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Mechanism-based inactivation of glutamate decarboxylase and aspartate aminotransferase with L-serine-O-sulfate

Hiroshi Ueno
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MECHANISM-BASED INACTIVATION OF GLUTAMATE DECARBOXYLASE
AND ASPARTATE AMINOTRANSFERASE WITH L-SERINE-O-SULFATE

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Mechanism-based inactivation of glutamate decarboxylase and aspartate aminotransferase with L-serine-O-sulfate

by

Hiroshi Ueno

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>METHODS</td>
<td>37</td>
</tr>
<tr>
<td>RESULTS</td>
<td>66</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>208</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>231</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>233</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>243</td>
</tr>
</tbody>
</table>
To my parents,
Fujio & Haruko UENO
for their life-time support.
INTRODUCTION

Enzymes dependent upon the vitamin B₆-containing coenzyme pyridoxal phosphate (PLP) have been studied extensively during the past three decades. Their mechanisms of action are well-understood (Metzler, 1977; Walsh, 1979). During recent years, new types of inhibitors have been designed for PLP-dependent enzymes. These inhibitors are called mechanism-based inhibitors, suicide substrates, enzyme-activated irreversible inhibitors, or \( k_{\text{cat}} \) inactivators. A large number of reviews about mechanism-based inactivation have been published (Abeles & Maycock, 1976; Rando, 1974a, 1975; Bey, 1981; Seiler et al., 1978; Kalman, 1979; Sandler, 1980; Walsh, 1978).

The following criteria outline the characteristics of suicide inactivators: (1) The inactivators themselves are relatively unreactive; however, they become reactive in the active site of the target enzyme. (2) During the inactivation, a normal catalytic process of the enzyme is required. (3) Inactivation is irreversible and depends upon the concentration of inhibitor and the time. The design of more specific and efficient inhibitors has been the primary goal of many researchers. PLP-dependent enzymes are known to produce key biological metabolites such as amino acids and biogenic amines, which can act as neurotransmitters. Abnormal amounts of these metabolites can result from malfunctions of PLP-dependent enzymes. Drugs designed for specific target enzymes may be able to control the abnormal amounts of these metabolites or to block a specific metabolic pathway. Possible applications for medical and pharmaceutical fields have been explored. For example, suicide inhibitors for D-amino acid aminotransferase could be new antibacterial
drugs (Soper & Manning, 1978). Inhibition of this enzyme in bacteria is lethal. Mechanism-based inactivators for γ-aminobutyric acid aminotransferase may be used as drugs to treat Huntington's chorea. In this disease, a low level of γ-aminobutyric acid (GABA) is found in the brain (Perry et al., 1973; Bird, 1980; Prusiner, 1981). Specific inhibition of GABA-transaminase raises the GABA level into the normal range.

There are two types of mechanism-based inactivators. The β-substituted amino acids can undergo enzyme-catalyzed β-elimination to form a reactive intermediate that inactivates the enzyme. The olefinic or acetylenic amino acids such as propargylglycine and vinylglycine can be modified by the active site residues of the enzymes to form an inactivating species.

In this dissertation, the biochemical action between one mechanism-based inhibitor and its target enzymes is described. Bacterial glutamate decarboxylase (GAD) and cytosolic aspartate aminotransferase (AAT) of pig heart have been used as the target enzymes. The amino acid, L-serine-O-sulfate is the mechanism-based inactivator. This is an example of the first type of inhibitor which has a β-substituent that can be eliminated. Both enzymes contain pyridoxal phosphate in their active sites and their properties are well-understood. The three-dimensional structure of AAT is now known (Arnone et al., 1982), and the mechanism of inactivation is discussed at the molecular level.

Treatment with base of glutamate decarboxylase inactivated by serine sulfate released an interesting compound (Likos & Metzler, 1976). Characterization of this compound and of its derivatives was begun by
Likos but was not completed (Likos, 1977). Further characterization of this compound and proof of the structure are reported here. Experiments similar to those with GAD have been conducted with AAT and serine sulfate. The reaction products have also been characterized using various chromatographic techniques, mass spectroscopy, chemical modification, and high resolution nuclear magnetic resonance spectroscopy.

Puzzling observations by Morino et al. (1974) concerning the reaction of AAT with β-chloroalanine have been reproduced with AAT. Total characterization of compound released by base and its derivatives has allowed us to explain Morino's results as well as the nature of the mechanism-based inactivation of GAD and AAT. A new mechanism of the inactivation of these two enzymes is proposed. The possible application of this mechanism to other PLP enzymes is also discussed. A protein which participates in the inactivation of GAD with serine sulfate was found. Its isolation and partial characterization are also described.
LITERATURE REVIEW

Glutamate Decarboxylase

L-Glutamate decarboxylase (GAD) from _E. coli_ (EC 4.1.1.15) catalyzes the following reaction (1):

\[
\text{L-glutamate} \rightarrow \text{\(\gamma\)-aminobutyric acid} + \text{CO}_2 \quad (1)
\]

This enzyme was first purified by Shukuya and Schwert (1960a) in a 90% homogeneous form. They showed that GAD contained tightly bound pyridoxal 5'-phosphate (PLP) as a coenzyme and had a molecular weight of 300,000. The enzyme had a pH optimum around 3.8, and the _K_m_ for glutamate was \(8.2 \times 10^{-4}\) M. By their preparation, 100 g of wet cells gave 60 mg of homogeneous enzyme. Shukuya and Schwert (1960b) also observed that the enzyme exhibited a sharp pH-dependent spectral transition centered at pH 5.6. The enzyme absorbed light maximally at 420 nm at low pH and at 340 nm at high pH. The steepness of the pH transition curve suggested the presence of some cooperative interaction between the dissociable groups in the enzyme. At least four protons were simultaneously dissociated during above transition. The enzyme was active at low pH, and its absorption at 420 nm was due to the formation of a protonated Schiff base between PLP and an \(\varepsilon\)-amino group of a lysine residue (Anderson & Chang, 1965). The 420 nm form was reduced by sodium borohydride (NaBH₄), the reduced form exhibiting an absorbance maximum at 330 nm. The 340 nm high pH form was resistant to NaBH₄ and PLP was released from the protein upon a further increase in pH (Anderson & Chang, 1965). The 340 nm species is thought to be a geminally substituted form arising by addition of some group X to the 4' carbon of the Schiff base (O'Leary, 1971; O'Leary & Brummund, 1974).
Huntley and Metzler (1967a) reported circular dichroism (CD) studies of GAD. A large positive CD was observed at 420 nm, but little CD was seen at 340 nm. The 330 nm reduced form was optically active. However, addition of solid urea (final concentration 8 M) to the 330 nm form eliminated the CD without changing the light absorption.

Shukuya and Schwert reported anion activation of GAD (1960a). Chloride gave the greatest effect of activation of the enzyme followed by bromide, sulfate, and phosphate. Acetate inactivated the enzyme. Low temperature inactivation was also observed by Shukuya and Schwert (1960c).

The enzyme was crystallized by adding ammonium sulfate (Strausbauch et al., 1967). The crystalline enzyme was used in the study of some chemical and physical properties of GAD, such as the molecular weight, association-dissociation properties, subunit structure, amino acid composition, and amino acid sequence of the active site, and also for electron microscopy (Strausbauch & Fischer, 1970a, 1970b; Tikhonenko et al., 1968; To, 1971). Tikhonenko et al. (1968) found that the enzyme had a hexameric structure and could be disrupted by dilution at low temperature.

Strausbauch and Fischer (1970a) confirmed that the molecular weight of the active enzyme was 310,000 and that for the subunit was 50,000. There was no evidence for nonidentical subunits. Therefore, bacterial GAD is assumed to have six identical subunits. The quaternary structure was investigated by To using electron microscopy techniques (1971). He found the enzyme a hexamer of D3 symmetry in an octahedral arrangement. An amino terminal methionyl residue and a Lys-His-Thr sequence at the carboxyl end were found in GAD (Strausbauch & Fischer, 1970a). Sodium
borohydride-treated enzyme was digested with trypsin and the peptide containing the active site lysine was shown to be Ser-Ile-Ser-Ala-Ser-Gly-His-Lys*-Phe (Strausbauch & Fischer, 1970b). The complete sequence has not been determined yet. Sukhareva and Tikhonenko (1972) further investigated the association-dissociation behavior of bacterial GAD. They found a number of factors which influence the reactions: the temperature, pH of the solution, concentration of enzyme protein and coenzyme, and the structure of the aqueous phase. Low temperature, high pH, or low concentration of enzyme or coenzyme led to dissociation of the enzyme. The dissociated enzyme was inactive.

Dunathan (1966, 1971) proposed a general concept for pyridoxal 5'-phosphate dependent enzyme chemistry. He stated that for an α-bond in the substrate to be labilized it must be perpendicular to the aromatic system of the pyridoxal ring. This stereochemical arrangement causes a gain in delocalization energy which aids in the bond breaking process. GAD is not the exception. The breaking bond between the α-carboxyl and the α-carbon of substrate is believed to be perpendicular to the conjugated system. This leads us to the important conclusion that the α-proton should not be abstractable during the decarboxylation reaction of GAD. Experiments using D₂O as a solvent or using α-methylglutamate as a substrate supported the above concept (Mandeles et al., 1954; Yamada & O'Leary, 1977; Huntley & Hetzler, 1967b, 1968; Sukhareva & Torchinsky, 1966). The incorporation of a single atom of deuterium into the product, γ-aminobutyric acid was found when glutamate and GAD were incubated in D₂O. If the α-proton were abstracted during the decarboxylation
reaction, the incorporation of two deuterium atoms into the product would be expected (Yamada & O'Leary, 1977). A substrate lacking an α-proton, α-methylglutamate was reacted with GAD. Normal decarboxylation was observed (Huntley & Metzler, 1967b, 1968; Sukhareva & Torchinsky, 1966). The decarboxylation of glutamate and of α-methylglutamate have been determined to occur with retention of configuration in D2O (Yamada & O'Leary, 1978). Two other PLP-dependent decarboxylases, tyrosine decarboxylase (Belleau & Burba, 1960) and Lysine decarboxylase (Leistner & Spenser, 1975) also showed retention of configuration.

The decarboxylation product of GAD with L-glutamate is γ-aminobutyrate (GABA), an important inhibitory neurotransmitter. Recently Ebadi (1981) reviewed the significant role of pyridoxal phosphate in central nerve system. An accompanying critique was written by Metzler (1981).

GAD has been isolated from other sources than bacteria. Roberts group purified mouse brain GAD in homogeneous form (Wu et al., 1973). Maitre et al. (1978) purified human and rat brain GAD. Haber et al. (1970) isolated the enzyme from mouse kidney. Some differences have been seen between the mammalian and bacterial enzymes. Distinguishing differences were in the molecular weight, N-terminal amino acid residues, pH optimum, and the number of subunits. The molecular weight for the mammalian enzyme was about 140,000. It is a dimer, and its pH optimum is around neutrality. The N-terminal amino acid residues were alanine for the human and rat enzymes (Maitre et al., 1978). The mouse brain enzyme was inhibited by Cl⁻ (Susz et al., 1966). The enzyme isolated from
crustacean axons showed an activation by $K^+$, but did not show any effect of anions (Molinoff & Kravitz, 1969). Haber et al. (1970) reported that the properties of GAD from mouse kidney were somehow similar to those of bacterial GAD.

The stereospecificity of the rat brain GAD during the decarboxylation was studied by Bouclier et al. (1979). Like the bacterial enzyme, rat GAD catalyzed the reaction of L-glutamate with retention of configuration.

**Cytosolic Aspartate Aminotransferase of Pig Heart**

Aspartate aminotransferase (AAT) (EC. 2.6.1.1) catalyzes the following reaction (2):

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \underset{\text{oxaloacetate} + \text{L-glutamate}}{\longrightarrow}$$ (2)

Highly purified enzymes have been isolated from various sources: a) pig heart (Jenkins & D'Ari, 1966; Martinez-Carrion et al., 1967; Michuda & Martinez-Carrion, 1969; Morino et al., 1977; Scardi et al., 1963); beef liver (Morino et al., 1963; Wada & Snell, 1962); c) chicken heart (Bertland & Kaplan, 1968, 1970; Gehring et al., 1977a; Sonderegger et al., 1977); d) rat heart (Morino et al., 1964); e) human heart (Teranishi et al., 1978); f) chicken liver (Schrawder & Martinez-Carrion, 1973; g) beef kidney (Scandurra & Cannella, 1972); h) *Escherichia coli* (Mavrides & Orr, 1974, 1975).

AAT exists as two isoenzymes; cytosolic and mitochondrial (Boyd, 1961; Katunuma et al., 1962). In 1958, the first pure cytosolic enzyme was reported by Jenkins et al. and by Lis. The cytosolic enzyme is cationic and the mitochondrial enzyme is anionic. The former isoenzyme has an isoelectric point of about 5.0 and the latter of about 6.8. Later,
subforms of AAT, α, β, and γ, were found and were separated by gel electrophoresis (Martinez-Carrion et al., 1965, 1967). Their physical properties (molecular weight, amino acid sequence) are similar; however, the activity and charges are different. The most cationic α form is the most active form. The β subform is half active, and γ subform is the least active and cationic.

The α subform of cytosolic AAT from pig heart has been studied most extensively. The enzyme is a dimer having a molecular weight of 46,344 per subunit. Each subunit contains one molecule of the coenzyme pyridoxal phosphate at the active site. It is linked as a Schiff base with the ε-amino group of Lys-258. The primary structure of the cytosolic isoenzyme was reported independently by Ovchinnikov et al. (1973) and Doonan et al. (1975). The sequence of the cytosolic isoenzyme from chicken heart has been reported also (Shlyapnikov et al., 1979). Recently, the mitochondrial isoenzyme has been completely sequenced by Wada and associates (Kagamiyama et al., 1980a, 1980b; Sakakibara et al., 1980).

Cytosolic AAT has 412 amino acid residues while the mitochondrial AAT has 11 residues less. A comparison between these two isoenzymes based upon primary structure has been made (Kagamiyama et al., 1980b). There is a 48% sequence homology (equivalent to 195 positions) with minimum average base change per codon of DNA of 0.72. The substitutions of amino acid residues at 116 positions are all explained by single base changes in the codons. This suggests the evolution of two isoenzymes from a common ancestral gene. As is indicated by the appearance of immunocrossover
between these two isoenzymes, most of the homologous regions in the sequence are located in the interior of the molecules, whereas the parts with dissimilar sequences occupy the surfaces of these isoenzymes (Kagamiyama et al., 1980b).

Active site sequences of other vitamin B₆ dependent enzymes have been reported: alanine aminotransferase from pig heart (Tanase et al., 1979); tryptophanase decarboxylase from E. coli (Kagamiyama et al., 1970); arginine decarboxylase (Boeker et al., 1971); lysine decarboxylase (Sabo & Fischer, 1974); glutamate decarboxylase from _E. coli_ (Strausbauch & Fischer, 1970b). Also, D-amino acid transaminase from _Bacillus sphaericus_ has been sequencing (private communication with Dr. James M. Manning, The Rockefeller University, New York).

The common sequence [Ser-X-X-Lys*] is found at the active sites of all of these enzymes. In the case of AAT, Ser-255 binds to the phosphate group of the coenzyme (Arnone et al., 1982). Thus, it is likely that the common serine residue acts the same way in the other PLP enzymes.

AAT from various sources has been crystallized: cytosolic isozyme from pig heart (Wada & Morino, 1964; Arnone et al., 1977); from beef heart (Wada & Morino, 1964); from beef liver (Morino et al., 1963); from chicken heart (Bertland & Kaplan, 1968; Borisov et al., 1978); mitochondrial isozyme from chicken heart (Gehring et al., 1977a). Single crystals suitable for X-ray crystallographic studies have been grown from solutions of polyethylene glycol (Arnone et al., 1977, Metzler et al., 1979) as described by McPherson (1976). Several groups are currently studying X-ray crystallography of AAT (Arnone et al., 1977, 1982; Gehring et al.,
Crystals of cAAT belong to the space group P2_12_12_1 with unit cell dimensions a = 125.1 Å, b = 130.8 Å, and c = 55.7 Å (Arnone et al., 1977). Both cytosolic and mitochondrial isoenzymes have extremely similar structure (Ford et al., 1980; Arnone et al., 1982). There are three distinct parts in common: a large central domain constructed from residues 75-300, a smaller peripheral domain composed of residues from the COOH-terminal and NH_2-terminal peptides, and a linker region between the domains. The coenzyme is bound in the active site by residues from the large domain. The coenzyme phosphate group is in contact with side chains of Arg-266, Ser-255, Ser-257, Thr-109, Tyr-70* (* indicates the residue from other subunit), and the NH_2-terminal end of the 108-123 helix. Besides the above residues and Lys-258, the coenzyme ring interacts with Ala-224, Tyr-225, and Asp-222. The negatively charged carboxylate group of Asp-222 neutralizes the positive charge on the ring nitrogen, and Tyr-225 interacts with the 3-hydroxyl group and with the internal aldimine NH^+ group. Difference electron density studies show that the guanidium groups of Arg-386 and Arg-292* interact with the carboxyl groups of the substrates (α-methylaspartate or α-ketoglutarate).

As predicted by Karpeisky and Ivanov (1966), rotation (by about 30°) of the coenzyme ring when the α-methylaspartate-aldimine complex was formed has been demonstrated by difference electron density studies.

The 3.5 Å difference electron density map of the β subform versus the α subform showed that one subunit of β subform contained a modified
coenzyme and the other subunit appeared to be unaltered (Arnone et al., 1982). The modification of one subunit supported the observation that the β form is half as active as the α subform. A dicarboxylic acid-like inhibitor is assumed to cause the modification found in the α subform (Metzler et al., 1981).

Crystals of AAT have also been studied by spectroscopic methods. Light spectra of crystals of enzymes, enzyme-substrate or enzyme-inhibitor complexes have been reported by various groups (Metzler et al., 1978, 1979; Eichele et al., 1978, 1979; Makarov et al., 1981).

As in solution, the two principal absorption bands, at 430 nm and 363 nm, are in a pH-dependent equilibrium. The spectra of the crystals of pig heart cytosolic AAT were measured with the light beam of the spectrophotometer parallel to the b-axis of the orthorhombic crystals. The plane of polarization of the light used for the measurement is parallel to either the a or the c axis. The polarization ratio, the ratio of absorbance with the plane of polarization parallel to the c-axis (c-polarization) to that in the a-polarization, were measured. Surprisingly, large changes in polarization ratios were obtained from the spectra of α-methylaspartate or other substrate–enzyme crystalline complexes. Rotation of the coenzyme ring in the complex compared to native is suggested strongly by these experiments (Metzler et al., 1978).

**Mechanism-Based Inactivation of Glutamate Decarboxylase**

Bacterial glutamate decarboxylase (GAD) is inhibited by various substrate analogs, such as α-ketoglutarate, glutarate, α-methylglutarate (Lupo & Halpern, 1970), L-isoglutamine, aliphatic dicarboxylic acids
(Fonda, 1972a, 1972b), tricarboxylic acid cycle intermediates (Gerig & Kwock, 1979), or bromopyruvate (Fonda & DeGreolla, 1974). The above inhibitors act on GAD competitively with respect to glutamate.

Jung et al. (1978) showed that bacterial GAD was irreversibly inactivated by (R)-(−)4-aminohex-5-ynoic acid. It has been known that 4-aminohex-5-ynoic acid is a mechanism-based inactivator. It irreversibly inhibits both bacterial and mammalian γ-aminobutyric acid transaminases (Jung & Metcalf, 1975; Jung et al., 1977; Bouclier et al., 1979).

