acids results in the formation of a compound with spectral and electrophoretic properties identical with those of **2**. On the other hand, no reaction was observed with 3–20 mM DL-α-methylserine *O*-sulfate.

Synthetic **2**. In 1979 Schnackerz et al. reported the synthesis of the following product of an aldol condensation between pyridoxal phosphate and pyruvate followed by dehydration.



2 (neutral pH form)

We noticed that the reported spectral properties of this compound were similar to those of our isolated compound **2**. Synthesis of this and of the corresponding dephosphorylated compound by the published procedures (Schnackerz et al., 1979) and comparison of spectral properties and high-voltage electrophoresis showed conclusively the identity of the two synthetic compounds with **2** and **3**, respectively. The identity was further substantiated by comparison of synthetic **2** and of several of its derivatives with the corresponding compounds obtained by reaction of serine sulfate with either glutamate decarboxylase or cytosolic aspartate aminotransferase.

Pyruvate is an expected product from serine sulfate and could conceivably be converted to **2** nonenzymatically by NaOH. Therefore we mixed 10^{-4} M pyridoxal phosphate with 10^{-4}–10^{-3} M sodium pyruvate at pH 8 and then raised the pH to 11. No **2** was formed.

Properties of Compound **2**. The electrophoretic mobilities of compounds **2** and **3** were compared with those of the reference compounds pyridoxal phosphate, pyridoxamine phosphate, pyridoxal, and pyridoxamine (Figure 2). An important fact is that **2** migrates more rapidly toward the anode than does pyridoxal phosphate at all values of pH from 3.3 to 10. Synthetic **2** mixed with **2** from either glutamate decarboxylase or aspartate aminotransferase (Ueno et al., 1982) migrated as a single spot at pH 6.5.

The absorption spectrum of **2** from glutamate decarboxylase was identical with that of synthetic **2** (Figure 3; Table I). The change in spectrum with pH indicated the existence of three

Table I: Peak Positions in Electronic Absorption Spectra

compound	band maxima (nm) cationic ring	dipolar ionic ring	anionic ring
2 from GAD[a]	~325 (sh)	413	426
	282	289	296
2, synthetic	~323 (sh)	411[b]	426[b]
	284	286	295
		244	
3 from GAD[a]	283	407	420
		311	296
3, synthetic	283	410	425
		310	296
5 from GAD[a]	286	313	296
5 from AAT[b,c]	283	311	295
5, synthetic[b]	284	311	295

[a] From glutamate decarboxylase. [b] Evaluated by computer-assisted fitting with log-normal curves (Harris et al., 1976). [c] From aspartate aminotransferase (Ueno et al., 1982).

Table II: Values of pK Determined Spectrophotometrically

compound	pK values at 25 °C
2, synthetic	3.93, 8.70
2 from glutamate decarboxylase; duplicate titrations	3.93, 8.74 3.98, 8.69
3, synthetic	
estimated for open form 3a	3.7, 8.2[a]
estimated for cyclic form 3c	4.5, 8.7[b]
5, synthetic	4.77, 9.17
from aspartate aminotransferase	4.8,[b] 9.3[b]

[a] Based on data above 345 nm. [b] Based on the assumption that 3 contains 33% 3a and 67% 3c and that spectra of the ionic forms of **2** and 3a are identical.

spectrally distinct ionic forms separated by pK values of 3.9 and 8.7. The pK values of isolated and synthetic **2** were also in good agreement (Table II). Sharp isosbestic points were present in a series of spectra at varying pH indicating a high degree of spectral purity.

Proton NMR spectra were obtained on samples of **2** isolated from both glutamate decarboxylase and aspartate aminotransferase in the range pD 1.2–2.5. They are shown in Figure 4 along with that of synthetic **2**. Additional spectra were recorded at neutral and alkaline values of pD. The proton NMR data are summarized in Table III. The ^{13}C NMR spectrum of synthetic **2** is shown Figure 5. It was not possible to obtain comparable spectra on the isolated samples because the amounts were too small.

The proton NMR spectrum of **2** at low pH is striking (Figure 4). The sharp resonances of the 6-H (8.30 ppm) and 2′-CH_3 protons (2.72 ppm) and the doublet at 5.09 ppm representing the 5′-CH_2 split by phosphorus are anticipated. For example, the corresponding positions for pyridoxamine phosphate are 8.36, 2.75, and 5.16 ppm (doublet), respectively. The pair of coupled doublets at 7.79 and 7.35 ppm must represent the 4′ and β protons. The coupling constant of 16.6 Hz, observed for synthetic **2** and for both isolated samples of **2**, shows that the configuration about the 4′–β C=C bond is trans.

