Physicochemical studies on the active site of glutamic acid decarboxylase

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PHYSICOCHEMICAL STUDIES ON THE ACTIVE SITE OF
GLUTAMIC ACID DECARBOXYLASE

by

Thomas Edward Huntley

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

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Dean of Graduate College

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Of Science and Technology
Ames, Iowa

1970
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INTRODUCTION

During the last ten years great advances have been made in the understanding of protein structure and the mechanism of enzyme action. Complete determination of the three-dimensional structure of proteins by X-ray analysis is now becoming almost routine and has confirmed most of the inferences drawn from the more esoteric methods of study. It is, in fact, possible that the days of tremendous excitement and discovery are over in this field and that protein chemistry in the next decade will be relegated to that area of science where, like engineering, the major effort is directed towards information of a more practical nature. One is reminded, however, that Alexander could scarcely imagine much of the world he thought he had conquered.

The answers to the two basic questions of enzyme chemistry still remain obscure. First, what are the forces and their relative importance which determine the tertiary structure of proteins and second, how does a large protein molecule grasp a relatively small substrate molecule, tear it apart and rearrange its molecular structure? X-ray analysis may tell us where a particular atom or functional group is located, but cannot by itself say why it is located there. X-ray analysis can also only give us fragmentary information about the kinetic aspects of enzyme action and protein structure. How flexible is a particular protein structure? What is the three
dimensional structure of each of the transition states of the enzyme substrate complexes? Work will continue in these areas for many years to come.

There are many methods used to study protein tertiary structure but those proteins having enzymic activity provide one additional weapon with which to attack the problem. Indeed, measurements of enzymic activity offer perhaps the most sensitive indications of small conformational changes in the protein. In the case of those enzymes which are dependent on low molecular weight coenzymes for their activity, the cofactor's role in the reaction can also be used as an important tool. It seems logical then, if one wished to study protein conformation, that this study would be greatly simplified by choosing a protein which has enzymic activity and is dependent on a coenzyme for this activity.

Enzymes containing the cofactor pyridoxal (vitamin B₆) offer perhaps the best systems for studying the above mentioned two basic problems: protein tertiary structure and the interaction of substrate molecules with proteins. The advantages of pyridoxal dependent enzymes are manifold. The coenzyme is stable over a wide pH range, is commercially available, and has a distinctive absorption spectrum in the visible region which often changes upon combination with the protein and also during the reaction with the substrate. And of great importance, non-enzymic model systems of the catalytic reac-
tion, involving pyridoxal and amino acids, are not only available, but are in fact fairly well understood and therefore one can concentrate on the effect of the protein on the reaction.

Amino acid decarboxylases, which with one exception require pyridoxal for activity, offer the further advantage of a relatively simple assay (CO$_2$ evolution) and a nearly quantitative conversion of reactant to products. Associated with the advantages of an essentially irreversible reaction are the disadvantages of the difficulty in studying equilibrium conditions.

Several excellent reviews of the literature on amino acid decarboxylation have appeared, the most recent being that of Guirard and Snell (1). Decarboxylases have also been covered in a review on pyridoxal phosphate by Fasella (2). A short discussion of what is known about amino acid decarboxylation with particular emphasis on glutamic acid decarboxylase (GAD) follows.

The presence of amines such as putrescine, tyramine, cadaverine, and histamine in decaying natural materials and their origin from specific amino acids was recognized in the early 1940's. Gale (3) demonstrated that the formation of these amines was due to the presence of specific amino acid decarboxylases$^1$ which catalyze reactions of the following

$^1$ For the purposes of this paper, the term decarboxylase will refer only to amino acid decarboxylases.
Decarboxylases have been found in bacteria, plants, and in animal tissue but considerably more is known about the bacterial enzymes than those from the other sources. In general, the bacterial decarboxylases are inducible, that is the formation of the enzyme is dependent upon the presence of the specific amino acid in the bacterial growth medium. In the absence of the amino acid, the bacteria produces little or no decarboxylase specific for that amino acid. Some strains of bacteria have the potential to produce more than one decarboxylase. A particular decarboxylase may amount to as much as two or three percent, by weight, of a bacterial acetone powder preparation. Since most bacterial decarboxylases have an enzymic activity maximum between pH 2 and 6, they can be assayed by measuring the evolution of carbon dioxide in a Warburg apparatus. As the decarboxylation reactions proceed essentially to completion, these enzymes can be used for the quantitative determination of an amino acid and if the enzyme preparation is pure the amino acid may be assayed in the presence of other amino acids. These enzymes have also been used to prepare D amino acids from the D,L mixtures.