The inhibition of bacterial GAD was specifically caused by the (R)-isomer, but not the (S)-isomer of 4-aminohex-5-ynoic acid. Jung et al. (1978) suggested that the reaction mechanism involved the abstraction of an α-proton from the substrate (Scheme I). Upon addition of 4-aminohex-5-ynoic acid, a decrease of the absorption of the native enzyme at 415 nm and an increase of absorption at 330 nm were observed. Radioactively labelled substrate was incorporated, one molecule of substrate apparently going into each pyridoxal phosphate binding site. The actual inactivated form was believed to be either a conjugated allene (I₄) or a ketimine with α-acetylenic group (I₅). The structure of the inactivated form has not been determined yet.

Interesting results came from studies of mammalian GAD with 4-aminohex-5-ynoic acid (Bouclier et al., 1979). The mammalian enzyme showed a pattern of inactivation similar to that of the bacterial enzyme. It was specifically inactivated by the (S)-isomer, but not by the (R)-isomer. It appeared that no conversion from (S) to (R) occurred during the inactivation. The mammalian enzyme also showed an abstraction
Scheme I.
of the \(\alpha\)-proton from the inhibitor. The stereochemistry of the inhibitors is of interest. The \(\alpha\)-proton of the \((R)\)-isomer of 4-amino-hex-5-ynoic acid and the \(\alpha\)-carboxyl group of L-glutamate presumably had the same orientation. Thus, the stereospecificity of bacterial GAD against L-glutamate and \((R)\)-4-aminohex-5-ynoic acid were the same in agreement with Dunathan's general hypothesis (1971). However, mammalian GAD catalyzed decarboxylation of L-glutamate and deprotonation of \((S)\)-4-aminohex-5-ynoic acid. The \(\alpha\)-carboxyl group of L-glutamate and the \(\alpha\)-proton removed from \((S)\)-4-aminohex-5-ynoic acid did not have the same orientation. One must ask why the mammalian enzyme catalyzed the elimination of the \(\alpha\)-proton from the \((S)\)-isomer. One explanation given by Bouclier et al. (1979) was that mammalian GAD, like an amino-transferase, is capable of abstracting an \(\alpha\)-hydrogen from L-glutamate or form the corresponding position in the \((S)\)-isomer of the inhibitor.

Other possible mechanism-based inhibitors for GAD were listed in the review by Jung et al. (1980). However, detailed chemistry has not been published yet.

In 1971, Sukhareva and Braunshtein reported that bacterial GAD was inactivated by L-serine-O-sulfate. Later, Likos & Metzler showed that L-serine-O-sulfate was a mechanism based inhibitor for bacterial GAD (Likos & Metzler, 1976; Likos, 1977). The enzyme showed an absorption change from 420 nm to 335 nm upon addition of serine sulfate. A yellow low molecular weight compound was released when the 335 nm form was treated with alkali at pH 11. The yellow product was neither pyridoxal phosphate nor pyridoxamine phosphate. The identity of the yellow product
remained unclear. Likos and Metzler also reported that GAD was inactivated with β-chloroalanine in a manner similar to that by serine sulfate. Base treatment of the β-chloroalanine inactivated enzyme gave the same yellow product. The results suggested that the enzyme catalyzed the β-elimination reaction with either serine sulfate or β-chloroalanine and that the inactivation occurred after the β-elimination step. The mechanism that is usually proposed for the mechanism-based inactivation of glutamate decarboxylase with serine sulfate is shown in Scheme II. In step b, the abstraction of the α-hydrogen atom of a substrate occurs. The ε-amino group of Lys-258 might function as a base participating in the abstraction of the α-hydrogen atom. In step c, the β-elimination occurs to form a highly reactive α-aminoacrylate Schiff base intermediate. Then, the ε-amino group of Lys attacks the β-position of the substrate to form a covalent linkage. Both serine sulfate and β-chloroalanine are known as mechanism-based inactivators for aspartate aminotransferase (see next section). However, the mechanism shown in Scheme II does not explain the formation of compound 2.

**Mechanism-Based Inactivation of Cytosolic Aspartate Aminotransferase**

Cytosolic aspartate aminotransferase (cAAT) is irreversibly inactivated by various mechanism-based inhibitors such as L-serine-O-sulfate, β-haloalanines, vinylglycine, β-methyleneaspartate, α-amino-γ-methoxybutenoate, or propargylglycine (Scheme III).

Inactivation of cAAT by serine sulfate was first reported by John & Fasella (1969). Incubation of cAAT with serine sulfate at pH 6.5 releases
\[ \text{Scheme III.} \]
equimolar amounts of puruvate, sulfate, and ammonia. Three reactions were found to compete: transamination, β-elimination, and inactivation. The enzyme was practically inactivated during the first few min. After one hour, 99% of the catalytic activity was lost. The β-elimination occurred with $K_m = 70 \text{ mM}$ and $k_{cat} = 12 \text{ s}^{-1}$. A broad spectral band at 340-360 nm was observed for the inactivated enzyme. Using uniformly labelled $^{14}$C serine sulfate, it was shown that a three-carbon residue from the inhibitor became stably bound to the protein. Later John et al. (1973) isolated the labeled peptide. The peptide was prepared by digesting the inactivated enzyme with thermolysin, by treating with iodoacetate, and by successive chromatography on Sephadex G-25, SP-Sephadex, and paper (overall yield, 60%). The amino acid sequence of the isolated peptide was reported as Phe-Ser-(carboxymethylcysteine, Gly)-Asn. The peptide was not susceptible to Edman degradation, except for the first two NH$_2$-terminal residues, Phe-Ser. The COOH-terminal position contained asparagine.

Both cytosolic and mitochondrial AAT undergo irreversible inactivation during an α, β-elimination reaction with β-chloro-L-alanine (Morino & Okamoto, 1972; Morino et al., 1974). During the inactivation, many molecules of pyruvate, Cl$^-$, and NH$_4^+$ are formed for every molecule of enzyme inactivated. Over 95% of the enzyme is inactivated within 2 min. A rate enhancement by formate ion was found during the inactivation. In the presence of 3.2 M formate at pH 7.8, the normal absorption band of the enzyme at 362 nm shifted to 345 nm. Also, the pK value of the native enzyme was increased from 6.3 to 7.3 by the formate.
Upon addition of β-chloroalanine in the presence of 3.2 M formate, the 345 nm peak disappeared and a new peak corresponding to the inactivated form of the enzyme appeared at 333 nm. Reduction of the 333 nm form with borohydride gave a band at 325 nm. When radioactive chloroalanine was used it was shown that this 325 nm form contained a stably bound 3-carbon residue. Enzymatic digestion of this 325 nm form gave a peptide fragment containing both the labeled 3-carbon residue and the pyridoxal phosphate coenzyme. The labeled Lys-258 was identified from peptide studies with both cytosolic and mitochondrial AATs inactivated by β-chloroalanine (Morino & Okamoto, 1973; Morino & Tanase, 1978). Tryptic hydrolysis of both isoenzymes gave 25 residue fragments. Further digestion with chymotrypsin gave Ser-Lys*-Asn-Phe and Ala-Lys*-Asn-Met from cytosolic and mitochondrial AATs, respectively (Lys* = modified Lys). Interestingly, these modified peptides from tryptic and chymotryptic digestion displayed a positive circular dichroism (CD). The corresponding peptide fragments from native enzyme treated with borohydride did not give any CD.

Similar inactivation by β-chloroalanine was observed with alanine transaminase (Golichowski & Jenkins, 1978; Morino et al., 1979). In this case, a negative CD was observed for the inactivated enzyme. The spectral change observed during the inactivation was similar to that with AAT. The band at 427 nm disappeared and an inactivated form absorbing around 335 nm appeared. Upon longer incubation (over 6 h), a 435 nm band appeared, and the intensity of 335 nm decreased greatly.

Morino et al. (1974) made another interesting observation concerning the reaction of cAAT with β-chloroalanine. In the presence of 3 M
formate, the 333 nm form was converted to a form absorbing at 455 nm over a period of 20 h. The structure of this form was completely unclear. Removal of formate by dialysis produced a drastic change in the spectrum, giving rise to three absorption bands at 333, 375, and 420 nm. This spectral change was reversible. Addition of formate to this solution gave back the 455 nm band. This observation was interpreted as indicating that formate was bound to a discrete subsite within the active site which normally binds the distal carboxyl group of a natural dicarboxylic acid substrate.

Another surprising observation made by Morino et al. (1974), came from borohydride reduction experiments. After the reduction, the 333 nm form remained at the same position; however, the 455 nm band shifted to 365 nm. This 365 nm form released the radioactive label and chromophore when treated with 7 M guanidine HCl after borohydride reduction. Before denaturation, the radioactive label and chromophore were tied to the protein. However, over 80% of the radioactivity was found in the supernatant when the 20 h, 455 nm, incubation product, was reduced and denatured. The authors left the chemical nature of the 455 and 365 nm species uncertain. A similar spectral change was reported for alanine transaminase during longer incubations with β-chloroalanine by Golichowski and Jenkins (1978). A new band at 435 nm appeared over a period of 6 h. Again, the chemical nature of the compound giving rise to this band remained uncertain.

Cytosolic aspartate aminotransferase is also inactivated by other β-haloalanines. Morino and Okamoto (1970) reported the inactivation
reaction with β-bromoalanine. Silverman and Abeles described the reaction of cAAT with β-polyhaloalanine (1976). Enzyme was irreversibly inactivated by β-fluoroalanine and β-chloroalanine, but not by β, β-dichloroalanine or β, β, β-trifluoroalanine. Dichloroalanine did not bind to the enzyme, however. Trifluoroalanine bound, but did not inactivate. The authors suggested that the slow rate of α-proton removal from trifluoroalanine by cAAT might be the reason that it was not an effective inactivator. The proposed mechanism of inactivation of AAT with β-haloalanine or serine sulfate is shown in Scheme IV.

The role of formate is interesting. It enhances the rate of inactivation of cAAT with β-chloroalanine. Morino et al. (1974) proposed that formate ion was involved in a charge relay system in the active site. The authors concluded that the formate ion replaced the distal carboxyl group of the substrate and that this carboxyl interacted with an active site histidyl residue necessary for a charge relay system. However, recent X-ray crystallographic studies show that no histidyl residue is present in the substrate-binding region (Metzler et al., 1981).

Irreversible inactivation of aspartate aminotransferase with vinylglycine was reported by Rando (1974b), and Gehring et al. (1977b). Inactivation was time dependent, and the enzyme was protected by the presence of aspartate. Mercaptoethanol did not have any effect on inactivation. Inactivated enzyme absorbed light at 335 nm. This absorption band did not shift position either upon treatment with borohydride or upon change of pH (Rando did not state the pH range). Rando (1974b) suggested that the 335 nm band represented a pyridoxamine
Scheme IV.

$\text{IV}_a$

$X = \text{OSO}_3^-, \text{Cl}, \text{Br}, \text{F}$

$\text{IV}_b$

$\text{IV}_c$

$\text{IV}_d$

$\text{IV}_e$
derivative. Gehring et al. (1977b) showed that inactivation was 100% complete when α-ketoglutarate was present. The 335 nm form released its chromophore when treated with 10% trichloroacetic acid or 0.1 M sodium hydroxide. The chromophore released was identified as PLP by absorption spectroscopy and by high voltage paper electrophoresis. A chymotryptic peptide containing the radioactive label and chromophore was isolated and analyzed for amino acid content. The results showed equimolar amounts of Asp, Ser, Phe, and a peak that comigrated with homoserine lactone. From the known sequence, the peptide sequence was matched with an active site tetrapeptide. Dansylation, digestion with aminopeptidase N, and digestion with carboxypeptidase C confirmed the sequence, Ser-Lys*-Asn-Phe. As with β-chloroalanine, Lys-258 was the actual base modified. Gehring et al. (1977b) did not treat the inactivated enzyme with borohydride before the cyanogen bromide digestion. The proposed mechanism of inactivation of AAT with vinylglycine is shown in Scheme V. The pathway is quite analogous to the one with serine sulfate and β-chloroalanine (see Scheme IV). An α, β-unsaturated intermediate (V₄) similar to the α-aminoacrylate Schiff base intermediate (IV₄) was proposed. The β-position of the substrate is then presumably attacked by the ε-amino group of Lys-258 and covalently modified.

Recently Cooper et al. (1982) showed that β-methyleneaspartate acts as mechanism-based inactivator upon aspartate aminotransferase. This compound has been used in the study of the carbon skeleton rearrangement during a reaction catalyzed by the vitamin B₁₂-dependent enzyme, methylaspartate mutase (Barker et al., 1958). Incubation of cytosolic
Scheme V.
aspartate aminotransferase with β-methyleneaspartate results in a time-dependent loss of enzyme activity. It is accompanied by the loss of the absorption maximum at 430 nm of native enzyme and the appearance of a new peak at 335 nm. The latter peak is also seen for other mechanism-based inhibitors described in this section. Incubation of the enzyme with β-methyleneaspartate in the presence of 60 mM 2-mercaptoethanol, dithiothreitol, or glutathione does not reverse the inactivation. Cooper et al. proposed a possible mechanism; 1) β-methyleneaspartate reacts with pyridoxal phosphate at the active site yielding an aldimine which is converted to a ketimine. 2) Nucleophilic attack on the methylene carbon of the conjugated ketimine by residue X of the enzyme leads to an inactive form. Inhibition constant ($K_i$) of around 3 mM for β-methyleneaspartate suggests strong binding of this compound to the enzyme. Inactivated enzyme released pyridoxamine phosphate when treated with a high phosphate concentration under condition used to convert holo-enzyme to apo-enzyme (Bertland & Kaplan, 1968). The formation of pyridoxamine phosphate was also observed after the inactivation of the enzyme with 2-amino-4-methoxy-3-butenoic acid (Rando et al., 1976). Unfortunately in both cases the evidence for the quantitative release of pyridoxamine phosphate from the inactivated enzyme is weak. Cooper et al. also observed that the inactivation was not 100% complete.

The naturally occurring bacterial toxin, L-2-amino-4-methoxy-trans-3-butenoic acid (IIId; AMB) was shown to inactivate AAT in a manner similar to that of vinylglycine (Rando et al., 1976; Rando, 1974b). Inactivated enzyme absorbed light at 350 nm, a position which was independent of pH.
over the pH range 4-9. The proposed mechanism of the inactivation is shown in Scheme VI. The introduction of a poor leaving group (−OCH₃) increased the structural complexity of this inhibitor over that of vinylglycine. Isomerization of the double bond from β, γ to α, β was quite unfavorable in this case. Thus, the nucleophilic attack was more favorable at the γ-position than at the β-position. Because the −OCH₃ group was a poor leaving group, step c might be necessary before the release of methanol could occur. AMB also inactivates sheep liver threonine dehydratase and tryptophan synthetase (Miles, 1975a, b). Miles discussed a similar mechanism for tryptophan synthetase (1978).

Another type of mechanism-based inactivation of aspartate aminotransferase was observed with L-propargylglycine (IIIₑ; Tanase & Morino, 1976). A possible mechanism is shown in Scheme VII. The key step is the formation of a β-carbanionic intermediate, VIIₑ. This is presumably converted to the reactive allene intermediate, VIIᵋ, which is then attacked by Lys-258 to give inactivated form, VIIₑ. The importance of abstraction of the α-proton during inactivation with propargylglycine was suggested by Marcotte and Walsh (1975). Marcotte and Walsh found that those enzymes that released β-protons were inactivated by propargylglycine. Such enzymes are cystathionine γ-synthetase, alanine aminotransferase, γ-cystathionase (Abeles & Walsh, 1973; Washtien & Abeles, 1977).

Tanase and Morino (1976) found that propargylglycine alone did not inactivate either cytosolic or mitochondrial aspartate aminotransferases. However, in the presence of α-ketoacid, such as α-ketoglutarate, or pyruvate, gradual inactivation of AAT was observed with propargylglycine.
Scheme VI.
Scheme VII.
Inactivation was enhanced in the presence of 3 M formate. The formate effect was more drastic on the cytosolic than on the mitochondrial isoenzyme. This observation was analogous to that on the reaction with ß-chloroalanine (Morino et al., 1974). Light absorption of the native enzyme at 362 nm shifted to 340 nm and 385 nm after the inactivation. A negative CD at 340 nm and a positive CD at 385 nm were also observed. Alanine aminotransferase was found to exhibit only one absorption band at 325 nm when it was inactivated by propargylglycine (Marcotte & Walsh, 1975).

Evidence For the Existence of Reactive Intermediates During Mechanism-Based Inactivation Reactions of Pyridoxal Phosphate Dependent Enzymes

Cavallini et al. (1973) reported a protective effect of thiosulfate upon the inactivation of aspartate aminotransferase by serine-O-sulfate. Incubation of AAT with serine sulfate in the presence of thiosulfate did not lead to inactivation. Instead, cysteine-S-sulfonate was found as a product. The following scheme (VIII) was suggested by Cavallini et al. to explain this fact. The reactive intermediate (VIII_a), an α-amino-acrylate Schiff base, was trapped by the attack of the thiol group of thiosulfate before it could be modified by reaction with the active site residue, Lys-258. The trapped form (VIII_b) was spontaneously converted to the native form (VIII_c) and the product, cysteine-S-sulfonate.

Soper and Manning (1978, 1981) reported a mechanism-based inactivation of bacterial D-amino acid transaminase with ß-haloalanines. Experiments to trap the key intermediate in the reaction were carried out (Soper & Manning, 1978). When the enzyme was incubated with ß-chloro-D-
Scheme VIII.
alanine in the presence of β-mercaptoethylamine, it was protected from inactivation. The substrate continued to be consumed, but no pyruvate was formed. The authors isolated the products and identified a major product as S-(β-aminoethyl)cysteine and a minor one as Δ1-thiomorpholine-2-carboxylic acid. An α-aminoacrylate Schiff base was proposed as the reactive intermediate. The proposed scheme for the inactivation reaction of D-amino acid transaminase is shown in Scheme IX.

The formation of an α-aminoacrylate Schiff base has been proposed not only during mechanism-based inactivation, but also as a normal part of some pyridoxal-linked enzymatic reactions (Snell & DiMari, 1970; Davis & Metzler, 1972). Davis and Metzler reviewed the spectrophotometric evidences for the α-aminoacrylate Schiff base. The peak at 470 nm was assigned to α-aminoacrylate Schiff base by Soda (1967) and by Cook and Wedding (1976) in the reaction of O-acetylserine sulfhydrylase with O-acetyl-L-serine. A 455 nm band was reported by Tokushige et al. (1968) and Robinowitz et al. (1973) for aminocrotonate in the reaction of threonine dehydrase with L-threonine. Tryptophan synthetase with L-serine gave a spectrum which clearly showed a strong shoulder at 468 nm, representing an α-aminoacrylate intermediate (Goldberg & Baldwin, 1967). A peak position around 450-470 nm is reasonably assigned to α-aminoacrylate intermediates; however, no direct observation of this intermediate has been reported during mechanism-based inactivation reactions. The problem may be that the lifetime of α-aminoacrylate Schiff base may not be long enough. Recently Schnackerz et al. (1979) did observe the formation of an α-aminoacrylate Schiff base with bacterial
Scheme IX.
D-serine dehydratase. Stopped-flow techniques were used to observe a transient 455 nm form. The half-life time of the absorbance at 455 nm was 6.5 ms in the reaction of bacterial D-serine dehydratase with D-serine. Moreover, Schnackerz et al. (1979) synthesized a transient-state analog of the α-aminoacrylate-pyridoxal phosphate Schiff base (X) and observed the formation of a 460 nm peak when the analog was incubated with the apo-D-serine dehydratase. The analog was bound so tightly that treatment of the complex by dialysis or gel filtration did not release the analog. Yang et al. (1975) made complexes with apo-aspartate aminotransferase and 4-vinyl, and 4-ethynyl analogs of pyridoxal phosphate. None of the analogs showed absorption around 450-470 nm. It was presumably because they lack a second conjugated double bond outside the ring and also perhaps because of the absence of a nitrogen atom in their bond system.