Besides the major species of **2**, there is a second form present at low pH, as is indicated by the coupled doublets at 7.03 and 6.67 ppm. These have a coupling constant of 16.1 Hz. Integration of the 4′-H and β doublets indicates a ratio of the

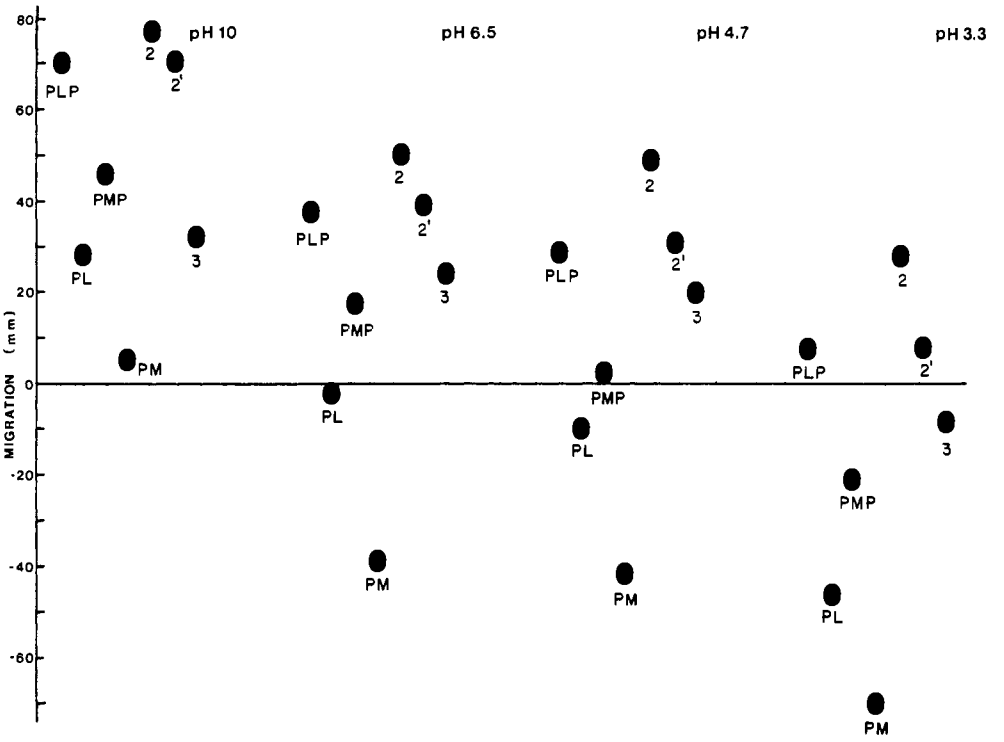


FIGURE 2: High-voltage electrophoresis of **2** and related compounds at four values of pH. Abbreviations: PLP, pyridoxal phosphate; PL, pyridoxal; PMP, pyridoxamine phosphate; PM, pyridoxamine; **2** and **3**, compounds **2** and **3**; **2'**, compound **2** after hydrolysis in NaOH.

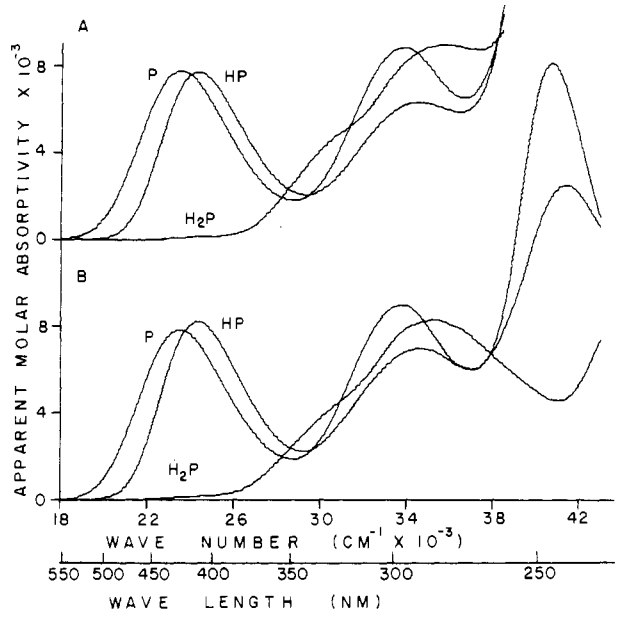


FIGURE 3: Absorption spectra of individual ionic species of keto acid **2**. (A) Compound isolated from reaction of glutamate decarboxylase with serine sulfate. (B) Synthetic compound. H_2P, low pH cationic ring form; HP, neutral form with dipolar ionic ring; P, high pH anionic ring form. The phosphate and carboxyl groups have been ignored, as their dissociation has almost no effect on the spectra.