Bellamy and Gunsalus (4) showed that maximum bacterial production of tyrosine decarboxylase was dependent not only
upon the presence of tyrosine in the growth media but also upon the presence of pyridoxine (Fig. 1). They also found that the small amount of tyrosine decarboxylase activity in cells grown on a pyridoxine deficient media could be greatly increased by addition of pyridoxal and adenosine triphosphate indicating that the phosphorylated form of the coenzyme was the active species (5). Cells containing the tyrosine apodecarboxylase have been used as a sensitive assay for the coenzyme. Baddiley and Gaie (6) confirmed this work and showed that the lysine, arginine and ornithine decarboxylases also require pyridoxal-5-phosphate (PLP). GAD was originally thought not to contain PLP (3) but Roberts (7) showed that certain types of inhibition could be prevented or reversed by PLP and more recently Shukuya and Schwert (8,9,10) have purified GAD and shown that it contains tightly bound PLP. Bacterial histidine decarboxylase is unique in that it does not contain PLP (11,12), but has pyruvic acid as the cofactor (13).

The bacterial decarboxylases are stereospecific for the L form of the amino acid and in general are specific for one amino acid. All of the substrate amino acids mentioned previously have two polar ends. A decarboxylase has been isolated from several bacteria which can decarboxylate leucine, valine, isoleucine, norvaline and α-amino-η-butyric acid (14). The enzyme has a broad pH optimum centered at pH 7 and can be
Fig. 1. Structures of pyridoxal and its analogs
induced by the presence of any one of the above hydrophobic amino acids. The $K_m$ is of the same order of magnitude for each of the substrates.

**Mechanism of decarboxylation**

Werle and Koch (15) found traces of histamine after incubating histidine with PLP. They proposed a mechanism involving a Schiffs' base between PLP and histidine as shown in the following scheme.

![Scheme A](image)

Braunstein and Shemyakin (16) and Metzler, Ikawa, and Snell (17) in their general mechanisms for pyridoxal catalysis and Westheimer, as reported by Mandeles et al. (18), independently proposed a somewhat more direct mechanism.
in which the $\alpha$-proton of the amino acid is not labilized (Fig. 2).

The Werle and Kocn mechanism would require the incorporation of two atoms of deuterium into the product amine if the reaction were run in $D_2O$, while the Braunstein-Snell mechanism would lead to the incorporation of only one atom of deuterium in the product. Mandeles, et al. (18) examined the reactions of three decarboxylases in $D_2O$ and found that all lead to the incorporation of one gram atom of deuterium per mole of product amine.

Weissbach, et al. (19) found that DOPA decarboxylase would decarboxylate $\alpha$-methylDOPA which contains no $\alpha$-proton. Huntley and Metzier (20, 21, and this manuscript) reported that GAD would decarboxylate $\alpha$-methylglutamate ($\alpha$MG).

\[
\begin{align*}
\text{CH}_3 & \quad \text{R-COOH} & \quad \text{CH}_2 & \quad \text{R-}\text{H} + \text{CO}_2 \\
\text{NH}_2 & \quad \text{NH}_2 & \quad \text{NH}_2 & \quad \text{NH}_2
\end{align*}
\]

(1a)

The results with deuterium and the decarboxylation of $\alpha$-methyl amino acids indicate that the $\alpha$-proton (or the $\alpha$-methyl group) is not labilized during the reaction and therefore the Braunstein-Snell mechanism is correct, at least in this respect.