One of the direct ways to learn the nature of the inactivated enzyme-substrate complexes is to determine the structural arrangement of the complexes. X-ray crystallography, of course, is the most powerful tool
for this purpose. Experiments to obtain difference density maps are in progress in Arnone's laboratory using AAT crystals which are modified with serine sulfate (private communication with Dr. A. Arnone, University of Iowa). Chemical degradation of the inactive enzymes has been used as the most successful approach to investigate the structure of the inactivated form. Washtien and Abeles (1977) reported the mechanism-based inactivation of γ-cystathionase with propargylglycine. The acid hydrolysis of labelled γ-cystathionase gave a product identified as 2-amino-4-ketopentanoic acid (XI\textsubscript{b}). Its formation is shown in Scheme XI.
Wang et al. (1981) carried out similar experiments on the product of serine transhydroxymethylase with D-fluoroalanine. Two radioactive compounds were isolated after the treatment of the complex with 6M urea, KBH₄, then acid hydrolysis. The products were identified as lanthionine and S-carboxyhydroxyethylcysteine by comparing the migration on TLC with that of synthetic reference compounds. The residue involved in inactivation was postulated to be cysteine. Base treatment of the inactivated complex of glutamate decarboxylase with serine sulfate is another example (Likos & Metzler, 1976). The yellow colored chromophore released is characterized and is described in this thesis.
METHODS

Materials

All chemicals used were of reagent grade. L-serine-O-sulfate was synthesized according to the procedure of Tudball (1962). The purity and authenticity were checked using high voltage electrophoresis and NMR. Synthetic 4-[2-methyl-3-hydroxy-5-(phosphoxymethyl)-4-pyridinyl]-2-oxo-3-butenolic acid 2, and synthetic 4-[2-methyl-3-hydroxy-5-(hydroxymethyl)-4-pyridinyl]-2-oxo-3-butenolic acid 3, were prepared by John Likos (Monsanto Co., St. Louis, Mo.) according to the procedures described by Schnackerz et al. (1979). Synthetic 5, the NaBH₄ reduction product of 3, was also prepared by J. Likos in a crystalline form.

Absorption Spectra

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 baseline errors and small amounts of turbidity. The data were replotted automatically as apparent molar absorptivity or absorbance vs. wave number (Johnson & Metzler, 1970; Metzler et al., 1973). The method of Nagano and Metzler (1967) was used to evaluate pK values, to plot spectrophotometric titration curves and to calculate the spectra of the individual ionic forms of the compounds studied. A Cary 219 spectrophotometer was also used.

Nuclear Magnetic Resonance Spectra

Spectra were recorded using a Bruker WM300WB spectrometer equipped with a wide bore 300 MHz superconductive magnet, variable temperature...
control unit, Diablo 44d disk, Aspect 2000 computer with 48K (24-bit words) memory, and an advanced software, DISNMR. Samples for $^1H$ nuclear magnetic resonance (NMR) spectra were typically 0.2-40 mM in chromophore concentration (total amount 0.1-20 mmol). High quality NMR tubes (5 mm inner diameter, #527-PP, Wilmad Glass Co.) were used. The volume of the samples was limited to 0.5 mL. Deuterium oxide was used as an internal lock signal and solvent; to avoid an undesirably high HDO peak, "100 % D$_2$O" was used. Special care was taken in handling the 100 % D$_2$O. The necessary volume of D$_2$O was taken up into a well-dried syringe which had been filled with dry N$_2$ gas. Chemical shifts were recorded in parts per million (ppm) relative to an external standard present in a capillary tube. A disposable micropipette of 5-10 µL volume was used as a capillary. It was filled with deuterated chloroform containing 1 % of tetramethyilsilane (TMS) and was then sealed by torch. The external standard exhibited one major peak for TMS at 0.0 ppm and two small peaks for CHCl$_3$ at 1.5 and 7.2 ppm. Typically 16 K words of computer memory were used for $^1H$ measurements giving a digital resolution of 0.49 Hz. A 30° flip angle (2.1 µs pulse) was employed with a delay time of 1.0 s while the water peak was decoupled. When decoupling was not carried out, the delay time was shortened to 0.1 s.

Carbon 13 NMR spectra were recorded at 75.5 MHz using 10 mm tubes. Broad band decoupling (1-2 Watts) was employed during the measurements unless otherwise indicated. An external capillary containing dioxane was used as a standard. Observed chemical shifts were made relative to TMS by adding 66.5 ppm. Typically 32 K words of computer memory giving a
digital resolution of 1.22 Hz, a 60° flip angle (15 μs pulse) and an 0.2 s delay time were employed. To assign the $^{13}$C resonances, broad band decoupled, gated decoupled, or specific proton decoupled spectra were taken and compared.

The value of pH was estimated by adding 0.41 to the reading on a Radiometer model PHM 64 pH meter. The pH of the sample was adjusted by adding DCl (20% in D$_2$O) or NaOD (40% in D$_2$O).

Other NMR instruments such as JOEL FX90Q, Bruker WH90, Varian HA-100, Hitachi-Perkin Elmer R20B, or Varian A-60 were also used.

**Amino Acid Analysis**

Amino acid analysis was performed on a Durrum D 400 analyzer by W. Harris after hydrolysis of the samples in 6N HCl at 110°C for 22 h.

**Gas Chromatography**

Gas chromatography was carried out with a Packard-Becker model 419 chromatograph. The instrument had a flame ionization detector. The 2.4 m x 2 mm glass column was packed with 3% Dexil 300 on Chromosorb W AW DMCS 100/120 mesh. The column temperature was programmed from 120 to 300 °C at 15 °C/min. The injection port temperature was 260 °C, and the detector temperature was 310 °C. The flow rates of H$_2$, air, and the carrier gas (Ar) were 25, 250, and 20 mL/min, respectively. Experiments were also done in Professor J. Fritz's laboratory using a Tracor 550 gas chromatograph with a Tracor 702 N-P detector. An OV1 column (1/4" x 6') was used.

**Mass Spectra**

Mass spectra were measured by S. Vesey and J. Bean at the ISU Chemistry Instrument Services using a Finnigan 4000 GC-MS data system or an AEI MS-902 mass spectrometer.
Enzymes

L-Glutamate decarboxylase was isolated from Escherichia coli (ATCC 11246) as described by Fonda and DeGrella (1974). The following improvements were done. The final two steps make use of columns of DEAE-Sephadex A50 and Sephacryl S200, respectively (Figures 1 & 2). It was found that during the first of these steps, a protein "factor" running behind the glutamate decarboxylase was sometimes separated from the enzyme (Figure 1). It was necessary to add this factor back to the enzyme before a rapid reaction with serine sulfate could be observed. Molar concentrations of the enzyme active sites were calculated from the heights of the absorption bands at 420 nm at pH 4.6 assuming a molar absorptivity of 10,000 (Fonda, 1971). Enzyme activity was determined using a Gilson differential respirometer (Fonda, 1971).

The α subform of cytosolic aspartate aminotransferase was isolated from pig hearts as described by Martinez-Carrion et al. (1967; see also Yang & Metzler, 1979).

Partial Purification of Protein Factor

Fractions numbers 90-160 following the glutamate decarboxylase on the DEAE-Sephadex A50 column (Figure 1) were pooled and concentrated. The concentrate was passed through a column of Sephacryl S200 using the same conditions as were used for the final step in the purification of the glutamate decarboxylase (Yang & Metzler, 1979). Using the assay described in the following section, three major protein bands were separated; protein factor was located in the third band (Figure 3).
Figure 1. Chromatography of Glutamate Decarboxylase and Protein Factor on DEAE-Sephadex A50

Solid line shows the elution profile followed at 280 nm, and the solid line with open circles indicates the protein factor activity (tubes no. 90-160). Glutamate decarboxylase activity was found in tubes no. 78-85.
DEAE SEPHADEX A50 ELUTION DIAGRAM

PROTEIN FACTOR, TOTAL UNITS

TUBE NO.

A 280NM

1.00

0.50

0.00

0

40

80

120

160

0

100

200

300

400

500

600
Figure 2. Chromatography of Glutamate Decarboxylase on Sephacryl S200

Glutamate decarboxylase was prepared by column chromatography on DEAE-Sephadex A-50. Absorbance at 280 nm is plotted versus tube number. Glutamate decarboxylase activity was found in tubes 53-60.
SEPHACRYL S200
ELUTION DIAGRAM

A 280 nm

TUBE NO.

0.00
0.40
0.80
1.20
1.60
2.00

GAD

D-
Molecular Weight Estimate for the Protein Factor

The molecular weight of the partially purified protein factor was estimated by electrophoresis in 10% acrylamide gel in the presence of sodium dodecyl sulfate. The gel was calibrated with four proteins; ribonuclease A (Mr = 13,700), chymotrypsinogen (Mr = 25,000), ovalbumin (Mr = 43,000), and bovine serum albumin (Mr = 67,000). The migration of proteins (as Rf) was plotted versus log Mr (Figure 4). Here Rf is defined as the mobility of a protein divided by the mobility of the marker dye, bromophenol blue. The position of this dye was marked on the gel prior to staining with Coomassie blue.

Assay for the Protein Factor

Protein factor that had been partially purified by chromatography as described above was tested with highly purified glutamate decarboxylase. Glutamate decarboxylase (37.2 nmol) in acetate buffer (0.1 M, K+ salt, pH 4.6) and L-serine-O-sulfate (17.8 μmol) were mixed in a cuvette in a volume of 0.62 mL at 25 °C. The absorbance (A₀ = 0.60) at 417 nm did not change significantly with time. A portion of protein factor (5-30 μL volume) was added and the absorbance change at 417 nm was recorded versus time (Figure 5). The final (infinite time) absorbance A∞ for the biphasic reaction was estimated (A∞ = 0.016) and a graph of log₁₀ (A₉ - Aₜ) against time (Figure 6) was prepared where A₉ was the absorbance at time t after addition of the protein factor. The apparent first order rate constants (k) for the second phase of the reaction were
Figure 3. Chromatography of the protein factor on Sephacryl S200

The eluent was monitored at 280 nm (solid line). The line with the open circles indicates the protein factor activity.
SEPHACRYL S200 ELUTION DIAGRAM

PROTEIN FACTOR, TOTAL UNITS

TUBE NO.

40 60 80 100 120 140

A 280nm
Figure 4. Molecular weight of protein factor by gel electrophoresis in the presence of sodium dodecyl sulfate

Open circles represent known marker proteins. 1 represents glutamate decarboxylase. 2 represents protein factor.
Figure 5. Effect of protein factor on reaction of glutamate decarboxylase with serine sulfate

Glutamate decarboxylase (37.2 nmol) and L-serine-O-sulfate (17.8 µmol) were mixed with various amounts of the protein factor in the cuvettes to adjust the final volume to 0.62 mL. The amounts of the protein factor added are indicated in units of microliters. Initial ($A_0$) and final ($A_\infty$) absorbances were 0.016 and 0.016, respectively. The absorbance at 417 nm is plotted versus time.
PROTEIN FACTOR ASSAY

TIME (MIN)

0.00  4.00  8.00  12.00  16.00  20.00
Figure 6. Semilogarithmic plot of the changes in absorbance at 417 nm with time.

Numbers indicate the amount of protein factor added in microliters.
PROTEIN FACTOR ASSAY

LOG[(A_t - A_0)]

TIME (MIN)

0.00  4.00  8.00  12.00  16.00  20.00
estimated from the slopes (slope = \(-k/2.303\)) in the above graph (Figure 6). These rate constants are plotted against the amount of protein factor in Figure 7. A unit of protein factor activity was defined as follows (3):

\[
1 \text{ unit} = \frac{10}{t_{1/2}} = \frac{10k}{\ln 2} = 14.4\ k, \quad (3)
\]

where \(k\) was the apparent first order rate constant for the second phase of the decay with the specific amount of glutamate decarboxylase (37.2 nmol).

**Preparation of the Initial Product (1) of Reaction of Serine Sulfate with Glutamate Decarboxylase**

In a typical preparation, 52.5 mL of 6.1 \(\times\) 10\(^{-5}\) M glutamate decarboxylase (160 mg of protein; 3.2 \(\mu\)mol) 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 final 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 in the preceding section. 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-liter portion of redistilled water.
Figure 7. The apparent rate constant versus the amount of protein factor

The apparent first order rate constants for the second phase of the reaction were obtained from the slopes in Figure 6.
Preparation of the Products la, lb, and lc of Reaction of Serine Sulfate
With Cytosolic Aspartate Aminotransferase

In a typical reaction, a 25 mL volume of 2.8 x 10^-4 M aspartate
aminotransferase (316 mg of protein, 6.8 μmol of pyridoxal phosphate) in
0.04 M sodium acetate buffer, pH 5.4, was used. To this was added 70 mg
of solid L-serine-O-sulfate to give a concentration of 12.6 mM. After 10
min at room temperature, the enzyme was converted almost quantitatively
to the inactive form la. Over a period of 20 h at room temperature, the
absorption maximum shifted from 336 nm to 455 nm (form lb). This form
was stable as long as the solution was not dialyzed. However, dialysis
against water for 8 h caused the disappearance of the 455 nm band and the
formation of lc, a form characterized by a 430 nm absorption band at pH
5.4 and a 360 nm band at pH 8 (Morino et al., 1974).

Preparation of the Low Molecular Weight Yellow Product 2

The pH of a solution of compounds 1, la, lb, or lc 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. When the volume of the protein solution had been reduced
to 1-2 mL, it was diluted with 3-5 mL of 0.05 N KOH or water and again
ultrafiltered. This "washing" was then repeated two 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 (see Table I), was passed through a thoroughly
washed 0.7 x 6 cm column of Dowex 50 (x8, 100-200 mesh) ion exchange
resin in the H⁺ form. The resin was repeatedly treated alternatively
<table>
<thead>
<tr>
<th>Volume (mL)</th>
<th>Absorbance A (nm)</th>
<th>mol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30</td>
<td>0.35 (420)</td>
<td>1.05</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0.25 (420)</td>
<td>0.74</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.20 (420)</td>
<td>0.61</td>
</tr>
<tr>
<td>5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>1.13 (311)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<sup>a</sup>The molar absorptivity, $\varepsilon = 10,000$ for native GAD was used for the calculation.

<sup>b</sup>GAD = glutamate decarboxylase.

<sup>c</sup>Compound 5 purified by gel filtration on BioGel P2.
Table Ib

Yields and Apparent Molar Absorptivities of Compounds Derived From Serine Sulfate-treated Enzyme

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>Volume (mL)</th>
<th>Absorbance A (λ, nm)</th>
<th>μ mol</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-aspartate</td>
<td>aminotransferase</td>
<td>24.5</td>
<td>18.24 (280)</td>
<td>65.50a</td>
<td>6.82</td>
</tr>
<tr>
<td>2</td>
<td>20 h dialysis of 1a vs H₂O, then to pH 11.5</td>
<td>32</td>
<td>15.1 (280)</td>
<td>71</td>
<td>7.81b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.46 (415)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>After ultra-filtration</td>
<td>83</td>
<td>0.53 (415)</td>
<td>7.81</td>
<td>5.63</td>
</tr>
<tr>
<td>3</td>
<td>After alkaline phosphatase</td>
<td>83</td>
<td>0.525 (310)</td>
<td>7.20c</td>
<td>6.1</td>
</tr>
<tr>
<td>5</td>
<td>Treated with NaBH₄, then acidified</td>
<td>88</td>
<td>0.590 (282)</td>
<td>8.42d</td>
<td>6.17</td>
</tr>
<tr>
<td>5</td>
<td>Chromatographed on DEAE-Sephadex</td>
<td>24</td>
<td>1.313 (308)</td>
<td>9.10</td>
<td>3.46</td>
</tr>
<tr>
<td>5</td>
<td>Passed through Biogel P2 column</td>
<td>12.4</td>
<td>2.48 (312)</td>
<td>9.10</td>
<td>3.38</td>
</tr>
</tbody>
</table>

aFrom Furbish et al. (1969). See also Yang and Metzler (1979).

bBased on pure synthetic 2.

cBased on pure synthetic 3.

dBased on pure synthetic 5.
avoid contamination of NMR samples in the aromatic region. 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.

Another procedure developed by R. Feldhaus in this laboratory made use of SP-Sephadex (SP-G25, 40-120 mesh beads) in the H⁺ form. This was prepared by treating the beads with 0.2 M HCl for 10 min and washing quickly with water. The treatment was repeated a second time. An 0.8 x 15 cm column was prepared and washed with about 6 L of water. Compound 2 from 100 mg of enzyme prepared with a minimum of KOH was passed through the column which was then washed with 20 mL of water. The combined effluent was lyophilized and taken up in D₂O for NMR spectroscopy. Compound 2 prepared in this way was free of aromatic impurities; however, a number of impurities with upfield resonances, presumably sugar residues from the Sephadex gel, were detected by NMR.

Two alternative methods were also developed for preparation of 2.

(1) The pH of an inactivated enzyme solution was raised to 11.0 by the addition of 0.1 N KOH. Then a solid trichloroacetic acid was added to precipitate the protein. Over 90 % of the chromophore remained in the supernatant. It was necessary to use homogeneous enzyme for this experiment. Otherwise some proteins remained in the supernatant after the trichloroacetic acid precipitation. (2) Compound 2 was also isolated by chromatography on Sephadex G25 (1.5 x 46 cm). Compound 1 at pH 11 (0.2 μmol) was applied to the column of Sephadex G25, and was eluted with 0.05 N KOH. Only about 30-40 % of the chromophore was
(0.2 μmol) was applied to the column of Sephadex G25, and was eluted with 0.05 N KOH. Only about 30-40% of the chromophore was recovered without overlap with the protein absorption band. Thus, the separation was not satisfactory.

The purity of 2 was checked by either high voltage electrophoresis (HVE) or by thin layer chromatography (TLC). Sheets (23 x 57 cm) of Whatman 3mm paper were used for HVE with 0.1 M pyridine-acetate buffer, pH 6.5 as the electrolyte. HVE was conducted for 20 min at 2000 V. Under these conditions 2 had the greatest electrophoretic mobility and moved 6.5 cm toward the anode. The sample could be located by spraying a test strip of the electropherogram with Gibbs reagent (2,6-dichloro-quinone-4-chloroimide) or by its distinct orange fluorescence when illuminated with ultraviolet light (Mineralight UVS 11). Silica gel plates from EM Merck were used for ascending thin layer chromatography with a 1-butanol-acetic acid-water (4:1:1) solvent. A weak yellow band which gave a positive result for Gibbs reagent and a characteristic fluorescence was observed at 0.25 cm (Rf = 0.015).

Preparation of 3 by Dephosphorylation of 2

Solutions of 2 at pH 11 as obtained by ultrafiltration of the inactivated and base-treated enzymes were adjusted to pH 8.1 with 0.05 N HCl. Approximately 0.03 mg of alkaline phosphatase from E. coli 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 x
68 cm column of BioGel P2 with water as the eluant. Compound 3 emerged shortly after the void volume in a sharp band. 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.