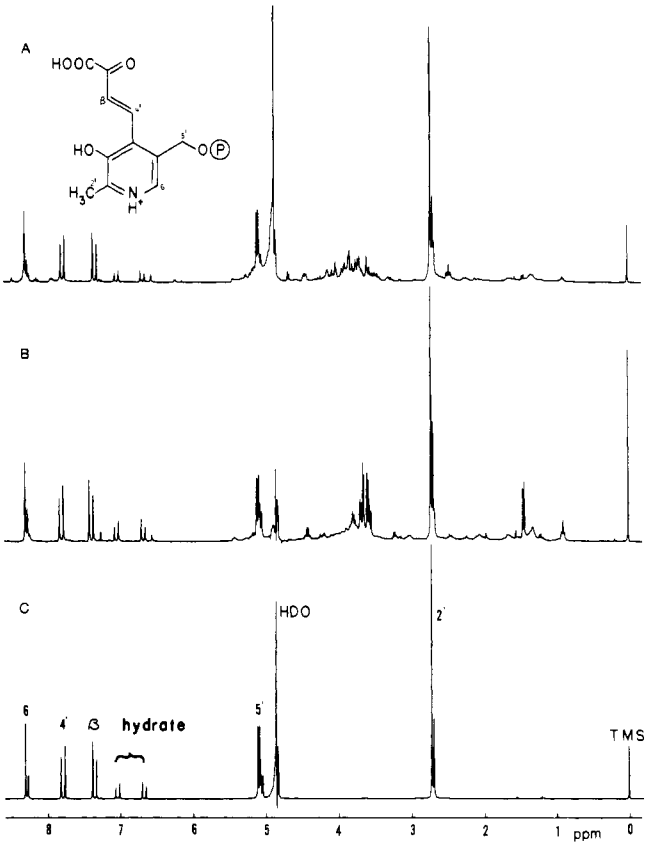


FIGURE 4: Proton NMR spectra of **2** obtained on a 300-MHz spectrometer at low pH at 24 °C. (A) Compound isolated from glutamate decarboxylase. (B) Compound isolated from aspartate aminotransferase. (C) Synthetic compound. The standard is Me_4Si (TMS).

major form to this minor form of 3.3 to 1. Resonances for the 6, 2', and 5' protons of the minor form are also present and in the same ratio (Table III). The most obvious possibility is that the minor form is the covalent hydrate of **2** and that the hydration is at the carbonyl group. This interpretation is supported by the ^{13}C NMR spectrum of synthetic **2** (Figure 5) where the resonance for the hydrated carbonyl is seen clearly at 91.7 ppm. Again the relative intensities of the two bands indicate a hydration ratio of 2.7:1. This value is less reliable than that from the proton NMR. Resonances of the hydrate are also seen for the other carbon atoms with the exception of C-5 and C-5'. Hydration ratios estimated from these varied between 1.9 (COO^-) and 3.5 (C-4). Both ^{13}C and

Table III: Chemical Shifts of Resonances in Proton NMR Spectra

	pD 2.48			pD 7.2			pD 12.4 (40 °C)		
compound **2**	ppm	*J* (Hz)	rel area	ppm	*J* (Hz)	rel area	ppm	*J* (Hz)	rel area
2'-CH_3 ketone[a]	2.72		3.32	2.49		3.25	2.28		3.66
hydrate[a]	2.68		1.04						
4'-H ketone	7.82 7.76	16.6	0.95	7.80 7.75	16.1	1.15	7.92 7.87	16.1	1.00
hydrate	7.06 7.01	16.1	0.29						
5'-CH_2 ketone	5.10 5.08	7.81	2.20	5.00 4.97	5.0	2.28	4.78 4.76	4.4	2.34
hydrate	5.06 5.04	7.8	0.71						
6-H ketone	8.30		(1.00)	7.64		(1.00)	7.51[b]		2.13[c]
hydrate	8.27		0.30						
β-H ketone	7.38 7.33	16.6	0.92	7.72 7.67	16.1	0.82	7.57 7.51		
hydrate	6.70 6.65	16.1	0.28						

[a] The ratio of ketone/hydrate is 3.26/1 at 24 °C as estimated from the β-hydrogen peaks. [b] Overlapped. [c] 6-H + β-H.