Kalyankar and Snell (22) have investigated the nonenzymic decarboxylation of amino acids by pyridoxal. To avoid complicating side reactions, i.e. transamination, they used $\alpha$-dialkyl amino acids and found that two reaction occur simultaneously; 1) the expected decarboxylation (Equation 1a) and 2) a decar-
Fig. 2. Mechanism of decarboxylation as proposed by Braunstein and Snell
boxylation dependent transamination (Equation 2).

\[
\begin{align*}
\text{CH}_3 & \text{R-C-COOH} + \text{N}2 \text{t} \text{CO}_2 \\
\text{NH}_2 & \rightarrow \text{CH}_3 \text{R-C}_2 \text{O} + \text{NH}_2 \text{CH}_2 + \text{CO}_2
\end{align*}
\] (2)

They proposed a single mechanism for both these reactions involving, at the point of divergence, a common ambident carbanion intermediate. As shown in Fig. 3, after Schiff's base formation between the amino acid and PL (structure VIII), this aldime loses \text{CO}_2 and is converted to intermediate IX. Migration of the free electron pair to the \(\alpha\)-carbon of the amino acid leads to structure X and reaction A, while stabilization of the free electron pair on the formyl carbon of the PL moiety would lead to structure XI and reaction B. Kalayankar and Snell pointed out that structures IX, X, and XI are merely resonance forms of the same bidendate carbanion. At the time they proposed this mechanism, no enzymic examples of a reaction of type B were known.

Mandeles and Hanke (23) demonstrated that the incorporation of the single deuterium into the product amine during enzymic decarboxylation of glutamic acid was stereospecific. They prepared the mono-deuterated product, gama-aminobutyric acid-\(4\)-\text{d}_1 (GABA-\text{d}_1), in two ways. First, by enzymic decarboxylation of glutamate in \(D_2O\), and second, by decarboxylation of a racemic mixture of \(D,L\)-glutamic acid-\(2\)-\text{d}_1 (note that only
Fig. 3. Mechanism of non-enzymic decarboxylation and decarboxylation-dependent transamination as proposed by Kalyankar and Snell (22)
the L isomer would react with the decarboxylase) in $H_2O$.

Incubation of GABA-$d_1$, prepared by the first method with GAD in $H_2O$ led to exchange of the deuterium with the medium. Incubation of GABA-$d_1$ prepared by the second method led to no exchange with the medium. These results are summarized in the following scheme.

Path 1

\[
\text{R-C-H,COOH}_{\text{NH}_2} \stackrel{\text{D}_2\text{O}}{\text{GAD}} \rightarrow \text{R-C-H,D}_{\text{NH}_2} \stackrel{\text{H}_2\text{O}}{\text{GAD}} \rightarrow \text{R-C-H,2}_{\text{NH}_2}
\]

Path 2

\[
\text{R-C-D,COOH}^1_{\text{NH}_2} \stackrel{\text{H}_2\text{O}}{\text{GAD}} \rightarrow \text{R-C-D,H}_{\text{NH}_2} \stackrel{\text{H}_2\text{O}}{\text{GAD}} \rightarrow \text{R-C-D,H}_{\text{NH}_2}
\]

Scheme B

Belleau and Burba (24) have investigated the stereochemistry of the enzymic decarboxylation of tyrosine. They first prepared the two forms of $\alpha$-deutero-tyramine by asymmetric synthesis (structures XII and XIII).

\[
\text{XII} \quad \text{XIII}
\]

They then found that the two enantiomers were oxidized at different rates by the enzyme monoamine oxidase. Preparation of $\alpha$-deutero-tyramine by treatment of tyrosine with tyrosine $^1$Prepared by incubation of glutamate with amino acid racemase in $D_2O$.}
decarboxylase in D$_2$O, followed by oxidation with the amine oxidase and comparison with the rates of oxidation with the $\alpha$-deuterotyramines of known configuration led to the conclusion that the enzymic decarboxylation proceeded with retention of configuration (Equation 3).

$$\text{HO-CH}_2\text{G-COOH} \quad \xrightarrow{D_2O} \quad \text{HO-CH}_2\text{G-D}$$

(3)

It seems likely that the mechanism of all enzymic amino acid decarboxylations would be similar and therefore each would proceed with retention of configuration.

Koppelman, Mandeles, and Hanke (25) have shown that the enzymic decarboxylations of glutamate and lysine are reversible. After incubating GABA and H$^{14}$O$_3^-$ with GAD, they isolated small amounts of radioactive glutamate. They attempted to determine the equilibrium concentration of glutamate by decarboxylating glutamate in the presence of radioactive carbonate and measuring the concentration of labeled glutamate at various times. What they expected was that the concentration of labeled glutamate would start at zero and then increase to some equilibrium value at longer incubation times. They observed the opposite. Shortly after addition of the enzyme, the amount of radioactive glutamate was at its highest value, and it then decreased to the equilibrium value at longer times. Koppelman, et al believe that this result can only be explained...
by a "decarboxylation step distinct from and much more rapid than the formation of the amine". This exchange reaction (Equation 4) apparently means that, after elimination, the carboxyl group can dissociate from the enzyme surface and be replaced by a second molecule of CO₂ faster than a proton can be placed on the ambident carbanion intermediate.