**Preparation of 4 By the Reduction of 2**

Compound 2 was reduced with sodium borohydride at pH 8. The reduction reaction was monitored 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.

**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 or borohydride 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% (see Table 1).

**Preparation of 6 by Dephosphorylation of 4**

The pH of a solution of 4 was adjusted to 8-9, then *E. coli* alkaline phosphatase (about 0.03 mg) was added. The dephosphorylation reaction was run overnight at room temperature; then the solution was lyophilized.

**Alkaline Hydrolysis of 2**

To 200 µL of a solution containing 18 nmol of GAD 2 in a 1.1 x 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 by V. Chen according to Yang and Metzler (1979). The concentration was made as 6.4 mg/mL (1.4 x 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 equivalents per equivalent 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).

**Preparation of Model Compounds**

Commercially available compounds such as pyridoxal, pyridoxamine, pyridoxine, and dipeptides were used without further purification. Reduced Schiff bases of pyridoxal with alanine, isopropylamine, and ethanolamine were synthesized and crystallized as follows. Pyridoxal.HCl (3.7 mmol) was mixed with 5.7 mmol of amine in 20 mL methanol at pH 10. Solid sodium borohydride was added until yellow color diminished, then pH was lowered to 5 with 6N HCl. After dryness, the product was redissolved in 10 mL of water and loaded on CG 50 column (1.5 x 45 cm) eluted with water. About 1.5 L of water was required to elute pure product off the column. The product was monitored by its absorption at
326 nm at neutral pH. The pooled fraction was concentrated to about 20 mL and the product was crystallized from ethanol/water. Purity was checked by TLC, high voltage electrophoresis, or NMR spectrum. Reduced ethanolamine-pyridoxal Schiff base gave $R_f = 0.24$ (TLC), mobility of 14 cm toward anode (electrophoresis).

**Permethylation of Model Compounds**

The compounds were subjected to permethylation, first described by Hakomori (1964) and reviewed by Morris (1980).

To avoid forming quaternary ammonium salts of the free amino group during methylation (described later), the compound was acetylated first. Four volumes of dry methanol were mixed with one volume of acetic anhydride. To 100–500 nmol of model compound, 0.5 mL of the above mixture was added. The mixture was allowed to stand at room temperature for 3–4 h. Then the reagents were removed in vacuo.

N-, O-, or S-permethylation is done with methyl iodide, using the methylsulfinylmethide carbanion as a catalyst. The carbanion is produced as follows: An amount of NaH/oil dispersion containing 120 mg (5 mmol) NaH is rinsed 3 times with hexane. Five mL of dry dimethylsulfoxide is added and the suspension is heated under nitrogen until evolution of hydrogen has ceased. The resulting clear solution has 1 mmol/mL of DMSO$^-$ and is kept for several weeks stored under nitrogen at 0 °C.

The acetylated compound (about 10 μmol) is dissolved in 50 μL of DMSO. Under nitrogen, 50 μL of the carbanion solution is added (about a 5-fold molar excess). After 10 min, 8 μL of methyl iodide is added under nitrogen (10 fold molar excess). The reaction proceeds at room
temperature for 5 min. I have varied this reaction time from 1 min to 12 h and found that 5-10 min was optimal. The reaction is terminated by the addition of 1 mL of water. The permethylated compound is extracted by shaking with 1 mL of chloroform and removing the water layer. The chloroform layer is then washed 3 times with 1 mL of water, and introduced into the GC-MS spectrometer. Overreaction causes methylation of the ring nitrogen which becomes a quaternary ammonium ion and therefore not extractable with chloroform.
RESULTS

Reaction of Glutamate Decarboxylase With Serine Sulfate

The reaction of glutamate decarboxylase with serine sulfate was extensively investigated by Likos (1977). Likos described the reaction of serine-O-sulfate (12.5 mM) and glutamate decarboxylase (4.1 x 10^{-5} M) which had been purified through the step of chromatography on DEAE-Sephadex A50 were incubated in 0.05 M sodium acetate buffer, pH 4.6, at 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 five minutes. Less than 10 % remained after 90 min and the enzyme was totally inactivated in 4 h. Preliminary kinetic studies have been also reported by Likos (1977).

Protein factor

Several batches of enzyme which I prepared reacted just as described in the preceding paragraph. However, later I obtained a presumably more highly purified enzyme that failed to react with serine sulfate. At this point, I began to add back other fractions from the DEAE-Sephadex A50 column and discovered that a fraction running behind the glutamate decarboxylase (Figure 1) enhanced dramatically the rate of reaction with serine sulfate. While the kinetics of the inactivation reaction were biphasic (Figures 5 & 6), the rate was determined largely by the concentration of the factor present in this fraction. An assay for the factor was devised and a partial purification was achieved as described
in METHODS. The partially purified factor has an absorption maximum at 280 nm. It is destroyed by boiling. It can be concentrated using an Amicon PM10 ultrafiltration membrane and is not lost upon prolonged dialysis against acetate buffer, pH 4.6. The factor activity was not removed when a solution of the factor was mixed vigorously with 1/5 volume of activated charcoal for 10 min. The presence of up to 0.2 M ethylenediaminetetraacetic acid (EDTA) did not inhibit the factor activity significantly. Treatment of the factor with trypsin appeared to lead to loss of the factor activity. A carbohydrate color test was carried out (Table II). A significant increase in absorbance at 490 nm was observed using the factor isolated from chromatography on DEAE-Sephadex A50. The results indicate that the factor is neither a nucleotide nor a metal related compound. It is likely that the factor is a protein with molecular weight greater than 10,000. However, it is not clear whether or not the factor contains some kind of carbohydrate.

Additional evidence was given by the dialysis experiment (Table III). The reaction of glutamate decarboxylase and serine sulfate was carried out in a dialysis tube in the presence of the factor either inside or outside of the dialysis membrane. After 4 h dialysis, the spectra of the enzyme solution were measured, and the decreases of 420 nm absorption bands were recorded. The results were as follows. (a) Glutamate decarboxylase dialyzed against buffer which contained serine sulfate alone or serine sulfate plus the factor decreased in absorption at 420 nm only 10-15 % compared to the original height. (b) Glutamate decarboxylase together with the factor dialyzed against serine sulfate-
Table II
Carbohydrate Color Test

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Absorbance at 490 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O + phenolᵇ</td>
<td>0.11</td>
</tr>
<tr>
<td>2ｃ + H₂O</td>
<td>0.13</td>
</tr>
<tr>
<td>2ｃ + phenol</td>
<td>0.76</td>
</tr>
<tr>
<td>GADᵈ + phenol</td>
<td>0.21</td>
</tr>
<tr>
<td>PFEᵉ + phenol</td>
<td>0.50</td>
</tr>
</tbody>
</table>

ᵇ0.5 mL of sample was mixed with 0.5 mL of 5% phenol (5 g/100 mL of H₂O). To the mixture was added 2.5 mL of concentrated A₂SO₄ and the solution was mixed vigorously. Then, the absorbance at 490 nm was measured.

ᵇ5% phenol.
ᶜ60 nmol of 2.
ᵈGlutamate decarboxylase.
ᵉProtein factor; isolated by chromatography on DEAE-Sephadex A50.
Table III

Dialysis Experiment

<table>
<thead>
<tr>
<th>Inside dialysis tube</th>
<th>Outside dialysis tube</th>
<th>% Completion of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADc (0.15 μmol)</td>
<td>Serine sulfate (20 mg)</td>
<td>15</td>
</tr>
<tr>
<td>GAD + PFd (0.2 mL)</td>
<td>Serine sulfate</td>
<td>94</td>
</tr>
<tr>
<td>GAD</td>
<td>Serine sulfate</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>+ PF (1 mL)</td>
<td></td>
</tr>
</tbody>
</table>

3 mL of 0.1 M potassium acetate buffer, pH 4.6, was used as dialysis buffer. % completion of reaction was calculated based upon the change of absorbance at 420 nm. Dialysis was conducted for 4 h at 4 °C. Glutamate decarboxylase. Protein factor.
containing buffer decreased its spectrum by 94%. The results suggest that the factor has a high molecular weight and interacts directly with glutamate decarboxylase. When the protein factor and serine sulfate were added together to the enzyme, the results were as shown in Figures 5 & 6. However, if the protein factor was preincubated with the GAD for 10 min prior to addition of serine sulfate only the slow phase of the biphasic reaction was observed.

The protein factor was subjected to electrophoresis in polyacrylamide gels with and without sodium dodecyl sulfate (SDS) (Figures 8 & 9). The gels were stained with Coomassie brilliant blue R-250. One major band was shown using either type of electrophoresis. The apparent molecular weight of the SDS denatured factor was estimated as 32,800 (Figure 4). The molecular weight of GAD estimated using the same method was 44,000 (Figure 4). This value is close to 45,000-48,000, reported by Strausbauch and Fischer (1970a) using a similar method. The partially purified factor had no effect on the electrophoretic mobility of glutamate decarboxylase if both were mixed prior to the electrophoresis (Figure 9) and had only a small effect on the enzymatic activity of glutamate decarboxylase acting on its normal substrate.

Properties of GAD 1

Likos investigated the properties of the enzyme inactivated with serine sulfate and his description is as follows. 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.
Figure 8. Sodium dodecyl sulfate polyacrylamide slab gel patterns of the glutamate decarboxylase and the protein factor

10% gel was prepared (see text).

a. Glutamate decarboxylase purified on DEAE-Sephadex A50 (20-30 µg).

b. Glutamate decarboxylase purified on Sephacryl S200 (5-10 µg).

c. Protein factor purified on Sephacryl S200 (30-50 µg).

d. Protein factor purified on Sephacryl S200 (30-50 µg). Prepared separately from c.

Both c and d were overloaded intentionally in order to see the contaminations. The molecular weight indicated for glutamate decarboxylase and protein factor were obtained from separate experiments.
7.5 % gel was prepared (see text).

a. Glutamate decarboxylase purified on Sephacryl S200 (5 μg)
   (1, hexamer; 2, probably dimer; 3, probably monomer).

b. Protein factor purified on Sephacryl S200 (5 μg).

c. Glutamate decarboxylase (5 μg) and protein factor (5 μg)
   were mixed together prior to gel electrophoresis.
Dialysis of the inactivated enzyme (compound 1) against 0.05 M acetate buffer, pH 4.6, for 24 h did not alter the spectrum. Assuming that the molar absorptivity at 282 nm is the same as that of the holoenzyme (88,000 M⁻¹ cm⁻¹, based on monomer, Fonda, 1971), the molar absorptivity of the chromophore was estimated as 8.2 x 10³ 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 was 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 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.

Reaction of Cytosolic Aspartate Aminotransferase With Serine Sulfate

I found that the reaction product of aspartate aminotransferase with serine sulfate gave an identical product 2 with base. The use of aspartate aminotransferase for completion of the structure determination for compound 2 was attractive since it can be isolated in gram quantity.
In agreement with the report of John and Fasella (1969), treatment of aspartate aminotransferase (AAT) with L-serine-O-sulfate causes a rapid loss of the 430 nm absorption band present at low pH, appearance of a new band at 336 nm (Figure 10) and inactivation of the enzyme. With 10 mM serine sulfate, over 95% of the catalytic activity is lost within 5 min at 25 °C. I designate the product of this initial reaction 1a.

Over a 20 h period at room temperature, 1a is converted to a second inactive species 1b, which has an absorption maximum at 455 nm (Figure 10). This conversion occurs at either pH 5.4 or pH 8 and under either O2 or N2 at similar rates. However, at 5 °C 1a is quite stable and is transformed only very slowly into 1b. It should be noted that conversion of 1a to 1b never seems to be quite complete. A shoulder of variable height always remains at about 336 nm. Form 1b is presumably identical to the 455 nm form of aspartate aminotransferase formed from β-chloroalanine (Morino et al., 1974).

As reported by Morino et al. (1974), the spectrum of 1b is altered by dialysis against water, the absorption maximum shifting from 455 to 360 nm (1c, Figure 12). The process is reversible, dialysis against 3 M formate converting the 360 nm band (1c) back to 455 nm (1b). The 455 nm band is formed by reaction of the enzyme with serine sulfate at either pH 8.3 or pH 5.4. No change in spectrum with pH is seen. However, after dialysis against water the 360 nm band of 1c shifts reversibly to 430 nm when the pH is lowered to 5.4. Compound 1c has an absorption spectrum similar to that of the native enzyme, but it has no catalytic activity. When 1b is boiled, no chromophore is released.
Figure 10. Reaction of aspartate aminotransferase with serine sulfate

Formation of compound la.
A = An electronic spectrum of native enzyme.
B = An electronic spectrum of la, 10 min product.
C = An electronic spectrum of reduced la.
Figure 11. Reaction of aspartate aminotransferase with serine sulfate

Formation of compound 1b and 4.

A = An electronic spectrum of 1b, 20 h product.
B = An electronic spectrum of reduced 1b.
C = An electronic spectrum of 4, obtained heat treatment of reduced 1b.
Figure 12. Reaction of aspartate aminotransferase with serine sulfate

Formation of compound Ic.
A = An electronic spectrum of Ib.
B = An electronic spectrum of Ic at pH 5.4, obtained after dialysis of Ib against buffer.
C = An electronic spectrum of Ic at pH 8.45, obtained after dialysis of Ib against buffer.
However, after borohydride reduction a diffusible compound with an absorption maximum at 325 nm is freed from the protein by boiling (Figure 11).

Reaction of $\beta$ Subform of Aspartate Aminotransferase With Serine Sulfate

The $\beta$ subform of aspartate aminotransferase ($1.4 \times 10^{-4}$ M) was inactivated with serine sulfate (final concentration 12 mM) (Figure 13). The reaction was analogous to that of the $\beta$ subform. Initially, a 336 nm band (1a) appeared on the top of 340 nm band, then the formation of 455 nm form (1b) was observed over a 20 h period. The reduction of the 455 nm form by sodium borohydride at pH 5.3 gave two overlapping absorption bands at around 345 and 370 nm.

Properties of 1a

The absorption band of 1a at 336 nm is in the same position as that of the corresponding inactivated form of bacterial glutamate decarboxylase (1). Compound 1a is stable at low temperature and against prolonged (1 week) dialysis versus water at 4 °C. Treatment of 1a with solid sodium borohydride causes no change in the spectrum (Figure 10). Incubation of either 1a or reduced 1a with 0.3 mM pyridoxal phosphate did not cause any reactivation of the enzyme.

Heating 1a in a boiling water bath for 2 min caused denaturation of the protein and release of about 50% of the chromophore in a form absorbing at 403 nm (presumably 2). The same treatment of reduced 1a resulted in no release of chromophore. Treatment with sodium hydroxide of reduced 1a did not release 2. This is in agreement with the observation of Morino et al. (1974) that reduction fixes the chromophore to the protein. Heat denaturation of the native enzyme under the same
Figure 13. Reaction of \( \gamma \) subform of cytosolic aspartate aminotransferase with L-serine-O-sulfate

Solid serine sulfate (final concentration of 12 mM) was added to 400 \( \mu \)L of 1.4 \( \times 10^{-4} \) M aspartate aminotransferase in 0.04 M sodium acetate buffer, pH 5.1 at room temperature. (1) Absorption spectrum of native enzyme. (2) Spectrum of inactivated enzyme (1a'), 10 min after addition of serine sulfate. (3) Spectrum of inactivated enzyme (1b'), 20 h after addition of serine sulfate. (4) Spectrum of inactivated enzyme (1b'), treated with sodium borohydride.
conditions releases about 50% of the pyridoxal phosphate. Again, as is well-known, reduction fixes the coenzyme to the protein.

Properties of lb

The absorption spectrum of lb has a maximum at 455 nm whose position and intensity are constant from pH 5.4 to 8.3. It is devoid of circular dichroism. Dialysis against water or against 0.1 M acetate buffer, pH 5.4, or 0.1 M triethanolamine HCl buffer, pH 8.3, with or without addition of 0.3 mM pyridoxal phosphate causes no reactivation of the enzyme. Form lb is very stable in 0.06 M acetate buffer at pH 5.4 and rapidly forms large orange crystals in the presence of 8% polyethylene glycol of molecular weight 4000 (Metzler et al., 1979). However, the resulting crystals are not isomorphous with those of the native enzyme (A. Arnone, The University of Iowa, Iowa City, IA., private communication).

Dialysis against either 0.1 M acetate or triethanolamine buffers converted lb to lc. However, as reported by Morino et al. (1974), no change in spectrum was seen when lb was dialyzed against 3 M sodium formate.

Heating of lb in a boiling water bath releases only about 10% of the bound chromophore. However, when lb is heated in 3 M formate at pH 5.4, the chromophore is released quantitatively. The released material has an absorption maximum at 410 nm at pH 5.4 and its electronic spectrum at other pH values suggests that it is 2. Reduction with sodium borohydride gives a product with properties appropriate for 4.

Treatment of lb with either sodium borohydride or sodium cyano-borohydride shifts the absorption maximum from 455 nm to 361 nm (Figure
11). As reported by Morino et al. (1974), treatment with 6 M guanidine hydrochloride denatures the reduced 1b and releases about 80% of the chromophore as a low molecular weight material. This material, which we identify as compound 4, is obtained in a purer form if 1b is thoroughly dialyzed against water and is then heated in a boiling water bath.

Treatment of 1b with 9 mM β-mercaptoethanol or with 16 mM cysteine sulfinate slowly (overnight) converted it to products similar to that obtained by borohydride reduction with an absorption maximum at 360 nm and a negative circular dichroism band at about 365 nm. The conversion with β-mercaptoethanol was only about 50% complete.

Attempts were made to remove the chromophore from 1b by displacement with phosphate using the procedure employed for resolving the native enzyme of pyridoxamine phosphate (Scardi et al., 1963; Yang & Metzler, 1979). Warming at 37 °C with 1 M sodium phosphate at pH 5 did not alter the electronic and CD spectra of 1b during a 24 h period.

Properties of form 1c

The spectrum of the dialyzed form 1c resembles that of the native enzyme, the absorption band shifting from 430 nm at low pH to 360 nm at high pH (Figure 12). Form 1c is catalytically inactive and is converted to 2 when treated with NaOH. Heating of 1c in a boiling water bath also appears to produce 2 quantitatively.

Form 1c is converted back to 1b by the addition of ammonium formate at a concentration of 0.14 M or less. Reduction of 1c with sodium borohydride gives a band absorbing at 360 nm, just as does reduction of 1b. Upon heat denaturation compound 4 was apparently released.
Formation of Yellow Compound 2 From GAD 1

Likos (1977) showed the formation of the low molecular weight compound by treating the inactivated glutamate decarboxylase with base. When the pH of a solution of GAD 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 could be removed from the protein readily by ultrafiltration (Likos, 1977). I found that bringing a suspension of trichloroacetate-precipitated 1 to 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 Likos stated (1977) that 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. As shown by Likos, the position of the absorption band of 1 was not altered by 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 has been purified partially as described in METHODS.

Likos also observed that both β-chloroalanine and β-phosphoserine 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 to those of 2. On the other hand, I found no reaction with from 3 to 20 mM DL-α-methylserine-O-sulfate.

**Formation of 2 from Inactivated Aspartate Aminotransferase (Forms 1a, 1b, & 1c)**

Products 1a, 1b and 1c led to nearly quantitative release of the coenzyme as yellow compound 2 with the treatment of NaOH to raise the pH to about 11.