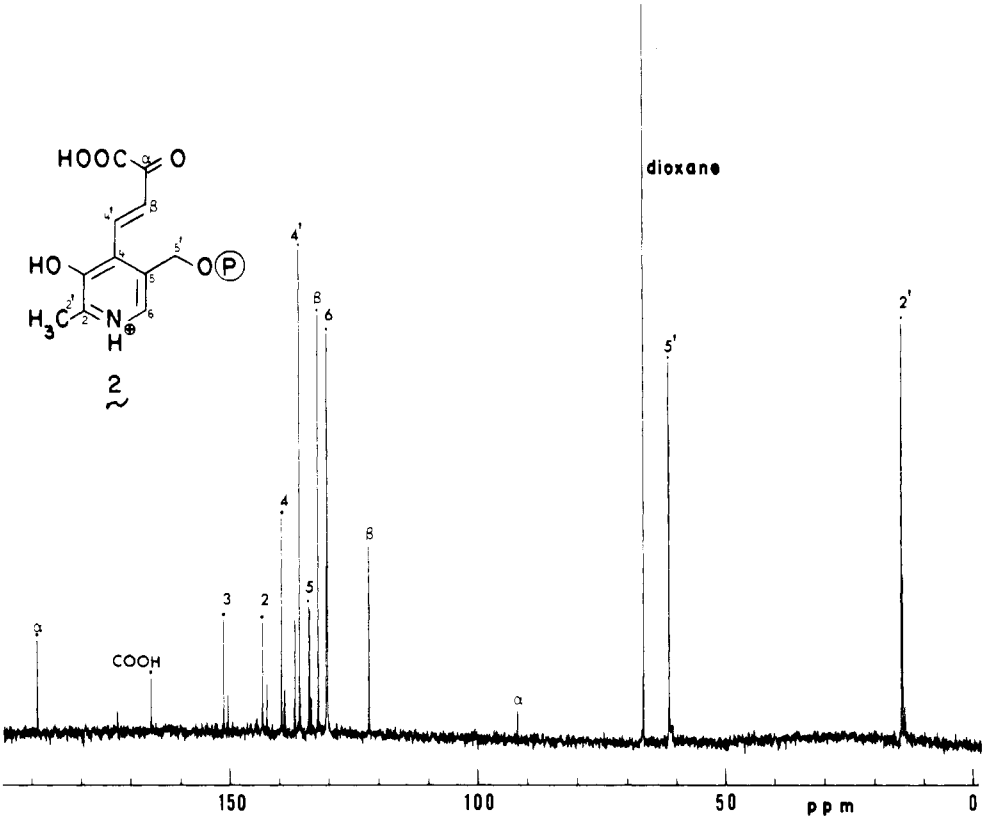


FIGURE 5: Natural abundance carbon-13 NMR spectrum of synthetic compound **2** at pH 1.5 (pD 1.9).

^{1}H (Table III) NMR spectra of **2** at neutral and alkaline pH values are also consistent with the assigned structure, but there is no evidence for any hydrate at these pH values.

As can be seen from Figure 2, the spectra of the isolated and synthetic **2** are identical except for the presence of resonances caused by impurities in isolated **2**. All of the protons in isolated **2** are observed, and the chemical shifts are the same within 0.01 ppm as those for synthetic **2**. However, the 4' proton of the ketone formed showed a very small pH variation being 0.02 ppm further downfield in the sample isolated from aspartate aminotransferase and measured at pD 1.2 than for synthetic **2** measured at pD 2.5. Likewise the β-proton doublet was shifted by about 0.04 ppm, presumably as a result of more complete protonation of the carboxyl group at the lower value of pD.

Hydrolysis of **2** *to Pyridoxal Phosphate.* When compound **2** was heated for 4 h at 110 °C at pH 12.5–13, it appeared to be reconverted to pyridoxal phosphate almost quantitatively as judged by three criteria. The absorption spectra at pH 1.7, 7.3, and 9.5 were almost the same as those of pyridoxal phosphate. The migration on electrophoresis at four values of pH was also the same as that of pyridoxal phosphate (Figure 2). Assay by reactivation of the apo form of aspartate aminotransferase (see Materials and Methods) regenerated 88% of the activity expected for an equivalent amount of pyridoxal phosphate.

Conversion of **2** *to Dephosphorylated and Reduced Derivatives.* Treatment of **2** with alkaline phosphatase from *E. coli* (see Materials and Methods) converted **2** to **3**, a compound with decreased electrophoretic mobility (Figure 2) and a

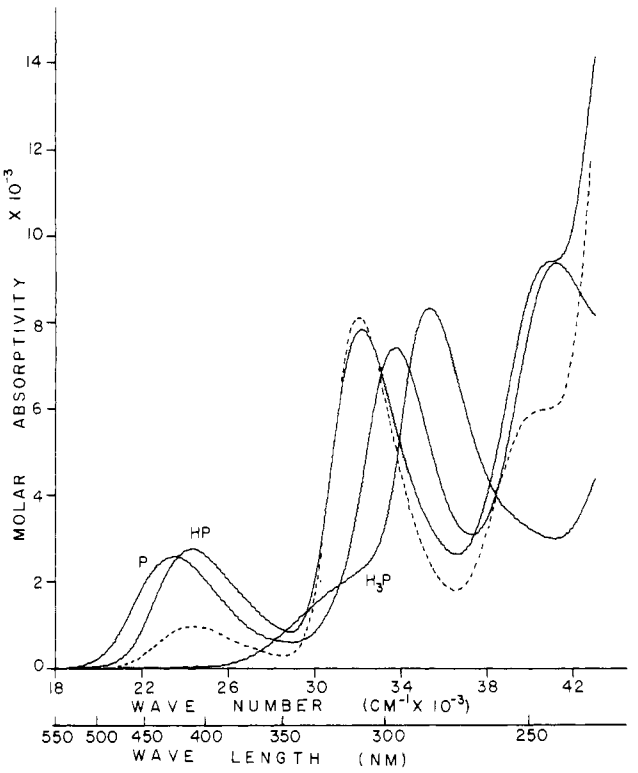


FIGURE 6: Absorption spectra of three ionic forms of synthetic compound **3** measured immediately after the pH was lowered from pH 11.9. H_3P, triprotonated form (cationic ring and undissociated carboxyl group); HP, monoprotonated form; P, unprotonated form. The dashed line represents the spectrum of **3** after standing for 4 h in 0.05 M phosphate buffer, pH 7.2.