\[
\begin{align*}
\text{R-CH-COOH} + \text{HCO}_3^- & \rightarrow \text{R-CH-COOH} + \text{HCO}_3^- \\
& \quad \text{NH}_2 \quad \text{NH}_2
\end{align*}
\]

From the equilibrium concentration of glutamate the authors calculated an equilibrium constant for the overall reaction (Equation 5) of 70 M.

\[
\frac{(\text{HCO}_3^-) (\text{R-CH-H})}{(\text{R-CH-COOH})} = 70 \text{ M}
\]

A fast CO₂ exchange reaction was also found with the lysine decarboxylation which had an equilibrium constant of 500 M for the overall reaction.

A common feature of all PLP enzyme reactions is the extension of the π system of a Schiffs' base between PLP and an amino acid. Perault, et al. (26) showed that this extension is accompanied by an important increase in the delocalization energy of the π system. Dunathan (27) pointed out that "if this gain in delocalization energy is to aid the bond breaking process, the transition state must assume a
geometry which places the bond to be broken in a plane perpendicular to that of the pyridoxal imine system. He adopted a schematic representation of the system in which the imine is viewed along the α-carbon to nitrogen bond and the atoms of the pyridine ring are indicated by a rectangular box (Fig. 4). Extending this idea, I suggest, in the case of decarboxylases where the proton is placed on the amino acid moiety with retention of configuration, that the proton donating group must not only be on the same side of the pyridine ring as the carboxylate binding site but also must be in approximately the same location (Fig. 4). Is it possible that the positively charged carboxylate binding site is also the proton donor, perhaps an epsilon amino group?

Properties of glutamate decarboxylase

Shukuya and Schwert (8) were the first to extensively purify a decarboxylase. They prepared GAD from E. coli in a 90 percent homogeneous form and found that it has a molecular weight of 300,000 with at least two moles of PLP per mole of enzyme. Strausbauch, et al (28) have recently crystallized the enzyme and found approximately five moles of PLP per mole of enzyme. Shukuya and Schwert (9) observed that the enzyme exhibits a sharply pH dependent spectral transition centered at pH 5.6 (Fig. 5). At the lower pH's the enzyme exhibits an absorption peak at 420 μ where, as the pH is raised, shifts to a peak at 340 μ. From the steepness of the titration
Fig. 4. Geometry of the active site of a decarboxylase

This arrangement has been suggested by Dunathan (27) and his schematic representations (A) can be slightly modified (B) to include the proton donor and the movement of the amino acid side chain and \( \alpha \)-proton into the plane of the pyridine ring as the reaction proceeds (see text).
Fig. 5. Absorption and circular dichroism of GAD
pH 5.0 solution is 0.1 M pyridine buffer
pH 6.3 solution is 0.1 M phosphate buffer
curve, the above authors calculated that the transition involved the simultaneous dissociation of at least four protons. They proposed, based on the spectral studies of pyridoxal imines by Metzler (29), that the 420 m\(\mu\) absorbing form is a Schiff's base between PLP and an epsilon amino group of a lysine residue. Anderson and Chang (30) confirmed this when they found that the 420 m\(\mu\) absorbing form of the enzyme could be reduced by NaBH\(_4\) and the expected PLP-lysine compound was isolated after hydrolysis of the reduced protein (Scheme C).

The nature of the 340 m\(\mu\) species, which does not react with NaBH\(_4\), is not known. Several structures have been proposed (9) including various ionic forms of the imine and a di-substituted compound in which the epsilon amino group of a second lysine residue is also bound to the formyl carbon to form the tetrahedral addition complex. It should be remembered however, that Heinert and Martell (31) have shown that a
Schiff's base in a hydrophobic environment absorbs near 330 μm in contrast to the 420 μm absorption in aqueous solution. It is possible that at the higher pH's there is a protein conformation change which places the PLP-lysine imine in a hydrophobic region of the protein where it is not available for reaction with NaBH₄.