**Properties of 2**

In 1979, Schnackerz et al. reported the synthesis of the product of an aldol condensation between pyridoxal phosphate and pyruvate followed by dehydration. Its structure (X) is given on p. 34.

Likos noticed the apparent similarity of this compound with 2 isolated from glutamate decarboxylase. He synthesized the compound and showed that its electronic spectrum was identical to that of 2. Its NMR spectrum obtained on a 60 MHz spectrometer was compared with that of 2 and obtained on a 90 MHz spectrometer shown in his Ph.D. dissertation (Likos, 1977). He found a close resemblance between these two NMR spectra; however, because of the lack of sensitivity available when the spectrum of isolated 2 was taken a number of details were unclear. The studies using synthetic 2 described here have been carried out in collaboration with Likos. I independently obtained the NMR spectra of isolated samples of 2 from glutamate decarboxylase and aspartate aminotransferase using a 300 MHz spectrometer. The NMR spectra of 2
isolated from the enzymes are compared with that of synthetic 2.
Electronic spectra have also been compared.

**Electronic Spectra**
Study of the absorption spectrum of synthetic 2 versus pH showed the existence of 3 ionic forms separated by pK values of 3.90 and 8.76 (Figure 14, Tables IV & V). The absorption spectra of 2 isolated from glutamate decarboxylase is also shown in Figure 14. The change in spectrum with pH indicated the existence of three spectrally distinct ionic forms separated by pK values of 4.0 and 8.7 (Table V). Peak positions of individual ionic forms are listed in Table IV. The pK values and light absorption spectral properties of isolated and synthetic 2 were in satisfactory agreement.

**NMR Spectra**
Proton and carbon 13 NMR spectra of synthetic 2 are shown in Figures 15 & 16. The chemical shifts are summarized in Tables VI & VII. The proton NMR spectrum of synthetic 2 at low pH is striking (Figure 15a). The sharp resonances of the 6-H (8.30 ppm) and 2'-CH3 protons (2.72 ppm) and the doublet at 5.09 ppm representing the 5'-CH2 split by phosphorous are anticipated for the proposed structure. For example, the corresponding positions for pyridoxamine phosphate are 8,30, 2.73, and 5.12 ppm (doublet) respectively (Table VIII). The pair of coupled doublets at 7.79 and 7.35 ppm must represent the 4' and 5 protons. The coupling constant of 16.6 Hz observed for synthetic 2 shows that the configuration about the 4'-5 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 (Figure 15a). These have a coupling constant of 16.1 Hz. Integration of
Figure 14. Absorption spectra of individual ionic species of the keto acid 2

A. Compound isolated from reaction of glutamate decarboxylase with serine sulfate (data taken from Likos, 1977).
B. Synthetic compound. (1, low pH form; 2, neutral pH form; 3, high pH form).
Table IV

Peak Positions in Electronic Absorption Spectra

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cationic ring</th>
<th>Dipolar ionic ring</th>
<th>Anionic ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (GAD)</td>
<td>325 (shoulder)</td>
<td>411</td>
<td>426</td>
</tr>
<tr>
<td></td>
<td>282</td>
<td>289</td>
<td>296</td>
</tr>
<tr>
<td>(Literature)b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>412</td>
<td>292</td>
<td>245</td>
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<td>3</td>
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</tr>
<tr>
<td>5</td>
<td>286</td>
<td>311</td>
<td>295</td>
</tr>
<tr>
<td>6</td>
<td>293</td>
<td>342</td>
<td>308</td>
</tr>
</tbody>
</table>

*aEvaluated by computer-assisted fitting with lognormal curves.

Harris et al. (1976).

bSchnackerz et al. (1979).
Table V

Values of pK Determined Spectrophotometrically

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK values at 25°</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 synthetic</td>
<td>3.93&lt;sup&gt;a&lt;/sup&gt; 8.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 from glutamate decarboxylase (duplicate titrations)</td>
<td>3.93&lt;sup&gt;b&lt;/sup&gt; 8.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 synthetic from glutamate decarboxylase</td>
<td>3.68&lt;sup&gt;a&lt;/sup&gt; 4.69&lt;sup&gt;a&lt;/sup&gt; 8.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 reduction product from synthetic 3</td>
<td>—</td>
</tr>
<tr>
<td>5 synthetic from aspartate aminotransferase</td>
<td>4.77&lt;sup&gt;a&lt;/sup&gt; 9.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 from aspartate aminotransferase</td>
<td>5.15&lt;sup&gt;d&lt;/sup&gt; 9.66&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>R. Feldhaus, unpublished results. Private communication, Iowa State University, Ames, Iowa.

<sup>b</sup>BJ. Likos (1977).

<sup>c</sup>Rapid titration. Subsequent slow changes in spectrum occur, presumably as a result of an enol-keto equilibrium.

<sup>d</sup>Estimated from titration curves but without computer-assisted evaluation.
Figure 15. Proton NMR spectra of synthetic 2 obtained on a 300 MHz spectrometer

About 2 mg of synthetic 2 was used.

a. \( pK_a = 2.5 \), 259 scans at 24 °C.
b. \( pK_a = 7.2 \), 322 scans at 24 °C.
c. \( pK_a = 12.5 \), 509 scans at 40 °C.
Hydrate

TMS

HDO

2'

5'

4'

6
Figure 15 (continued)
Figure 16. Natural abundance carbon-13 NMR spectra of synthetic 2 obtained on a 75.5 MHz spectrometer at 24 °C.

About 10 mg of synthetic 2 was used.

a. pH = 1.9, 199707 scans.
b. pH = 7.1, 88172 scans.
c. pH = 12.4, 192900 scans.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>75.473</td>
</tr>
<tr>
<td>SM</td>
<td>25000.000</td>
</tr>
<tr>
<td>TD</td>
<td>16384</td>
</tr>
<tr>
<td>NC</td>
<td>192900</td>
</tr>
<tr>
<td>PR</td>
<td>H1</td>
</tr>
<tr>
<td>P1</td>
<td>13585.239</td>
</tr>
<tr>
<td>PP1^CM</td>
<td>2.4002</td>
</tr>
<tr>
<td>CY</td>
<td>10000</td>
</tr>
<tr>
<td>MI</td>
<td>1.533</td>
</tr>
<tr>
<td>P2</td>
<td>0.00020</td>
</tr>
<tr>
<td>D1</td>
<td>0.00020</td>
</tr>
<tr>
<td>D2</td>
<td>5.000</td>
</tr>
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<td>FW</td>
<td>31300</td>
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<td>PW</td>
<td>5.0</td>
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<tr>
<td>CY</td>
<td>112.36</td>
</tr>
<tr>
<td>LB</td>
<td>1.0000</td>
</tr>
<tr>
<td>CY</td>
<td>25.000</td>
</tr>
<tr>
<td>SP</td>
<td>61364.512</td>
</tr>
<tr>
<td>IS</td>
<td>0</td>
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<tr>
<td>PC</td>
<td>1.000</td>
</tr>
<tr>
<td>DI</td>
<td>0.00020</td>
</tr>
<tr>
<td>P2</td>
<td>5.000</td>
</tr>
</tbody>
</table>

Figure 16 (continued)
### Table VI

Chemical Shifts of Resonances in Proton NMR Spectra

<table>
<thead>
<tr>
<th>Compound 2</th>
<th>pD 2.48\textsuperscript{a}</th>
<th>pD 7.2</th>
<th>pD 12.4 (40°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm relative area</td>
<td>ppm relative area</td>
<td>ppm relative area</td>
</tr>
<tr>
<td>2'-CH\textsubscript{3} Ketone</td>
<td>2.72</td>
<td>3.32</td>
<td>2.49</td>
</tr>
<tr>
<td>Hydrate</td>
<td>2.68</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>4'-H Ketone</td>
<td>7.82</td>
<td>0.95</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>7.76</td>
<td></td>
<td>7.75</td>
</tr>
<tr>
<td></td>
<td>J=16.6 Hz</td>
<td>J=16.1 Hz</td>
<td>J=16.1 Hz</td>
</tr>
<tr>
<td>Hydrate</td>
<td>7.06</td>
<td>0.29</td>
<td>7.01</td>
</tr>
<tr>
<td></td>
<td>J=16.1 Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-H Ketone</td>
<td>5.10</td>
<td>2.20</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>5.08</td>
<td></td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td>J=7.8 Hz</td>
<td>J=5.0 Hz</td>
<td>J=4.4 Hz</td>
</tr>
<tr>
<td>Hydrate</td>
<td>5.06</td>
<td>0.71</td>
<td>5.04</td>
</tr>
<tr>
<td></td>
<td>J=7.8 Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6'-H Ketone</td>
<td>8.30 (1.00)</td>
<td>7.64 (1.00)</td>
<td>7.51\textsuperscript{b}</td>
</tr>
<tr>
<td>Hydrate</td>
<td>8.27</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>(\beta)-H Ketone</td>
<td>7.38</td>
<td>0.92</td>
<td>7.72</td>
</tr>
<tr>
<td></td>
<td>7.33</td>
<td></td>
<td>7.67</td>
</tr>
<tr>
<td></td>
<td>J=16.6 Hz</td>
<td>J=16.1 Hz</td>
<td></td>
</tr>
<tr>
<td>Hydrate</td>
<td>6.70</td>
<td>0.28</td>
<td>6.65</td>
</tr>
<tr>
<td></td>
<td>J=16.1 Hz</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}The ratio of ketone: hydrate is 3.26: 1 at 24 °C.
\textsuperscript{b}Overlapped.
\textsuperscript{c}C6H + \(\beta\)H.
Table VII
Natural Abundance Carbon-13 NMR Spectra for Compound 2

<table>
<thead>
<tr>
<th>Ketone</th>
<th>Hydrate (Hydration ratio)</th>
<th>Chemical shift in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pD 1.94</td>
</tr>
<tr>
<td>C-2</td>
<td>143.2</td>
<td>142.3</td>
</tr>
<tr>
<td>C-3</td>
<td>151.1</td>
<td>150.2</td>
</tr>
<tr>
<td>C-4</td>
<td>139.3</td>
<td>138.7</td>
</tr>
<tr>
<td>C-5</td>
<td>133.7</td>
<td>133.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J=7.6 Hz</td>
</tr>
<tr>
<td>C-6b</td>
<td>130.1</td>
<td>130.2</td>
</tr>
<tr>
<td>C-2'c</td>
<td>14.3</td>
<td>14.1</td>
</tr>
<tr>
<td>C-4'd</td>
<td>135.7</td>
<td>136.6</td>
</tr>
<tr>
<td>C-5'a,e</td>
<td>61.3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J=4.6 Hz</td>
</tr>
<tr>
<td>COOH</td>
<td>165.8</td>
<td>172.5</td>
</tr>
<tr>
<td>C(o)</td>
<td>188.7</td>
<td>91.7</td>
</tr>
<tr>
<td>C(β)</td>
<td>131.9</td>
<td>121.7</td>
</tr>
</tbody>
</table>

\(^a^\)Doublets arising from adjacent \(^3\)P.
\(^b^\)Doublet in gated proton decoupled spectrum, \(^1\)J(CH) = 167 Hz.
\(^c^\)Quartet in gated proton decoupled spectrum, \(^1\)J(CH) = 130 Hz.
\(^d^\)Triplet in gated proton decoupled spectrum, \(^1\)J(CH) = 158 Hz.
\(^e^\)Triplet in gated proton decoupled spectrum, \(^1\)J(CH) = 147 Hz.
\(^f^\)Doublet in gated proton decoupled spectrum, \(^1\)J(CH) = 189 Hz.
Table VIII
Chemical shifts for proton and carbon-13 NMR spectra of pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Position</th>
<th>Chemical shifts (ppm, downfield from tetramethylsilane)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMP</td>
<td></td>
<td>Proton pH 2.6 pH 6-7 pH 12&lt;sup&gt;b&lt;/sup&gt; Carbon pH 1.6&lt;sup&gt;c&lt;/sup&gt; pH 7 pH 12.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>144.5 146.0 151.1 136.4 135.8 132.2</td>
</tr>
<tr>
<td>2'</td>
<td>2.73 2.46 2.24</td>
<td>16.3 16.4 20.3 138.2 134.0 137.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>154.7 163.7 161.2 138.2 134.0 137.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>138.2 134.0 137.5 138.2 134.0 137.5</td>
</tr>
<tr>
<td>4'</td>
<td>4.45 4.30 3.70</td>
<td>36.1 37.6 37.6 36.1 37.6 37.6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>136.4 135.8 132.2 136.4 135.8 132.2</td>
</tr>
<tr>
<td>5'</td>
<td>5.13 4.93 4.69</td>
<td>63.0 62.9 63.8 63.0 62.9 63.8</td>
</tr>
<tr>
<td></td>
<td>J = 7.8Hz J = 7.3Hz J = 4.4Hz</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.30 7.66 7.48</td>
<td>132.9 125.1 134.1 132.9 125.1 134.1</td>
</tr>
<tr>
<td>PLP</td>
<td>pH 2.6</td>
<td>pH 6-7</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>2.66</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ald)</td>
<td>10.50</td>
<td>10.28</td>
</tr>
<tr>
<td>4'</td>
<td>(hyd)</td>
<td>6.55</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>5.15</td>
<td>5.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.23</td>
<td>7.57</td>
</tr>
</tbody>
</table>

<sup>a</sup>Unless indicated otherwise, data were collected on a Bruker WM300 NMR spectrometer at 24 °C. The data are in satisfactory agreement with those reported by Korytnyky and Ahrens (1970), Mantsch and Smith (1979) and Harruff and Jenkins (1976). This table is prepared for "Transaminases", Metzler and Christen (1983).

<sup>b</sup>Temperature = 40 °C. At 24 °C the HDO peak overlaps the 5' doublet.

<sup>c</sup>Data of Mantsch and Smith (1979).

<sup>d</sup>Values for hydrate except as indicated for 4'-carbon.
the 4'-H and 8-H doublets indicates a ratio of the 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 VI). 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 16a) where the
resonance for the hydrated carbonyl is seen clearly at 91.7 ppm. Again
the relative area of the two peaks 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 5 and 5' (Table VII). Hydration ratios estimated from these varied
between 1.9 (COO') and 3.5 (C-4). The NMR spectra of 2 at neutral and
alkaline pH values (Figures 15b, c, 16b, c, Tables VI & VII) are also
consistent with the assigned structure but there is no evidence for any
hydrate at these pH values.

The reliability of the hydration ratio given by NMR spectroscopy can
be checked by comparison with the hydration ratios of pyridoxal
5'-phosphate obtained from both proton NMR spectra and electronic spectra
(Table IX). Reasonable agreement is seen between the two methods,
especially at low pH.

Proton NMR spectra of 2 isolated from both glutamate decarboxylase
(Figure 17, Table X) and aspartate aminotransferase (Figure 18, Table X)
were also obtained. Spectra from three separate preparations of 2
(Figure 18) showed different degrees of contamination. Compound 2 shown
in Figure 18a was prepared using insufficiently washed Dowex 50
Table IX
Hydration Ratio of Pyridoxal 5'-phosphate
Comparison between nmr and electronic spectra

<table>
<thead>
<tr>
<th>pH</th>
<th>nmr spectra(^a)</th>
<th>electronic spectra(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>4.15(^c) - 4.4(^d)</td>
<td></td>
</tr>
<tr>
<td>~ 2</td>
<td>3.8(^e)</td>
<td>3.2</td>
</tr>
<tr>
<td>~ 6.8</td>
<td>0.2(^f) - 0.3(^g)</td>
<td>0.5</td>
</tr>
<tr>
<td>&gt; 12</td>
<td>0.01(^f) - 0.04(^h)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\(^a\) Measured with the Bruker WM300 WB spectrometer at room temperature. 1-10 mg of pyridoxal phosphate was dissolved in about 1 mL of D\(_2\)O. The pD value was adjusted by the addition of DCl or NaOD.

\(^b\) Data taken from Harris et al. (1976).

\(^c\) Averaged data from 2', 5', & 6 protons (3.7 - 4.7).

\(^d\) Averaged data from 2', 5', & 6 protons (3.55 - 4.9).

\(^e\) Averaged data from 2', 4', & 5' protons (3.2 - 4.45).

\(^f\) Taken from 4'- proton.

\(^g\) Taken from 6- proton.

\(^h\) Taken from 2'- proton.
Figure 17. Proton NMR spectrum of 2 isolated from glutamate decarboxylase obtained on a 300 MHz spectrometer at 24 °C

About 10 µmol of compound 2 was used at pH = 3.4, 236 scans.
CO hydrate

HDO

TMS
Table X
Comparison of Chemical Shifts\(^a\) of 2 isolated from GAD and AAT with synthetic 2

<table>
<thead>
<tr>
<th></th>
<th>GAD 9.7 (\mu)mol pD=2.36</th>
<th>AAT 3.0 (\mu)mol pD=1.21</th>
<th>Synthetic 10.6 (\mu)mol pD=2.48</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2'-\text{CH}_3) Ketone</td>
<td>2.71</td>
<td>2.72</td>
<td>2.72</td>
</tr>
<tr>
<td>Hydrate</td>
<td>2.68 (23%)</td>
<td>2.69 (28%)</td>
<td>2.68 (24%)</td>
</tr>
<tr>
<td>(4'-\text{H}) Ketone</td>
<td>7.81 J=16.6 Hz</td>
<td>7.84 J=16.6 Hz</td>
<td>7.82 J=16.6 Hz</td>
</tr>
<tr>
<td>Hydrate</td>
<td>7.06 J=16.1 Hz</td>
<td>7.07 J=16.1 Hz (30%)</td>
<td>7.06 J=16.1 Hz (23%)</td>
</tr>
<tr>
<td>(5'-\text{H}) Ketone</td>
<td>5.10 J=7.8 Hz</td>
<td>5.11 J=7.8 Hz</td>
<td>5.10 J=7.8 Hz</td>
</tr>
<tr>
<td>Hydrate</td>
<td>5.06 J=7.8 Hz (31%)</td>
<td>5.07 J=7.8 Hz (37%)</td>
<td>5.06 J=7.8 Hz (24%)</td>
</tr>
<tr>
<td>(6'-\text{H}) Ketone</td>
<td>8.30</td>
<td>8.31</td>
<td>8.30</td>
</tr>
<tr>
<td>Hydrate</td>
<td>8.27 (23%)</td>
<td>8.28 (29%)</td>
<td>8.27 (23%)</td>
</tr>
<tr>
<td>(8'-\text{H}) Ketone</td>
<td>7.36 J=16.6 Hz</td>
<td>7.42 J=16.6 Hz</td>
<td>7.38 J=16.6 Hz</td>
</tr>
<tr>
<td>Hydrate</td>
<td>6.70 J=16.6 Hz (19%)</td>
<td>6.71 J=16.6 Hz</td>
<td>6.70 J=16.1 Hz (23%)</td>
</tr>
</tbody>
</table>

\(^a\)Chemical shifts are all relative to tetramethylsilane.
Figure 18. Proton NMR spectra of 2 isolated from aspartate aminotransferase obtained on a 300 MHz spectrometer at 24 °C

a. About 4 μmol of 2 passed through Dowex 50 (H⁺ form) resin which was improperly washed (303 scans).
b. About 3 μmol of 2 passed through SP-Sephadex (H⁺ form) (310 scans).
c. About 2 μmol of 2 passed through Dowex 50 (H⁺ form) resin which was washed thoroughly (236 scans).
Figure 18 (continued)
Figure 18 (continued)
resin. It is obvious that the aromatic region (6.8-8.5 ppm) is highly contaminated with impurities from the Dowex resin. Preparation of 2 using SP-Sephadex gave a cleaner spectrum at this region, however, more contamination was observed at 3.5-4 ppm (Figure 18b). The impurities are probably sugar residues hydrolyzed off by base from the Sephadex gel. When carefully washed Dowex resin was used, the cleanest spectrum was obtained (Figure 18c). The aromatic region is expanded to show the detail of the coupling pattern (Figure 19). Each pair of doublets show an identical coupling of 16.6 Hz. Irradiation experiments showed that there is no interaction between two sets of coupled peaks (Figure 20) suggesting that each set belongs to a different species.