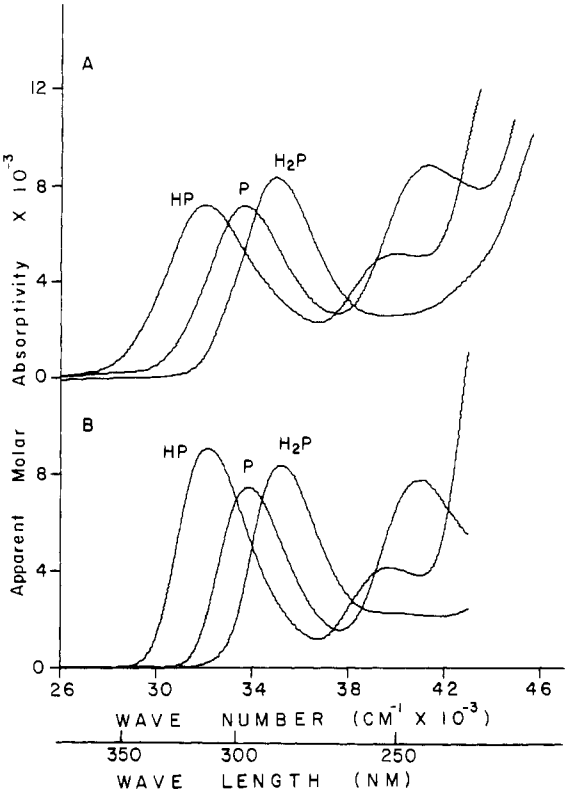
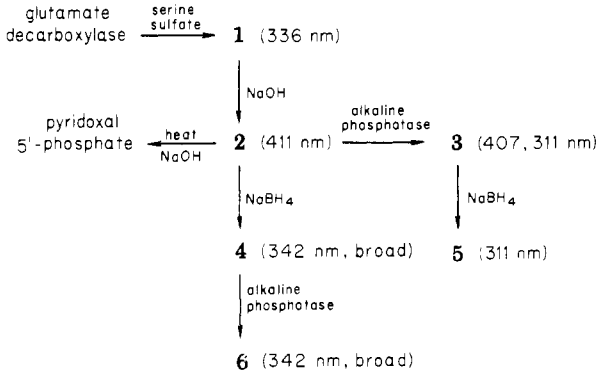


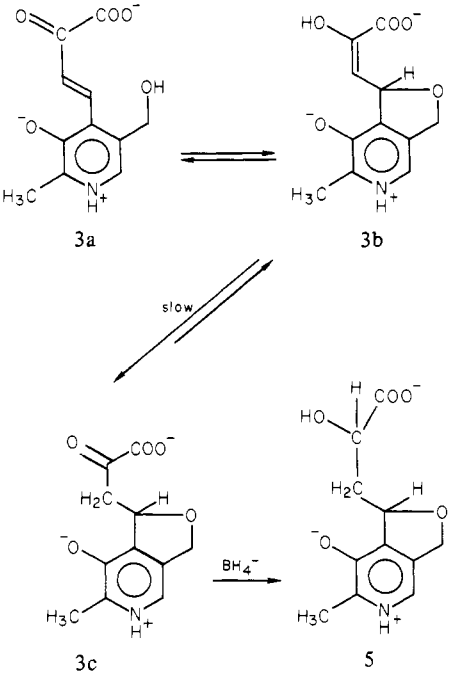
FIGURE 7: Absorption spectra of three ionic forms of compound **5**. (A) Prepared from **2** isolated from glutamate decarboxylase. (B) Prepared from synthetic **3**. H_2P, cationic ring form; HP, dipolar ionic ring form; P, anionic ring form. The dissociation of the carboxyl group is ignored, as it has no effect on the spectra.

Scheme I[a]



[a] All peak positions are at neutral pH.

Scheme II



drastically altered electronic absorption spectrum (Figure 6). Reduction of **3** with sodium borohydride or sodium cyanoborohydride produced a new compound (**5**) with a spectrum typical of 3-hydroxypyridines but shifted to lower wavelengths (Figure 7) (Scheme I). Compounds **3** and **5** isolated from the enzyme have absorption spectra identical with those of synthetic **3** and **5** (Figures 6 and 7; Table I) and identical electrophoretic mobilities (Figure 2). However, the spectrum of the neutral form of **3** undergoes changes with time that depend on its previous history. These changes are related to the slow equilibration shown in the Scheme II. Proton NMR spectra are also identical for synthetic samples and for samples of **3** and **5** isolated from aspartate aminotransferase (Ueno, 1982).