Huntley and Metzler (32) simultaneously with Sukhareva and Torchinsky (33) have investigated the circular dichroic properties of the PLP absorption bands in GAD. Pyridoxal-5-phosphate is a symmetrical compound and consequently exhibits no optical activity. Any circular dichroism must therefore be due to the way in which the PLP is bound to the protein. Fig. 5, taken from the paper of Huntley and Metzler (32), shows, in agreement with Sukhareva and Torchinsky, that the 420 μm form of GAD has a large circular dichroic peak associated with it, while the high pH, 340 μm form is optically inactive. I also reported that the 330 μm absorbance peak of reduced GAD, formed by treatment of the native enzyme with NaBH₄, is optically active. However, addition of solid urea (final concentration, 8 M) to the reduced protein eliminates the PLP optical activity without changing the absorbance spectrum. Since urea, which destroys the conformational integrity of the protein, also causes loss of the induced circular dichroism of the covalently bound PLP moiety, the optical activity of the PLP absorption bands must be due only to non-covalent interactions and not to the covalent bonds.
Concentrations of PLP, PMP, GAD active sites, and protein

PLP and PMP concentrations were calculated from absorption measurements by using molar extinction coefficients of 6600 (388 μm) and 8600 (327 μm) respectively. These extinction coefficients were determined by Peterson and Sober (34).

The GAD active site concentrations were roughly determined from the absorption at 418 μm, using a molar extinction coefficient of 10,000. This coefficient was estimated by comparison of the absorbance of free PLP at 388 μm with the increase in absorbance at 418 μm when an excess of apoGAD was added to the PLP. This measurement was made difficult by increasing amounts of turbidity, with time, in all apoprotein preparations. Nevertheless, this crude estimate is considered sufficiently accurate for the type of experiments performed, the accuracy claimed, and the conclusions drawn.

Protein concentrations were estimated from the absorption at 280 and 260 μm using the equation of Warburg and Christian (35).

\[
\text{Protein concentration (mg/ml)} = 1.55D_{280} - 0.76D_{260}
\]

This again is merely a rough estimate, based on the percent tyrosine and tryptophan content of an "average" protein.

Purification of L-glutamic acid decarboxylase

Shukuya and Schwert's method (8), with slight modificatio-
tions, was used to purify L-glutamic acid decarboxylase from E. coli ATCC 11246. Cells were grown on a medium containing 0.25% ammonium sulfate, 0.5% K₂HPO₄, 0.6% yeast extract, 1.0% glucose, 1.0% L-glutamic acid, 10% (volume) tap water, and adjusted to pH 7 by addition of solid KOH. In general, three, one liter, portions of the medium were autoclaved (glucose autoclaved separately and added to the remainder aseptically) and each inoculated directly from the bacterial slant. After twelve hours of growth with vigorous aeration the three, one liter batches of inoculum were added to 135 liters of unsterilized growth media. The cells were grown with vigorous aeration with sufficient G. E. antifoam 60 to prevent excessive foaming. The cells were harvested by centrifugation between the twelfth and twentieth hours of growth.

The cell paste (approximately 500 gm) was then used immediately or frozen and stored at -20°C for later use. 250 gm of cell paste were disrupted by sonication (20 w/v % suspension of the cells in water) or by running a thick slurry of the cells through a French press at 18,000 lb/in² pressure. The cell debris was removed by centrifugation and discarded. To the supernatant (1300 ml in the case of the cells disrupted with the French press), a 2% aqueous protamine sulfate solution was added (400 ml) until precipitation ceased. The precipitate was removed by centrifugation and discarded. The purification procedure of Shukuya and Schwert was then
followed up to their first phosphate dialysis step. At this point, I dialyzed the enzyme preparation (200 ml) against three changes (3 liters each) of 0.1 M phosphate buffer at pH 6.0 for a total of eighteen hours. The solution was centrifuged and the highly turbid supernatant placed on a DEAE cellulose column (3.8 cm x 45 cm) previously equilibrated with the 0.1 M phosphate buffer. The column was washed with 0.1 M phosphate (600 ml) until all the turbid material was eluted. The enzyme was then eluted by a linear gradient between 0.1 M phosphate (800 ml) and 0.3 M phosphate (800 ml) also at pH 6.0. Fifteen ml fraction were collected and the decarboxylase containing tubes (fractions 12-43) were pooled and the enzyme precipitated by addition of sufficient (50.2 gm/100 ml) solid ammonium sulfate, at room temperature, to make a 70% saturated solution. The yellow precipitate was dissolved in the minimum amount (25 ml) of distilled water and a small amount of dark material was removed by centrifugation. The supernatant was then kept as the stock enzyme solution and was stable for at least one month at 4°C. The solution generally contained between 20 and 40 mg/ml of protein (35). In a typical preparation, starting with 250 gm of cell paste, 650 mg of protein were obtained with a specific activity of 11,200 l CO₂/10 min/mg protein at 36°C, somewhat higher than the 10,100 units reported by Shukuya and Schwert after the use of their first DEAE cellulose column.
Determination of GAD enzymic activity