The proton nmr spectra of 2 isolated from glutamate decarboxylase and aspartate aminotransferase are compared with that of synthetic 2 in Figure 21 and Table X. The identity of the three spectra is evidence that 2 from three different systems are the same.

**High voltage electrophoresis** The electrophoretic mobilities of 2 isolated from AAT and GAD and of synthetic 2 were compared with those of the reference compounds: pyridoxal phosphate, pyridoxamine phosphate, pyridoxal, and pyridoxamine. Likos showed at 4 different pH values (pH 3.3-10) that 2 isolated from glutamate decarboxylase migrated further toward the anode than did pyridoxal phosphate (Likos, 1977). I found that mixtures of any combination of 2 from glutamate decarboxylase, aspartate aminotransferase, or synthetic migrated as single spots at pH 6.5.
Figure 19. Proton NMR spectrum of 2 isolated from aspartate aminotransferase obtained on a 300 MHz spectrometer at 24 °C.

Aromatic region (6–8.5 ppm) is expanded (102 scans).
\[
\text{HOOC} \stackrel{\text{O}}{\text{O}}
\]

\[ J = 16.6 \text{ Hz} \]

hydrate

\[ \text{J = 16.6 Hz} \]

\[ \text{hydrate} \]
Figure 20. Proton NMR spectra of 2 isolated from aspartate aminotransferase obtained on a 300 MHz spectrometer

A. Upfield doublets (7.47 ppm) corresponding to 8-proton of keto acid of 2 were irradiated (arrow) (125 scans).
B. Downfield doublets (7.05 ppm) corresponding to the 4'-proton of hydrated 2 were irradiated (arrow) (234 scans).
C. No irradiation (246 scans).
Figure 21. 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.
Hydrolysis of 2 to pyridoxal phosphate  

Likos (1977) observed that 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. The incubation of apo-aspartate aminotransferase with an excess of hydrolyzed 2 gave enzymatic activity. This last experiment, however, was not quantitative. Therefore, I carried out a quantitative assay of apo-aspartate aminotransferase with the hydrolysis product of 2 (Figure 22). Known amounts of pyridoxal phosphate incubated with apo-aspartate aminotransferase gave a standard assay curve. From this curve, the concentration of pyridoxal phosphate in the hydrolysis product of 2 was calculated. The amount of pyridoxal phosphate found in the hydrolysis product of 2 was 2.8 nmol, and that from untreated 2 was 0.9 nmol (Table XI). These values corresponded to 88% conversion from 2 to pyridoxal phosphate by basic hydrolysis.

Likos demonstrated the dephosphorylation of compound 2 by alkaline phosphatase (1977). The dephosphorylated material (3) was further reduced by sodium borohydride to (5). I have studied these compounds further and have also investigated the reduced product (4) which retains the phosphate group as well as its dephosphorylated derivative (6). Schemes XII & XIII summarize the formation of 2 and of its derivatives as applied to glutamate decarboxylase and aspartate aminotransferase, respectively.
Figure 22. Identification of base-hydrolyzed product of 2 as pyridoxal phosphate using apoaspartate aminotransferase

Reconstitution experiment of apoaspartate aminotransferase and pyridoxal phosphate. Each assay contained 7.0 nmol of apoaspartate aminotransferase. Total amount of aspartate transferase activity (μmol/min) per assay tube is plotted versus the amount of added pyridoxal phosphate per tube. The values obtained from alkali-treated and untreated 2 (3.2 nmol) are indicated.
<table>
<thead>
<tr>
<th></th>
<th>Total activity per assay tube (μmol/min)</th>
<th>The amount of PLP found per tube (nmol)</th>
<th>Concentration of PLP (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9</td>
<td>0.9</td>
<td>1.8 x 10^-5 (28%)</td>
</tr>
<tr>
<td>Alkali-treated 2</td>
<td>10.1</td>
<td>2.8</td>
<td>5.6 x 10^-5 (88%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compound 2 was prepared as A<sub>400nm</sub> = 0.54 at pH 6.7. ε = 8,500 M<sup>-1</sup> cm<sup>-1</sup> was assumed. Concentration was 6.4 x 10^-5 M and 3.2 nmol was used for each assay.
Scheme XII.

Glutamate Decarboxylase → Serine sulfate → Protein factor → 1 (336 nm) → NaOH → 2 (407 nm) → alkaline phosphatase → 3 (313 nm) → NaBH₄ → 4 (342 nm) → alkaline phosphatase → 5 (313 nm) → NaBH₄ → 6 (342 nm)

[All peak positions are at neutral pH]
Aspartate Aminotransferase

Serine sulfate

Aspartate Aminotransferase → 1a (336 nm) → 1b (455 nm)

2 (407 nm)

alkaline phosphatase

3 (313 nm) NaBH₄

4 (342 nm) boiling NaBH₄

5 (312 nm) alkaline phosphatase

1c (430 nm, low pH) (360 nm, high pH)

[All peak positions are at neutral pH]

Scheme XIII.
The characterization of these derivatives of 2 provides additional chemical information concerning the structure of 2.

**Properties of 3**

When alkaline phosphatase from *E. coli* acted upon 2, a marked change in spectrum occurred over a time period of about 13 h. The band at 413 nm was sharply decreased while an increased absorption was seen at 310 nm. For the cationic, neutral, and anionic forms, respectively, strong bands appeared at 284, 313, and 297 nm. These are 7 to 12 nm lower than the positions for pyridoxine. It was noticed that the relative heights of the lower energy absorption bands (413 nm region) were much reduced relative to these in the high energy region (286-312 nm) compared to the spectrum of 2. The spectra were those of a 3-hydroxypyridine ring lacking a conjugated exocyclic double bond. A possible explanation was the 5'-hydroxyl group added to the double bond in a reaction comparable to the cyclization of pyridoxal to the hemiacetal.

I monitored the reaction of 2 with alkaline phosphatase by TLC and high voltage electrophoresis. Treatment of 2 for a short time (5 min) with alkaline phosphatase was carried out. The electronic spectrum was still almost like that of 2 (Figure 23). The migration of the product obtained from a short incubation (5 min) on TLC was the same as that of the overnight product ($R_f = 0.03$). However, the following differences were observed. A blue fluorescence was observed for the 5 min product on TLC, while the overnight product gave a yellowish-green fluorescence. On high voltage electrophoresis, however, the migration of 5 min product did
Figure 23. Reaction of 2 isolated from aspartate aminotransferase with *E. coli* alkaline phosphatase

The numbers give the time in minutes after addition of alkaline phosphatase (about 0.03 mg). The pH was 8 adjusted with 1N HCl at 24 °C.
differ from that of overnight product. A spot of mobility relative to PLP of about 0.85 appeared at pH 6.5 for the 5 min product; a spot of mobility relative to PLP of 0.65 was seen for the overnight product. The probable route of conversion from 2 to 3 by alkaline phosphatase is shown in Scheme XIV. Since under the same condition dephosphorylation of PLP was completed within 10 min, the formation of XIIIb may be very fast and the 5 min incubation product appears to correspond to this ring-open form. Conversion from the enol form (XIVc) to the ketone (XIVd) is believed to be the rate limiting (R. Feldhaus, Iowa State University, unpublished results).

NMR spectra The NMR spectrum of 3 at pH = 6.9 supports the proposed structure of XIVd (Figure 24, Table XII). The spectra of 3 isolated from aspartate aminotransferase are also shown (Figure 25).

Mass spectrum The mass spectrum of 3 shows a parent ion peak at m/e = 237 (C11H11NO5) while the base peak is at m/e = 149 (C8H7NO2) (Figure 26)

Properties of 4

Reaction of 2 with both sodium cyanoborohydride and sodium borodeuteride gives the identical reduction product, which is designated 4.

NMR spectra NMR spectra of synthetic 4 and of 4 derivatized from 2 isolated from aspartate aminotransferase are shown in Figures 27 & 28. Compound 4 was also prepared by using sodium borodeuteride (Figure 29). The chemical shifts of 4 are summarized in Tables XIII & XIV. The unique NMR properties of 4 are sets of doublets at δ = 5.03, 5.13, 6.70, and 6.86 ppm. The most up-field doublet represents the 5'-protons which are split by phosphorous (J = 7.8 Hz). The most down-field doublet (J = 16.6 Hz) is coupled with a neighboring doublet of doublet (J = 16.1 Hz &
Scheme XIV.

1. $\text{XIV}_a$ (2)
2. $\text{XIV}_b$
3. $\text{XIV}_c$
4. $\text{XIV}_d$ (3)
5. $\text{XIV}_e$ (5)

$\text{Alkaline phosphatase}$

$\text{NaBH}_4$
Figure 24. Proton NMR spectrum of synthetic 3 obtained on a 300 MHz spectrometer at 24 °C

About 10 mg of synthetic 3 at pD = 6.9 was used (384 scans).
Table XII

Proton NMR Spectrum of Synthetic Compound 3 at pH = 6.91

<table>
<thead>
<tr>
<th>Protons</th>
<th>Chemical Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-CH₃</td>
<td>2.46</td>
</tr>
<tr>
<td>4'-H</td>
<td>5.79</td>
</tr>
<tr>
<td></td>
<td>5.07</td>
</tr>
<tr>
<td>5'-CH₂</td>
<td>5.11</td>
</tr>
<tr>
<td></td>
<td>5.16</td>
</tr>
<tr>
<td></td>
<td>5.20</td>
</tr>
<tr>
<td>6-H</td>
<td>7.55</td>
</tr>
<tr>
<td>β-H</td>
<td>4.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Partially exchanged for 2H.
Figure 2.5. Proton NMR spectra of 3 isolated from aspartate aminotransferase obtained on a 300 MHz spectrometer at 24 °C

About 1 µmol of isolated 3 which had undergone gel filtration on a BioGel P2 column was used.

a. pD = 2 (1414 scans).
b. pD = 8.4 (2314 scans).
Figure 26. Mass spectrum of synthetic 3

The electron-impact mass spectrum was obtained at 20 eV. The temperature of the probe was quickly raised to 250 °C to obtain the stable parent ion peak.
Figure 27. Proton NMR spectra of synthetic 4 obtained on a 300 MHz spectrometer at 24 °C

About 10 μmol of synthetic 4 was used.

a. pD = 2.2 (66 scans).
b. pD = 6.95 (101 scans) at 40 °C.
c. pD = 12.95 (100 scans) at 50 °C.
Figure 27 (continued)
Figure 28. Proton NMR spectrum of 4 isolated from aspartate aminotransferase obtained on a 300 MHz spectrometer at 24 °C

Compound 4 (about 1 µmol) was prepared by reducing 2 isolated from aspartate aminotransferase with NaBH₄ (pD = 1, 363 scans).
Figure 29. Proton NMR spectrum of synthetic 4 obtained on a 300 MHz spectrometer at 24 °C

Synthetic 4 with one deuterium atom incorporated in the α-position (about 16 μmol) was prepared at pD = 2 (201 scans).
Table XIII

Chemical Shifts\(^a\) from \(^1\)H-NMR Spectrum of Compound 4

<table>
<thead>
<tr>
<th>Protons</th>
<th>Deuterated(^b)</th>
<th>Undeuterated compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PD = 1</td>
<td>PD = 2.2</td>
</tr>
<tr>
<td>2'-CH(_3)</td>
<td>2.67</td>
<td>2.66</td>
</tr>
<tr>
<td>4'-H</td>
<td>6.86 J=16.1Hz</td>
<td>6.84 J=17.6Hz</td>
</tr>
<tr>
<td></td>
<td>6.91</td>
<td>6.89</td>
</tr>
<tr>
<td>5'-CH(_2)</td>
<td>5.03 J=7.3Hz</td>
<td>5.01 J=7.8Hz</td>
</tr>
<tr>
<td></td>
<td>5.05</td>
<td>5.04</td>
</tr>
<tr>
<td>6-H</td>
<td>8.25</td>
<td>8.23</td>
</tr>
<tr>
<td>α-H</td>
<td>5.12 J=5.4Hz</td>
<td>4.57 J=6.8Hz</td>
</tr>
<tr>
<td></td>
<td>5.13</td>
<td></td>
</tr>
<tr>
<td>β-H</td>
<td>6.68 J=16.6Hz</td>
<td>6.67 J=5.4Hz</td>
</tr>
<tr>
<td></td>
<td>6.68</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>6.74</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td>6.74</td>
<td>6.74</td>
</tr>
</tbody>
</table>

\(^a\)At 25°C. Chemical shifts in ppm relative to TMS as an external standard.

\(^b\)One atom of \(^2\)H incorporated at C-α by reduction of 2 with sodium borodeuteride.
Table XIV
Proton NMR Spectrum of Compound 4 Isolated from Boiled Reduced 1b

<table>
<thead>
<tr>
<th>Protons</th>
<th>isolated 4(^a) PD = 1</th>
<th>PD = 3.7(50°C)</th>
<th>4 prepared from isolated 2 PD = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-'CH(_3)</td>
<td>2.67</td>
<td>2.59</td>
<td>2.63</td>
</tr>
<tr>
<td>4'-H</td>
<td>6.86 J=16.1Hz</td>
<td>6.72 J=17.1Hz</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>6.91</td>
<td>6.78</td>
<td>-b-</td>
</tr>
<tr>
<td>5'-CH(_2)</td>
<td>5.04 J=7.8Hz</td>
<td>4.95 J=7.8Hz</td>
<td>4.98 J=7.3Hz</td>
</tr>
<tr>
<td></td>
<td>5.07</td>
<td>4.98</td>
<td>5.00</td>
</tr>
<tr>
<td>6'-H</td>
<td>8.25</td>
<td>8.15</td>
<td>8.20</td>
</tr>
<tr>
<td>(\alpha)-H</td>
<td>5.13 J=5.4Hz</td>
<td>4.80 J=4.4Hz</td>
<td>5.08 J=3.9Hz</td>
</tr>
<tr>
<td></td>
<td>5.15</td>
<td>4.81</td>
<td>5.10</td>
</tr>
<tr>
<td>(\beta)-H</td>
<td>6.69 J=4.9Hz</td>
<td>6.56 J=5.4Hz</td>
<td>-b-</td>
</tr>
<tr>
<td></td>
<td>6.70</td>
<td>6.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.74 J=4.9Hz</td>
<td>6.62 J=4.9Hz</td>
<td>-b-</td>
</tr>
<tr>
<td></td>
<td>6.76</td>
<td>6.63</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Digital resolution = 0.5Hz, about 5 \(\mu\)mol for each sample.

\(^b\)Insufficient sensitivity to allow accurate determination of chemical shifts.
$J = 5.4 \text{ Hz})$. The former represents the 4'-proton and the latter represents the $\alpha$-proton which is coupled further with a more upfield single proton at $\delta = 5.13 \text{ ppm (} J = 5.4 \text{ Hz) at } \text{pD} = 2.2$ (Figure 27a). This single proton at $\delta = 5.13 \text{ ppm must be the } \alpha$-proton of 4. The spectrum of 4 prepared by reduction of 2 with sodium borodeuteride lacks the $\alpha$-proton peak and the associated coupling with $\beta$-proton (Figure 28).

As mentioned in the preceding paragraph, 4 is also produced by heat treatment of reduced 1b. The NMR spectrum of the released compound is shown in Figure 30. This compound is indistinguishable from 4 by three criteria: (a) NMR; Of special importance is the pair of coupled resonances with $J = 16.1$-$16.6 \text{ Hz representing the } 4'$ and $\beta$ protons of 4. It is evident that 4 exists as the major product and shows an extremely clean spectrum in the aromatic region. (b) Electrophoresis. High voltage electrophoresis of 4 was performed at 2000 V for 30 min (pH 6.5). A single spot migrating 5.9 cm toward anode has a closely similar migration to that of PLP. A mixture of isolated and synthetic 4 migrated as a single spot at pH 6.5. None of the sample of 4 gave a positive result against ninhydrin spray test. (c) Spectra. The absorption spectrum of 4 is rather complex. There appear to be absorption bands at 292 nm (acidic), 342 nm (neutral), and 328 nm (basic pH).

Properties of 5

Reduction of 3 with either sodium borohydride or sodium cyanoborohydride at neutral pH led to loss of the low energy absorption bands. A strong band remained at 284 nm at pH 2.2, 312 nm at pH 8.3, and 298 nm at pH 12.0. Spectra of 5 isolated from glutamate decarboxylase,
Figure 30. Proton NMR spectra of 4 isolated from 1b after its reduction with sodium borohydride and heat denaturation obtained on a 300 MHz spectrometer at 24 °C

a. About 5 μmol of compound was prepared at pH = 0.9 (4125 scans).
b. At pH = 3.7 (152 scans).
Figure 30 (continued)
aspartate aminotransferase, and of synthetic 5 are all identical (Figure 31, Table IV).

The mass spectrum of synthetic 5 shows a parent ion peak at m/e = 239 (C_{11}H_{13}NO_5) (Figure 32). The base peak at m/e = 164 (C_9H_10NO_2) results from a loss of [CO_2H-CHOH]^+. The fragmentation patterns of synthetic 3 (Figure 26) and 5 are similar, a fact that supports the existence of a cyclic structure in both compounds.

Compound 5 reacts only weakly with Gibbs reagent. Migration of 5 on high voltage electrophoresis at 2000 V, pH 6.5, for 30 min was 2.8 cm toward anode, about half of that of PLP.

**NMR spectra** The ^13C nmr spectra of synthetic 5 are simple (Figures 33 & 34) and are described in Table XV. The expected eleven carbon atoms are present. Some of the resonances are doubled in a manner indicative of the presence of a diastereomeric pair (Table XV). However, the crystalline synthetic 5 was clearly a single diastereomer. Complete assignments have been made and are consistent with the cyclic structure shown (Scheme XIII). Of particular importance is the fact that in gated proton decoupled spectra the 5' carbon appeared as a triplet with ^1J(CH) \sim 50 Hz and the 4' carbon as a doublet with ^1J(CH) \sim 146 Hz (Figure 34, Table XV). 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 (Figure 35, Table XVI). The ^1H-NMR spectra of 5 isolated from aspartate aminotransferase are shown in Figure 36 (Table XVI). The quality of these spectra is not excellent because of line
Figure 31. Absorption spectra of 5

A. Isolated from glutamate decarboxylase (1, pH = 1.2; 2, pH = 6.8; 3, pH = 11.0).
B. Isolated from aspartate aminotransferase (1, pH = 2.5; 2, pH = 8.1; 3, pH = 11.1).
C. Synthetic (1, low pH form; 2, neutral pH form; 3, high pH form; spectrum was recorded by R. Feldhaus).
Figure 32. Mass spectrum of synthetic 5

The electron-impact mass spectrum was obtained at 20 eV. The probe was quickly heated to 250 °C to obtain a stable parent ion peak.
Figure 33. Carbon-13 NMR spectra of synthetic 5 obtained on a 75.5 MHz spectrometer at 24 °C

Broad band proton decoupled spectra were recorded using about 10 mg of synthetic 5 in 2 mL of D$_2$O.

a. pD = 1.6 (17472 scans)
b. pD = 4.5 (46871 scans)
c. pD = 7.7 (27827 scans)
d. pD = 13.0 (40538 scans)
Figure 33 (continued)
Figure 33 (continued)
Figure 33 (continued)
Figure 34. Carbon-13 NMR spectra of synthetic 5 obtained on a 75.5 MHz spectrometer at 24 °C.