When **2** is dephosphorylated to **3**, the electronic spectrum changes drastically over a period of hours. However, pyridoxal 5′-phosphate is dephosphorylated under the same conditions within 10 min. When we examined **2** treated with alkaline phosphatase for only 10 min, the spectrum was identical with that of **2**, but the electrophoretic mobility at pH 6.5 was about 0.85 times that of pyridoxal-P rather than 0.65 for **3** (see Figure 2).

For the cationic, neutral, and anionic forms of **3**, respectively, strong bands appear at 284, 313, and 297 nm, respec-

tively, upon standing (Figure 6). These are 7–12 nm lower than the positions for pyridoxine. The spectra are those of a 3-hydroxypyridine ring lacking a conjugated exocyclic double bond. We suggest that the longer wavelength bands belong to the keto acid **3a** which is formed rapidly but that the 5′-hydroxyl group adds to the double bond in a reaction comparable to the cyclization of pyridoxal to the hemiacetal to give the keto acid **3c** via the intermediate enolic form **3b**. This interpretation is supported by the fact that **5**, which must have the closed ring structure shown, has absorption bands in nearly the same positions as those of **3**.

The spectra in Figure 6 were obtained by preparing a stock solution of **3** at pH 11.9, then diluting portions into suitable buffers, and recording the spectra quickly. Around neutral pH the spectrum changes with time. In 0.05 M phosphate buffer at pH 7.2, it reached the value shown by the dashed line in Figure 6. This spectrum is close to but not quite identical with the equilibrium value that would presumably be obtained if the compound were completely stable and were allowed to stand for a much longer time. We estimate that for this equilibrium value the absorbance at 410 nm is about 24% of that in the monoprotonated species shown in Figure 6. When a portion of **3** allowed to stand 19.5 h at pH 7.6 in phosphate buffer was adjusted to pH 1.2, the low shoulder at 345 nm fell to 36% of the value shown in Figure 6.

The ^{13}C NMR spectrum of **5** is simple. The expected 11-carbon atoms are present. Some of the resonances are doubled in a manner indicative of the presence of a diastereomeric pair. However, crystalline synthetic **5** was clearly a single diastereomer. Complete assignments have been made and are consistent with the cyclic structure shown. Of particular importance is the fact that in gated proton decoupled spectra the 5′ carbon appeared as a triplet with $^1J(CH) \sim 150$ Hz and the 4′ carbon as a doublet with $^1J(CH) \sim 146$ Hz. This suggests that two hydrogen atoms are bonded to C-5′ and only one to C-4′ in accord with the proposed cyclic structure. The proton NMR spectrum is more complex but is also in agreement with the proposed structure (Ueno, 1982).

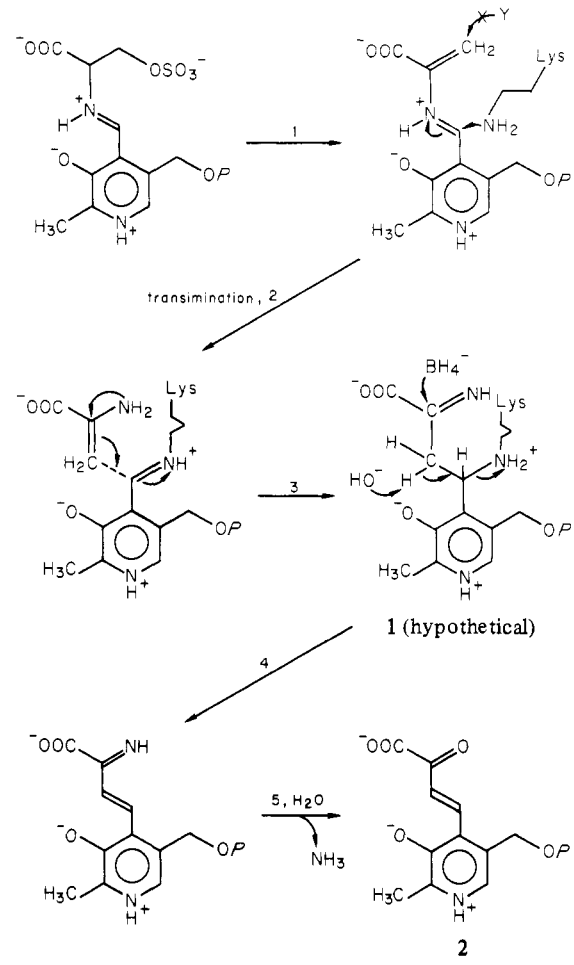
The mass spectrum of **5** shows a parent ion peak at m/e 239 ($C_{11}H_{13}NO_5$). The base peak at m/e 164 ($C_9H_{10}NO_2$) results from loss of $[CO_2H\text{–}CHOH]^+$. Synthetic **3** shows a parent ion peak at m/e 237 ($C_{11}H_{11}NO_5$) while the base peak is at m/e 149 ($C_8H_7NO_2$). The fragmentation patterns of **3** and **5** are similar, a fact that supports the existence of a cyclic structure in both compounds.