Gated proton decoupled spectra were recorded using about 10 mg of synthetic 5 in D₂O.
- pD = 1.6 (34425 scans).
- pD = 7.7 (38232 scans).
Figure 34 (continued)
Table XV
Natural Abundance $^{13}$C NMR Spectra for Synthetic Compound 5

<table>
<thead>
<tr>
<th>Chemical shift in ppm</th>
<th>pD = 1.2-1.6</th>
<th>4.51</th>
<th>7.71</th>
<th>13.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>144.9</td>
<td>143.6</td>
<td>143.2</td>
<td>148.0</td>
</tr>
<tr>
<td></td>
<td>144.8$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>147.2</td>
<td>149.3</td>
<td>156.7</td>
<td>155.4</td>
</tr>
<tr>
<td>C-4</td>
<td>142.3</td>
<td>142.6</td>
<td>142.6</td>
<td>136.0</td>
</tr>
<tr>
<td>C-5</td>
<td>137.3</td>
<td>137.1</td>
<td>135.5</td>
<td>133.4</td>
</tr>
<tr>
<td>C-6$^b$</td>
<td>125.1</td>
<td>122.8</td>
<td>117.3</td>
<td>124.3</td>
</tr>
<tr>
<td>C-2'$^c$</td>
<td>13.5</td>
<td>13.5</td>
<td>14.0</td>
<td>17.4</td>
</tr>
<tr>
<td>C-4'$^d$</td>
<td>66.6</td>
<td>68.0</td>
<td>69.1</td>
<td>69.5</td>
</tr>
<tr>
<td>C-5'$^e$</td>
<td>69.7</td>
<td>69.2</td>
<td>69.4</td>
<td>69.6</td>
</tr>
<tr>
<td></td>
<td>69.6$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COOH</td>
<td>176.6</td>
<td>179.4</td>
<td>180.6</td>
<td>181.0</td>
</tr>
<tr>
<td>C-α</td>
<td>78.9</td>
<td>79.3</td>
<td>78.0</td>
<td>80.1</td>
</tr>
<tr>
<td></td>
<td>78.5$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-β</td>
<td>35.4</td>
<td>36.8</td>
<td>36.9</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td>35.0$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Additional peaks observed in mixture of diastereoisomers.
$^b$Doublet in gated proton decoupled spectrum, $^1J(CH) = 191$ Hz.
$^c$Quartet in gated proton decoupled spectrum, $^1J(CH) = 131$ Hz.
$^d$Doublet in gated proton decoupled spectrum, $^1J(CH) = 146$ Hz.
$^e$Triplet in gated proton decoupled spectrum, $^1J(CH) = 130$ Hz.
$^f$Doublet in gated proton decoupled spectrum, $^1J(CH) = 154$ Hz.
$^g$Triplet in gated proton decoupled spectrum, $^1J(CH) = 131$ Hz.
broadening. One probable cause is that the sample contains
diastereomers. The chemical shifts of these diastereomers may differ
slightly which would cause such broadening. The $^1$H-NMR spectrum of
deuterated 5 is shown in Figure 37. The lack of the $\alpha$-proton around 4-5
ppm is evident.

It is interesting to note that the electronic spectrum of 5 closely
resembles that of isopyridoxal in all three states of protonation of the
ring (Table XVIII). Although the proton NMR spectra of isopyridoxal
(Figure 38, Table XVIII) are not identical with those of 5 (Figure 35,
Table XVI), the cyclic structure of both 5 and of isopyridoxal is evident
from these spectra. The two 5'-CH$_2$ protons are no longer equivalent and
appear as a pair of doublets ($J = 12$ Hz for 5 and $J = 15.6$ Hz for
isopyridoxal). The peaks of the lower doublet centered at 5.06 ppm for
5 (5.3-5.4 ppm for isopyridoxal) are split further ($J \sim 2$ Hz) by long
range coupling, probably to 4'-H. The 4'-proton of 5 appears as a pair
of doublets ($J \sim 2$ Hz) centered at 4.47 ppm and caused by coupling to the
two nonequivalent $\beta$-protons. The latter appears as two multiplets
centered at 1.94 and 2.12 ppm. The $\alpha$-proton forms two doublets at 4.13
ppm with $J \sim 2.4$ Hz.

Properties of 6

The electronic spectrum of 6 is identical to that of 4. The
absorption bands are at 289 nm (low pH), at 342 nm (neutral), and at 322
nm (high pH). Two pK values, 5.2 and 9.5, were estimated. Neither 4 nor
6 show any evidence of the cyclic structure proposed for 3 and 5.
Figure 35. Proton NMR spectra of synthetic 5 obtained on a 300 MHz spectrometer at 24 °C

a. About 5 mg of synthetic 5 at pD = 1.5 was used (1060 scans).
b. pD = 12.5 (10 scans).
Figure 35 (continued)
Table XVI

Chemical shifts of proton NMR spectra of 5

<table>
<thead>
<tr>
<th></th>
<th>Synthetic 5</th>
<th>5 isolated from aspartate aminotransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 1.5</td>
<td>pH 12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-CH₃</td>
<td>2.63</td>
<td></td>
</tr>
<tr>
<td>4'-H</td>
<td>5.74</td>
<td>5.42</td>
</tr>
<tr>
<td></td>
<td>J = 7.3 Hz</td>
<td>J = 7.8 Hz</td>
</tr>
<tr>
<td>5'-H</td>
<td>5.25</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>J = 13.0 Hz</td>
<td>J = 12.2 Hz</td>
</tr>
<tr>
<td>6-H</td>
<td>8.11</td>
<td>7.43</td>
</tr>
<tr>
<td>α-H</td>
<td>4.47</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>J = 4.4 Hz</td>
<td>J = 2 Hz</td>
</tr>
<tr>
<td></td>
<td>J = 11 Hz</td>
<td></td>
</tr>
<tr>
<td>β-H</td>
<td>2.33⁺</td>
<td>2.03⁻</td>
</tr>
</tbody>
</table>

⁺Multiplet, J ~3.5 Hz.
⁻Multiplet, J ~2 Hz.
Compound 5 was purified through DEAE-Sephadex column (eluted with ammonium bicarbonate buffer gradient) and BioGel P2 column (eluted with 0.02 M acetic acid) prior to nmr measurements.

a. About 10 μmol of 5 at pH = 2 was used (4463 scans).
b. About 1 μmol of 5 at pH = 5 was used.
c. About 1 μmol of 5 at pH = 10.5 was used (189 scans).
Figure 36 (continued)
Figure 37. Proton NMR spectrum of synthetic 5 obtained on a 300 MHz spectrometer at 24 °C

One deuterium atom was incorporated into the α-position of synthetic 5 with NaBD₄ at pD = 2 (643 scans).
Table XVII

Comparison of Absorption Spectra\(^d\) of 5 and Isopyridoxal

<table>
<thead>
<tr>
<th>Form</th>
<th>AAT 5 (^b)</th>
<th>Position nm</th>
<th>Width cm(^{-1}) x 10(^{-3})</th>
<th>Skewness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Synthetic 5(^c)</td>
<td>IsoPL</td>
<td>Synthetic 5</td>
</tr>
<tr>
<td>Cationic</td>
<td>286</td>
<td>35.24</td>
<td>3.24</td>
<td>3.39</td>
</tr>
<tr>
<td>Neutral</td>
<td>313</td>
<td>32.17</td>
<td>3.73</td>
<td>3.48</td>
</tr>
<tr>
<td>Anionic</td>
<td>298</td>
<td>33.85</td>
<td>3.65</td>
<td>3.40</td>
</tr>
</tbody>
</table>

\(^a\)All values are given after computer-assisted fitting with log normal curves.

\(^b\)Absorption spectra are obtained at three different pH values.

\(^c\)Recording of spectra and fitting with log normal curves were taken by R. Feldhaus.

\(^d\)Taken from library of absorption spectra in this laboratory. Calculated pK values are 4.14, 8.60, and 12.31.
Figure 38. Proton NMR spectra of isopyridoxal obtained on a 300 MHz spectrometer at 24 °C

About 2 mg of isopyridoxal was used.

a. $pD = 2.5$ (36 scans);
b. $pD = 6.8$ (56 scans);
c. $pD = 12.5$ (167 scans).
Figure 38 (continued)
Figure 38 (continued)
Table XVIII

Proton NMR Chemical Shifts of Isopyridoxal\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>2.48</th>
<th>6.81</th>
<th>11.91</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-H</td>
<td>2.69</td>
<td>2.51</td>
<td>2.35</td>
</tr>
<tr>
<td>4'-H</td>
<td>5.25 (J = 2 Hz)</td>
<td>5.09</td>
<td>5.02</td>
</tr>
<tr>
<td></td>
<td>5.30</td>
<td>5.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.39</td>
<td>5.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.44</td>
<td>5.29</td>
<td></td>
</tr>
<tr>
<td>5'-H</td>
<td>6.71</td>
<td>6.62</td>
<td>6.71</td>
</tr>
<tr>
<td>6-H</td>
<td>8.31</td>
<td>7.77</td>
<td>7.65</td>
</tr>
</tbody>
</table>

\textsuperscript{a}About 10 mg was dissolved into D\textsubscript{2}O and used for this purpose.
Loss of the ketone at the α-position in 4 and 6 would presumably make the cyclization unfavorable.

Chemical Modification of Model Compounds

Compound 2 isolated from both glutamate decarboxylase and aspartate aminotransferase have been submitted for mass spectrometry as a solid. Likos first attempted this approach and found an ion peak at 207 for compound 2 isolated from glutamate decarboxylase (unpublished results). There were no peaks above 207. Later I reproduced his observation, then found that the 207 peak originated from the oil of the vacuum pump in the system. Thus, I learned that 2 was not a good compound to study by mass spectrometry because of its poor volatility. I started to develop the chemical modification techniques such as acetylation, trimethylsilylation, trifluoroacetylation, or permethylation for use with vitamin B6 compounds. Thus, I could apply the technique to the compounds isolated from the enzymes. Since those modifications are known to make the nonvolatile compounds volatile and give good mass spectra. Among the model compounds used are pyridoxine, pyridoxamine, reduced Schiff bases of pyridoxal with alanine, isopropylamine and ethanolamine, and a dipeptide (Leu-Ala). The reduced Schiff bases with pyridoxal were prepared in crystalline forms, and their functional groups were modified with common techniques mentioned above. Permethylation first described by Hakomori (1964) in use of methylsulfinyl carbanion in dimethyl sulfoxide showed the most promising results which are described in the following sections.
Pyridoxine  Both proton and carbon-13 NMR spectra (Figure 39) show that methylation occurs at the 3', 4', and 5'-positions of pyridoxine. The GC-MS spectrum of this derivative showed the expected parent ion peak at 211 (C_{11}H_{17}NO_{3}, Figure 40).

Pyridoxamine  The methylation procedure employed here tends to give quaternary ammonium salt at 4'-position of pyridoxamine. To avoid this problem, pyridoxamine was acetylated first using acetic anhydride in methanol. In this way, acetylation occurs only upon the amino group. The GC-MS spectrum of the permethylated product of pyridoxamine shows a parent ion peak at 252 which is expected for C_{13}H_{20}N_{2}O_{3} (Figure 41).

Reduced alanine-pyridoxal Schiff base  The crystalline reduced alanine-pyridoxal Schiff base was of sufficient purity according to the both proton and carbon-13 NMR spectra (Figure 42). Its GC-MS spectrum indicates a parent ion peak at 296 (C_{15}H_{24}N_{2}O_{4}, Figure 43). Reduced Schiff base with one deuterium atom incorporated in its 4'-position was also synthesized in crystalline form. Its NMR spectra, both proton and carbon-13, indicated a pure preparation (Figure 44). The GC-MS spectrum of the methylation product of this deuterated Schiff base has peaks of one mass increment (m/e = 297) over (Figure 45) those of its undeuterated derivative (m/e = 296).

Reduced isopropylamine-pyridoxal Schiff base  The absorption spectrum of crystalline reduced isopropylamine-pyridoxal Schiff base gives absorption bands at 296 nm (acidic), at 321 nm (neutral) and at 309
Figure 39. NMR spectra of methylated product of pyridoxine

a. Proton nmr spectrum obtained on a 60 MHz spectrometer. About 10 mg of derivative was dissolved in CDC$_3$.

b. The carbon-13 nmr spectrum obtained on a 22.5 MHz spectrometer.
Figure 40. GC-MS spectrum of the methylated product from pyridoxine
Figure 41. GC-MS spectrum of permethylated acetylated pyridoxamine
Figure 42. NMR spectra of the reduced alanine-pyridoxal Schiff base

a. Proton NMR spectrum obtained on a 60 MHz spectrometer. Crystalline compound (10 mg) was dissolved in D2O.

b. Carbon-13 NMR spectrum obtained on a 22.5 MHz spectrometer.
Figure 43. The GC-MS spectrum of the methylated product of the reduced alanine-pyridoxal Schiff base
Figure 44. NMR spectra of reduced alanine-pyridoxal Schiff base with one deuterium atom incorporated into the 4'-position

a. Proton NMR spectrum obtained on a 90 MHz spectrometer. About 10 mg of compound was dissolved in D$_2$O.

b. Carbon-13 NMR spectra obtained on a 22.5 MHz spectrometer. Broadband proton decoupling was performed (15876 scans).
Figure 44 (continued)
Figure 45. The GC-MS spectrum of the methylated product of the reduced alanine-pyridoxal Schiff base with one deuterium atom incorporated into the 4'-position.

Ionization voltage 20 eV was employed.
nm (basic) (Figure 46). The GC-MS spectrum of this compound indicates a parent ion peak at 252 which corresponds to $C_{14}H_{24}N_{2}O_{2}$ (Figure 47).

**Reduced ethanolamine-pyridoxal Schiff base**

The crystalline reduced ethanolamine-pyridoxal Schiff base exhibits an absorption maximum at 295 nm (pH = 1.28), at 326 nm (pH = 6.58) and at 309 nm (pH = 10.05) (Figure 48). The GC-MS spectrum of the methylation product of this reduced ethanolamine-pyridoxal Schiff base gives a parent ion peak at 268 corresponding to an experimental formula of $C_{14}H_{24}N_{2}O_{3}$ (Figure 49).

**Permethylation of the dipeptide (Leu-Ala)**

The use of the mass spectrometer to identify small peptide attracts me in various ways. If a compound which I have isolated from enzyme systems contains a small peptide, identification of this peptide using a mass spectrometer may be helpful. Also, instead of running an amino acid analyzer to identify a residue X which might modify the coenzyme moiety, mass spectroscopy could be used. It might have some advantages over the use of amino acid analysis.

The GC-MS spectrum of the permethylated dipeptide of Leu-Ala shows a parent ion peak at 286 suggesting $C_{14}H_{26}N_{2}O_{4}$ (Figure 50).
Figure 46. Absorption spectrum of the reduced isopropylamine-pyridoxal Schiff base

1, pH = 1; 2, pH = 6; 3, pH = 12
Figure 47. The GC-MS spectrum of the methylated product of the reduced iso-propylamine-pyridoxal Schiff base
Figure 48. Absorption spectrum of the reduced ethanolamine-pyridoxal Schiff base

1, pH = 1.3; 2, pH = 6.6; 3, pH = 10.1
Figure 49. The GC-MS spectrum of the methylated product of the reduced ethanolamine-pyridoxal Schiff base.
Figure 50. GC-MS spectrum of the permethylated product of the dipeptide, Leu-Ala
A key step of many proposals for mechanism-based inactivation of pyridoxal phosphate-dependent enzymes is a nucleophilic attack of a residue X of the enzyme on the β-carbon of an α, β-unsaturated Schiff base such as α-aminoacrylate (Scheme XV). This has been accepted in general without any real proof. The results given in this dissertation show that the above assumption is wrong for both bacterial glutamate decarboxylase and for cytosolic aspartate aminotransferase of pig heart.

A key finding was the observation that a yellow compound 2 is released by NaOH from both glutamate decarboxylase (Likos & Metzler, 1976) and from aspartate aminotransferase inhibited by either L-serine-0-sulfate, β-chloroalanine or phosphoserine. This compound was identified by Likos (Monsanto Co., St. Louis, MO., private communication) as a known compound that had been synthesized by Schnackerz et al. (1979) through an aldol condensation between pyruvate and pyridoxal phosphate (Scheme X). Results presented in this dissertation verify this identity, show that the same compound is produced from aspartate aminotransferase and provide a detailed description of molecular properties.

Both synthetic and isolated 2 have been examined by NMR and Light absorption spectroscopy, thin layer chromatography and high voltage electrophoresis. The proton NMR spectrum of synthetic 2 is compared with that of 2 isolated from glutamate decarboxylase and from aspartate aminotransferase in Figure 21 and Table X. Over the pD range of 1.2–2.5, all three spectra agree within 0.07 ppm. None of the results raises any doubt of the identity of 2. The complete assignments of
Scheme XV.

$X_{V}^7$. 

Inactivation
chemical shifts of proton and carbon-13 NMR spectra (Tables VI, VII, X) confirm the structure of synthetic 2 as reported by Schnackerz et al. (1979; see Scheme XVI).

Compound 2, both synthetic and isolated, has been converted by dephosphorylation and reduction to a series of derivatives 3–6. Their structures and the identities of synthetic and isolated compounds are also established (Scheme XVI).

Compound 2 exists in two interconvertible forms at low pH (Figure 19). These are presumed to be the keto acid form and its covalent hydrate. The hydration ratio [hydrate]/[carbonyl] at pH 1.5 is about 0.30 for 2, but is about 3.2 for pyridoxal phosphate (Harris et al., 1976). 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 (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 pK values of 2 (3.98, 8.69) are both just 0.36 higher than for pyridoxal phosphate, but are still less than those of pyridoxine (5.00, 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 pK of the phosphate group has not been estimated because dissociation of that group has very little effect on the absorption spectrum.