Reduction of **2** by sodium borohydride or cyanoborohydride produces a new compound, **4**, with a somewhat complex absorption spectrum that more closely resembles that of pyridoxine than does **5**. Dephosphorylation of **4** with alkaline phosphatase produced **6**, a compound with properties very different from those of **5**. Compounds **4** and **6** are considered further in the following paper (Ueno et al., 1982). It is shown that **4** is the alcohol expected from reduction of the carbonyl group of **2** and that **6** is the dephosphorylation product. No ring closure occurs, and the positions of the major absorption bands almost coincide with those of pyridoxine.

Discussion

Many examples are known in which a mechanism-dependent enzyme inhibition occurs when a β substituent of an inhibitor can be eliminated to form a Schiff base of α-aminoacrylate or a related α,β-unsaturated amino acid or amine (step 1 of Scheme III; Miles & Meister, 1967; Relyea et al., 1974; John & Fasella, 1969; Fowler & John, 1972, 1981; Silverman & Abeles, 1976, 1981; Soper et al., 1977; Soper & Manning, 1978; Morino et al., 1974; 1979; Morino & Tanase, 1978; Wang & Walsh, 1978; Wang et al., 1981; Kallio et al., 1981; Bey, 1981). It has generally been assumed, without any real proof, that inactivation of the enzyme is a result of addition of a nucleophilic group from the enzyme to the β carbon of the double bond as indicated by the reaction marked with the X in Scheme III. While there is considerable evidence that this does occur with aspartate β-decarboxylase (Relyea et al., 1974), we propose that for glutamate decarboxylase the inactivation follows release of α-aminoacrylate from its Schiff base by "transimination" (step 2 of Scheme III). Inactivation results from nucleophilic attack by the β carbon of the aminoacrylate on the "internal" Schiff base of pyridoxal phosphate with a lysine side chain (step 3 of scheme III). The resulting product **1** that is covalently attached to the enzyme could be the imine shown or the corresponding carbonyl compound. Attack of the hydroxide ion on **1** (step 4, scheme III) would form either an imino intermediate which would be hydrolyzed (step 5) to **2** or **2** directly. Reduction of the C=NH or C=O bond of **1** by borohydride would prevent the removal of the adjacent carbon-bound proton by attack of OH^- and thereby prevent the formation of **2**. It would also link the chromophore tightly to the protein as has been observed.

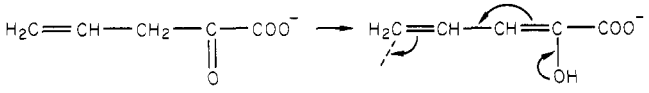
Scheme III



The postulated reaction depends upon the well-known fact that both the nitrogen and the β carbon of enamines such as aminoacrylic acid have nucleophilic properties (Dyke, 1973). The aminoacrylate formed in the transimination step need not leave the active site but may simply rotate around the bond to the carboxylate group, the latter remaining bound in the active site.

Essentially the same set of reactions has been established for another enzyme aspartate aminotransferase as is described

in the following paper (Ueno et al., 1982). A similar reaction has been proposed to explain the inhibition of glutamate decarboxylase in the brain by the convulsant amino acid allylglycine (R. Johnson, P. Marcotte, M. Chang, M. O'Leary, and C. Walsh, personal communication). This amino acid is apparently oxidized to 2-keto-4-pentenoate which exists in part as the enol:



Nucleophilic attack of the latter (as indicated by the curved arrows) would inactivate the enzyme in the same manner as does the attack of aminoacrylate shown in the preceding scheme.

The present findings suggest that the mechanisms proposed for various other "suicide inactivators" must be reconsidered. This includes all of these postulated to act via Schiff bases of aminoacrylate or other α,β-unsaturated amines or amino acids. A larger number of these inhibitors contain carbon–carbon double or triple bonds. For example, (4*R*)-4-L-aminohex-5-ynoic acid is an inhibitor of bacterial glutamate decarboxylase (Jung et al., 1978). The changes in spectrum caused by this compound are somewhat similar to those observed in this work. The proposed mechanisms of action seem entirely reasonable. However, our findings suggest a possible alternative mechanism.

Compound **2** is an α-keto acid with the carbonyl group conjugated with the vitamin B_6 ring. The absorption bands are shifted to longer wavelengths than those for pyridoxal phosphate in both neutral and anionic forms, but not for the cation. The absorption spectrum of the latter (Figure 3) is complex, possibly reflecting a nonplanar conformation. The NMR spectra at all pH values seem straightforward, however.

The hydration ratio [hydrate]/[carbonyl] at pH 1.5 is about 0.30 for **2**, but about 3.2 for pyridoxal phosphate. This difference is not surprising, as aldehydes are usually more hydrated than ketones. The hydration ratio for pyridoxal phosphate drops to 0.5 in the neutral form and to 0.09 in the anion form (Harris et al., 1976). It is understandable, therefore, that for **2** at neutral or basic pH, there is no detectable hydration as judged by the NMR spectrum. The electronic spectrum of **2** at low pH cannot be interpreted in a clear way to give a measure of the hydration ratio as it can for pyridoxal phosphate. In the latter case the measurement of the degree of hydration from NMR (H. Ueno, unpublished results) and electronic spectra (Harris et al., 1976) are in good quantitive agreement.

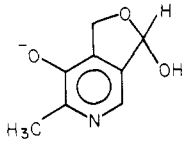
The p*K* values for **2** (3.98 and 8.70) are 0.36–0.37 higher than for pyridoxal phosphate but are still less than those of pyridoxine (5.00 and 8.97). Thus, the electron-withdrawing effect of the carbonyl group in **2** is less than that in pyridoxal phosphate but is still clearly evident. Note that the p*K* of the phosphate group has not been estimated because dissociation of that group has very little effect on the absorption spectrum.

The striking change in the spectrum in going from **2** to **3** and the slow changes in absorption spectra of **3** at neutral pH are strong evidence for the postulated cyclization and for a slowly reversible equilibrium between cyclic and open forms. We explain the changing spectra by assuming that at high pH a certain ratio of the anionic forms of **3a** and **3c** is established. From resolution with log–normal curves, it is estimated that about 33% is present in the open form and 67% as the cyclic species **3c**. When the pH is lowered to the region 5–8, the fractions of the open form **3a** and of the ring form **3c** do not change instantly. Resolution with log–normal curves shows that there is still about 34% **3a** at neutral pH. However, a small amount of enol **3b** is present, and this slowly changes over a period of hours into **3c** so that at equilibrium only about 8% of **3a** remains. The ketonization reaction is promoted strongly by inorganic phosphate.

At low pH, about 36% is present in the open form (Figure 6), again nearly the same fraction as at high pH. This fact suggested that the equilibration between open and closed forms does not occur at an appreciable rate at low pH. This was confirmed by the observation that a sample allowed to partially equilibrate at pH 7.6, when lowered to pH 1.2, contained only 9% of the open form, just what had been present at pH 7.6.

The proton NMR spectrum of **3** is interpretable in a straightforward way (Ueno, 1982). The β proton exchanges with solvent. This is expected for the initial product of the cyclization; **3b** is an enol that, upon ketonization, will incorporate solvent protons into the β position.

The isolation of **5** and the very different electronic spectra of **5** and **6** are further verification of the foregoing conclusions. It is also of interest that the electronic spectrum of **5** closely resembles that of isopyridoxal in all three states of protonation of the ring. Isopyridoxal exists almost entirely as the cyclic hemiacetal, a compound with a five-membered ring similar to that in **5**.



isopyridoxal (hemiacetal form)

The proton NMR spectrum of crystalline **5** confirms the cyclic structure. The two 5′-CH_2 protons are no longer equivalent and appear in the pH 12 spectrum as a pair of doublets with J = 12 Hz. The peaks of the lower doublet centered at 5.06 ppm are split further (J < 2 Hz) by long-range coupling, probably to the 4′-H. This interpretation is confirmed by the observation of a similar pattern for isopyridoxal where the splitting of the 4′-CH_2 can be detected (Ueno, 1982). The 4′ proton appears as a pair of doublets (J ~ 2 Hz) caused by coupling to the two nonequivalent β protons. The latter appear as two distinct multiplets centered at 1.94 and 2.12 ppm. The α proton forms two doublets at 4.13 ppm with J ~ 2.4 Hz.

A puzzling aspect of our findings is that glutamate decarboxylase apparently removes the proton from the α-carbon atom of the Schiff base of serine sulfate with the coenzyme rather than catalyzing decarboxylation. Perhaps the presence of the sulfate group in place of the carboxylate of the normal substrate leads to a distortion of the Schiff base conformation that makes removal of the α proton more likely than decarboxylation. In this context it is of interest that some inhibitors such as (4*R*)-4-aminohex-5-ynoic acid depend upon removal of an α proton rather than decarboxylation for their activation by the enzyme (Jung et al., 1978). However, this (*R*)-aminohexynoic acid is of the opposite chirality to L-serine *O*-sulfate, another puzzling fact.

Yet another puzzle is the role of the protein factor. Its explanation will clearly require more work. A possibility is that it forms a complex with the enzyme, inhibiting the release of α-aminoacrylate and making the inactivation event more probable.

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