Formation of 2 from both inactivated bacterial glutamate decarboxylase and from aspartate aminotransferase with serine sulfate
Scheme XVI.
treated with base suggests the new mechanism proposed in Scheme XVII. The formation of free aminoacrylate by the enzyme and the attack by the latter upon the 4'-carbon atom of the Schiff base are strongly indicated. Since 2 is synthesized by the aldol condensation of pyridoxal phosphate and pyruvate, analogous chemistry can occur in the active site of the enzyme. However, it is clear that most of the aminoacrylate formed is released and does not react with the Schiff base, since over 100 molecules of pyruvate and ammonia are produced before the enzyme is inactivated (Wang & Walsh, 1978; John & Fasella, 1969; Soper & Manning, 1978). The first structure shows α-aminooacrylate Schiff base intermediate (XVIIa). XVIIa can go through "transimination" to release free aminoacrylate and to form internal Schiff base (XVIIb). This step is reverse of the formation of external Schiff base with substrate. The carboxyl group of the aminoacrylate is bound to its binding site interacting with Arg-386 (AAT) and the eliminated sulfate presumably occupies the site of its initial binding on Arg*-292 (AAT) of other subunit. This binding of α-carboxyl group of free aminoacrylate allows a simple rotation through its C-α bond, which makes it possible that its β-carbon is ready for nucleophilic attack on carbon 4' of the coenzyme. It is a well-known fact that both the nitrogen and the β-carbon of enamines such as aminoacrylate have nucleophilic properties (Dyke, 1973). Protonation of the β-carbon during above process is probably the most favorable and produces pyruvate and ammonia spontaneously. The nucleophilic attack of β-carbon onto carbon 4' of the coenzyme is crucial in order to have a covalent adduct which produces
Scheme XVII
2 with base. The resulting adduct is proposed as the structure of 1 from glutamate decarboxylase and 1a from aspartate aminotransferase. It is likely that the coenzyme tilts forward toward the substrate during the addition as it does during the transimination of normal substrate by aspartate aminotransferase (Karpeisky & Ivanov, 1966; Ivanov & Karpeisky, 1969; Arnone et al., 1982). The formation of 2 from 1 treated with base can be explained by the abstraction of the β-proton followed by the elimination of the ε-amino group of lysine from the 4'-position of the coenzyme. The imino group at the α-position is hydrolyzed off by base to give the ketone 2. It is uncertain whether 1 contains the imino group as pictured or whether this has been hydrolyzed to a ketone with loss of ammonia. Likewise 1 (1a for aspartate aminotransferase) might be either the imine shown or the ketone. Treatment of 1 with sodium borohydride reduces the C=NH bond (or C=O bond) and decreases the acidity of the β-protons. This prevents the removal of the β-proton by attack of OH⁻ and prevents the formation of 2. The resulting complex (XVIIc) is stable and suitable for peptide work. Peptide work done by Morino's group (Morino & Okamoto, 1973; Morino & Tanase, 1978) presumably started from this complex (XVIIc), and found the Lys-258 as the modified residue. Formation of 2 from 1 is quite logical; however, formation of 2 from the previously proposed structure (XVd) is quite difficult. Base treatment of XVIIa would be expected to yield pyridoxamine phosphate (PMP) and possibly 3-hydroxybutyrate (Scheme XVIII).

Peptide work carried out by John et al. (1973) is of interest. Isolated peptide from aspartate aminotransferase reacted with serine
Scheme XVIII.
sulfate gave the possible sequence of Phe-Ser-(Carboxymethylcysteine, Gly)-Asn. The isolated peptide could represent the known active site sequence, Phe-Ser-Lys±-Asn. However, the finding of Gly in the isolated peptide is confusing. It might be possible that some of a longer peptide such as Phe-Ser-Lys*-Asn-Phe-Gly was present in the preparation which gave Gly as a part of the sequence. Carboxymethylcysteine might have been confused with the modified lysine residue.

An additional fact supporting the structure of 1 is its chirality. Morino and Okamoto (1973) and Morino and Tanase (1978) observed a positive circular dichroism for tryptic and chymotryptic peptides isolated from both β-chloroalanine-inactivated cytosolic and mitochondrial aspartate aminotransferase after reduction. The shortest fragments, which were tetrapeptides, retained the same circular dichroism observed for the intact inactivated enzyme. It is known that coenzyme having the chiral center at 4'-position has more influence to the circular dichroism. It is more attractive to propose the structure of 1 than that of β-substituted form (XVIIa), since reduced 1 has a chiral center at 4'-position. It is of interest that the circular dichroism of β-chloro-alanine-inactivated alanine aminotransferase is negative (Morino et al., 1979), a fact that may indicate the opposite chirality on 4'-position of the derivative analogous to 1 formed with that enzyme.

A puzzling aspect of the reaction of glutamate decarboxylase with serine sulfate is the finding of an α-carboxyl group in the structure of 2. Glutamate decarboxylase apparently abstracts the α-proton from 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. Since the enzyme is also inactivated by β-chloroalanine or phosphoserine in a similar manner and releases 2 with base treatment, this explanation is not totally satisfactory. It is interesting to recall that mammalian glutamate decarboxylase abstracts an α-proton from the mechanism-based inactivator, (S)-4-aminohex-5-ynoic acid (Jung et al., 1980). Jung et al. speculated that mammalian enzyme is capable of removing an α-proton from the substrate as do transaminases, while the bacterial enzyme cannot. However, in the light of the reactions of serine sulfate, β-chloroalanine and phosphoserine, with the bacterial enzyme, abstraction of the α-proton by bacterial enzyme cannot be explained by the above theory given by Jung et al.

Yet another puzzle is the role of the protein factor. It is not clear whether the protein factor is involved in the normal catalysis of bacterial glutamate decarboxylase. It is unlikely that the protein factor is a subunit of glutamate decarboxylase. However, the protein factor may be a protein which forms a complex with the enzyme, inhibits the release of α-aminoacrylate, and makes the inactivation event more probable. Or another explanation is that it forms a complex with the enzyme distorting its active site conformation and allowing the removal of the α-proton from serine sulfate to initiate the inactivation.

Of special interest for aspartate aminotransferase is the slow formation of the product 1b which absorbs at 455 nm. This appears to
be identical to the product formed from \( \beta \)-chloroalanine in the presence of 3.5 M formate (Morino et al., 1974) and may be closely related to a product formed slowly by the action of \( \beta \)-chloroalanine on alanine aminotransferase (Golichowski & Jenkins, 1978). When \( \text{Ib} \) is treated with sodium borohydride, the resulting reduced coenzyme derivative is released by heating as compound 4. This is quite analogous to findings by Morino et al. (1974) that 80% of \( ^{14} \text{C} \) label was lost from chloroalanine-inactivated enzymes (either cytosolic or mitochondrial) after reduction with \( \text{KBH}_4 \) and following denaturation by 6 M guanidine HCl. On this basis it is proposed that \( \text{Ib} \) is the keto acid 2 bound at the active site (Scheme XIX). The imino group that is pictured in this scheme as present in \( \text{Ia} \) must be lost by hydrolysis either prior to or immediately after elimination of the Lys-258 side chain from \( \text{Ia} \). Since \( \beta \)-protons immediately next to the imino or keto group of \( \text{Ia} \) should be quite acidic, a slow removal of one of these protons and elimination of the amino group of Lys-258 from the 4'-position are quite reasonable. The release of 2 by heating \( \text{Ib} \) prepared in 3 M formate strongly suggest the existence of 2 at the active site. The release of 2 is also observed when \( \text{Ib} \) is treated with base. Compound 2 must be very tightly bound to the active site in \( \text{Ib} \) probably through ionic interactions. Heat treatment of \( \text{Ib} \) does not release a significant amount of chromophore. However, reduced \( \text{Ib} \) releases compound 4 quantitatively by heat treatment. The identification of released chromophore as 4 was carried out as follows. The proton NMR spectrum of the released chromophore was compared with that of 4 isolated from aspartate aminotransferase and synthetic 4
Scheme XIX.
(Table XIV). The striking similarity of these NMR spectra identifies the released chromophore as 4. Further evidence is provided by the identical electronic spectra at all pH values and the identical migrations on paper electrophoresis. Electrophoresis provided an additional interesting conclusion: the released chromophore has a hydroxyl group at α-position. The imino nitrogen of 1a, which is pictured in Scheme XIX, must be lost sometime before the formation of 1b. Otherwise the released chromophore should have carried an NH3+ group at the α-position.

The nature of form 1c is not clear. Borohydride reduction does not fix the coenzyme to the protein. Therefore, 1c cannot be a Schiff base of 2 with Lys-258. It seems possible that the sulfate eliminated from the inhibitor remains bound in the active site in 1b, or is replaced by formate. Form 1c may have lost this sulfate and have undergone a change in protein conformation which allows the observed pH dependent change in absorption spectrum. It seems reasonable that the 430 nm band at low pH could be caused by 2 if the double-bond containing side chain were rotated out of coplanarity with the ring. Since treatment of 1c with heat or base releases 2, it is evident that 1c has 2 in its active site. Three absorption bands at 333, 375, and 420 nm which were observed by Morino et al. (1974) in the reaction of aspartate aminotransferase with β-chloroalanine in the absence of formate correspond to those of 1c. Compound 1c exhibits a peak around 370 nm at high pH; however, it is hard to see how a simple deprotonation could shift the band to a shorter wavelenth at high pH. A possible explanation is that at high pH some
nucleophile adds to the carbonyl group of the bound 2. This could be Lys-258 forming a carbinolamine rather than a Schiff base. The band at 333-335 nm observed in ic is interpreted as some remaining form ia. Since the slow conversion from ia to ib is suspected of never being complete, it is reasonable to see residual amount of ia in ic.

Both bacterial glutamate decarboxylase and cytosolic aspartate aminotransferase are inactivated in a similar manner by aminoacrylate generated in the active site discussed above (see Scheme XVII). A question of obvious interest is to what extent similar mechanism apply to other mechanism-based inactivation reactions. A strong similarity is expected in the reaction of mitochondrial aspartate aminotransferase and alanine aminotransferase with β-chloroalanine. In the case of the reaction, L-aspartate-β-decarboxylase with β-chloroalanine a considerably different chemistry is suggested (Tate et al., 1969; Relyea, et al., 1974). Relyea et al. (1974) found the 14C-labeled peptide as β-hydroxypyruvate through ester linkage to a glutamic acid residue at the active site of the enzyme.

The action of vinylglycine, a known mechanism-based inactivator for aspartate aminotransferase, can be explained by an alternative chemistry related to that described here (Scheme XX). An inactivated form (XXd or XXe) analogous to 1 or ia of serine sulfate is suggested. As Gehring et al. (1977b) and Rando (1974b) observed, an absorption maximum at 335 nm corresponding to the inactivated enzyme by vinylglycine shares the same absorption position with that of serine sulfate inactivated enzyme.
Scheme XX.
The only structural change between the active intermediate forms of the inhibitors is a methyl group at β-position of carbon-3. This change presumably affects the reactivity against base. No formation of a compound similar to 2 with base was reported by Gehring et al. for the vinylglycine complex; instead, pyridoxal phosphate is released. This seems contrary to our suggestions; however, 2 is very unstable in strong base and is converted to pyridoxal phosphate. Therefore, it is still possible that the 335 nm form is an adduct (XXd or XXe) which is analogous to la.

Recently Cooper et al. (1982) reported the mechanism-based inactivation of cytosolic aspartate aminotransferase with β-methylene-DL-aspartic acid (IIIc or XXIa). Interestingly the appearance of a new peak at 335 nm for the inactivated enzyme is same as for serine sulfate-inactivated aspartate aminotransferase. Therefore, it is likely that our mechanism applies for this inactivator (Scheme XXI). In the final structure (XXIg), which is proposed by using our mechanism, formation of 2 with base would be impossible because of the absence of a β-proton. However, like vinylglycine it may be able to release pyridoxal phosphate, but not pyridoxamine phosphate. Contrary to the author's expectation, Cooper et al. (1982) mentioned formation of pyridoxamine phosphate by deproteination of the inactivated enzyme. Pyridoxamine phosphate was judged by electrophoresis. Rando et al. observed the same result using 2-amino-4-methoxy-3-butenolic acid (1976). In both cases, the description of the quantity of pyridoxamine phosphate released was poor. Additionally, neither enzyme was inactivated 100%. Thus it still may be possible that a 1 type structure exists in this case. Since residual
Scheme XXI.
substrate-Schiff base may well produce pyridoxamine phosphate as a minor product.

Manning et al. (1968) reported the $\alpha, \beta$-elimination reaction of cytosolic aspartate aminotransferase of pig heart with $\beta$-chloroglutamic acid. The authors did not observe the inactivation of the enzyme. Possible intermediate (XXI$\alpha$) which possesses an $\alpha, \beta$-unsaturated bond is proposed by Manning et al. Similar intermediate is also proposed by Rando (1974b) and Gehring et al. (1977), and Cooper et al. (1982) for the mechanism-based inactivation of aspartate aminotransferase with vinylglycine and $\beta$-methyleneaspartate, respectively. Intermediate (see $V_d$) is quite analogous to that of $\beta$-chloroglutamate (XXI$\beta$). If an accepted mechanism which employs a Michael addition of group $X$ to the $\beta$-carbon is applied, intermediate form (XXI$\beta$) should be converted into the inactivated form (XXI$\gamma$). This step should be common for these inactivators; serine sulfate, $\beta$-chlooroalanine, vinylglycine, $\beta$-methyleneaspartate, and $\beta$-chloroglutamate. Only chloroglutamate, however, does not inactivate the enzyme. An obvious question is asked why chloroglutamate is the exception. Manning et al. (1968) simply stated the reason as enzyme specificity. A more likely explanation can be given from our findings. The proposed mechanism employed here requires: 1) transimination reaction, 2) formation of free aminoacrylate, 3) rotation around $\alpha$-carboxylate group, then 4) attack of $\beta$-carbon atom of aminoacrylate onto $4^\prime$-carbon of the coenzyme. In the case of chloroglutamate, the first two categories can be satisfied; however, the third category may not be met. Since two carboxylate groups of chloroglutamate fit into
Scheme XXII.
their own binding sites in which they interact with Arg-386 and Arg*-292, respectively, the rotation around α-carboxylate group may be prohibited. Because of this restriction of rotation, the attack of β-carbon atom upon 4'-carbon of the coenzyme ring is unfavorable. This explanation seems to be quite reasonable; however, two mechanism-based inactivators do not fit this explanation. These are β-fluorooxaloacetate and β-methyleneaspartate. The latter has already been discussed. The former is known to be a suicide substrate for mitochondrial aspartate aminotransferase (Kun et al., 1960). These two inactivators possess two carboxylate groups which are believed to interact with same arginine residues in the enzyme as shown above. The difference between these inactivators and chloroglutamate is the length of the carbon chain. In general, longer chain hydrocarbon is believed to have more flexibility than shorter chain one. If this is applied here, 4 carbon substrate should have more restriction of rotation than the 5 carbon substrate, chloroglutamate. This seems to be somewhat contradictory to our proposal. However, if the $K_i$ values for these mechanism-based inactivators are taken into account a more satisfactory explanation is obtained. Table XIX shows $K_i$ values for some of the inactivators. Chloroglutamate shows high $K_i$ value (>150 mM); however, methyleneaspartate has extremely small $K_i$ value (3 mM). In general, the $K_i$ value indicates the binding ability of the molecule to its target enzyme, methyleneaspartate is expected to stay at the active site longer than chloroglutamate. Since we propose free aminoacrylate generated in the active site as the form that inactivates the enzyme, the
### Table XIX

**List of $K_i$ values of mechanism-based inactivators for aspartate aminotransferase**

| Mechanism-based inhibitors       | "Possible structure for intermediate?"
<table>
<thead>
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<th></th>
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<tbody>
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<td>β-Chloroalanine</td>
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<tr>
<td>Phosphoserine</td>
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<tr>
<td>Vinylglycine</td>
<td><img src="image2" alt="Structure" /></td>
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<tr>
<td>β-methyleneaspartate</td>
<td>yes</td>
</tr>
<tr>
<td>β-Chloroglutamate</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td></td>
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<table>
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<th>Inactivation</th>
<th>$K_i$ (mM)</th>
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<tbody>
<tr>
<td>yes</td>
<td>70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>yes</td>
<td>200&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>yes</td>
<td>--</td>
</tr>
<tr>
<td>yes</td>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>no</td>
<td>&gt;150&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The value for serine sulfate (John & Fasella, 1969).
<sup>b</sup>The value for chloroalanine (Morino et al., 1974).
<sup>c</sup>$K_a$ value for chloroglutamate (Manning et al., 1968).
mechanism-based inactivator which shows stronger affinity for the enzyme is expected to be more effective for inactivation. Therefore, the action of chloroalanine can be explained by both restriction of its rotation and its poor binding ability to aspartate aminotransferase. It has been known that 4-carbon substrates are generally better substrate than 5-carbon substrates for aspartate aminotransferase (Ivanov, 1973). Thus, the above explanation is not surprising. Some research groups reported a protective effect of thiol reagents, and others did not see the protection during suicide inactivation. Manning and Soper (1978) and Cavallini et al. (1973) are among the former, and Silverman and Levy (1980) and Cooper et al. (1982) the latter. An explanation similar to the above satisfies the observations. Inactivators having high $K_i$ are trapped easier by thiol reagents than these having low $K_i$. If one talks about this in terms of turnover number/inactivation ratio, probably, the inactivators having a high ratio will be trapped by thiol reagents during inactivation more efficiently than these having a low ratio. In the case of aspartate aminotransferase, inactivators whose $K_i$ values are above 70 mM (e.g. serine sulfate) are expected to be trapped by a thiol reagent and those below 10 mM (e.g. $\beta$-methylenaspartate) are not.

Gamma-aminobutyrate aminotransferase of brain is inhibited by ethanolamine-O-sulfate (John & Fowler, 1976; Fowler & John, 1981). The inactivated enzyme has an absorption band at 330 nm but the 415 nm band of the native enzyme is only partially gone. This suggests that the chemistry of Scheme XVII might be correct for this enzyme. The ketimine product could be in equilibrium with some aldimine which may exhibit a
nm band. On the other hand the 415 nm band of the inhibited enzyme could resemble product 1b of the present study. The enzyme is also inhibited by 4-amino-5-halopentenoic acids (Silverman & Levy, 1980, 1981). The changes in absorption spectrum are very similar to those observed with ethanolamine-O-sulfate.
The mechanism-based inactivation of both glutamate decarboxylase (GAD) of *E. coli* (Sukhareva & Braunstein, 1971) and of cytosolic aspartate aminotransferase (AAT) by L-serine-O-sulfate (John & Fasella, 1969) has been reinvestigated. The 336 nm form of GAD (I) releases a low molecular weight yellow compound 2 at high pH (Likos & Metzler, 1976). The identical compound is released from AAT. Compound 2 is a known compound which had been synthesized previously (Schnackerz et al. 1979) by an aldol condensation between pyridoxal phosphate (PLP) and pyruvate. The identity of 2 was recognized by Likos (Monsanto Co., private communication). The characterization of 2 isolated from GAD and AAT and comparison with synthetic 2 by 300 MHz NMR spectroscopy is described. The structure of 1 is proposed in collaboration with Likos as an adduct formed by attack of the $\gamma$-carbon of $\alpha$-aminoacrylate on the "internal" Schiff base of PLP with a lysine side chain from the protein. Aminoacrylate is generated from serine sulfate by an enzyme-catalyzed $\beta$-elimination. Compound 2 is presumably released by attack of hydroxyl ion on the adduct 1. Release is prevented by treatment with NaBH$_4$ which is assumed to reduce the C=N or C=O linkage in 1 and thereby to lower the acidity of the $\beta$-proton removed in the formation of 2. A new "protein factor" was discovered. The protein factor is required for the inactivation of GAD. Its partial purification and properties are described. The formation of 2 indicates that GAD does not require decarboxylation during the inactivation with serine sulfate. As described in the reaction with chloroalanine (Morino et al., 1974), AAT
is inactivated over a 10 min period and the absorption maximum at pH 5.4
shifts from 430 nm to 336 nm (la). Upon prolonged standing, the peak
shifts again over a period of 20 h to 455 nm (lb), a behavior entirely
similar to that reported by Morino et al. for chloroalanine in the
presence of 3 M formate. When the 20 h product lb is reduced with NaBH₄
and then heated in a boiling water bath, a material which is identical to
the reduction product of 2 (4) is released. Therefore, the 20 h
product lb consists of 2 bound to the enzyme. Pathways for the formation
of the various compounds are proposed. These findings require a
reevaluation of the known mechanisms of action of many mechanism-based
inactivators of PLP-dependent enzymes.
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