GAD activity was measured by determining proton uptake with a Radiometer TTT1 pH Stat. Although the experimental development of this assay will not be discussed in detail, a brief summary follows.

Examination of the equation representing the decarboxylation reaction shows that a proton is required if the CO$_2$ is not converted to bicarbonate.

\[
\begin{align*}
\text{H} & \text{R-COO}^- + \text{H}^+ \rightarrow \text{R-C-H} + \text{CO}_2 \\
\text{NH}_3 & \text{NH}_3
\end{align*}
\] (7)

At a pH of 5 or less, no appreciable amount of bicarbonate can exist, hence any CO$_2$ formed will escape to the air (36). Therefore, as each mole of glutamic acid is decarboxylated, a mole of protons will be required. The reaction proceeds, then, with an increase in the pH, unless the required amount of protons is added during the reaction. The pH stat is, of course, the instrument designed to do just that. A Radiometer pH stat was set to maintain the pH of the media at its initial value and after addition of the enzyme, HCl was automatically added to the reaction mixture at the same rate as protons were used up and the amount of acid added was recorded on a time base scale. The sensitivity of a pH stat assay is inversely proportional to the buffer concentration. The standard assay medium consisted of 4 ml of a 0.01 M L-glutamic acid - 0.05 M KCl solution adjusted to pH 4.6 and contained
in a thermostated (generally 20°C) reaction vessel equipped with a magnetic stirrer. The titrant (in a 0.5 ml syringe) was 0.05 M HCl, and the reaction was initiated by addition of μl quantities of the enzyme. Pen response of the titrigraph was essentially linear for 8 to 10 minutes and the slope of the recorded line was used to calculate the moles of glutamate decarboxylated per unit of time.

Reproducibility of the assay, i.e., assay of identical aliquots of the enzyme, was excellent, always better than ± 3% and often ± 1% as shown in Table 1.

Table 1. Reproducibility of the pH stat assay

<table>
<thead>
<tr>
<th>10 μl enzyme</th>
<th>5 μl enzyme</th>
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<tr>
<td>Assay (slope, in/10 min)</td>
<td>Assay (slope, in/10 min)</td>
</tr>
<tr>
<td>1</td>
<td>6.01</td>
</tr>
<tr>
<td>2</td>
<td>6.08</td>
</tr>
<tr>
<td>3</td>
<td>6.05</td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Av</td>
<td>6.05 ± .02</td>
</tr>
</tbody>
</table>

Specific Activity (per 10 μl enzyme) 6.05

Specific Activity (per 10 μl enzyme) 6.26

\[
\frac{6.26 - 6.05}{6.05} \times 100 \approx 3.5 \%
\]

As shown in Table 1, there was a slight increase in specific activity at the lower concentration of enzyme. This increase became progressively worse as more dilute enzyme solutions were assayed and was attributed to a slight variation...
able, base line drift which, of course, would be most important at very low activities. This problem was obviated by working at concentrations of enzyme which gave slopes of 2 in 10 min or more.

As a further check on the reliability of the assay, a large amount of enzyme was added to a solution of L-glutamic acid (4 ml of 0.05 M) and the reaction allowed to go to completion. The moles of protons added to the solution by the titrator amounted to 103% of the moles of L-glutamate in the solution.

There are several advantages in the pH stat assay as compared with the manometric assays normally used. First, the convenience of an instrument which automatically records the data and plots its versus time, second, the high degree of reproducibility, often better than ±1% compared with the 5% usually found with manometry, and third, and most important, the pH stat assay medium contains no buffer ions other than the substrate, in contrast to the high buffer concentration necessary to keep the pH constant in the manometric assays. The marked effect of various buffer ions (c.f. Shukuya and Schwert, 8, and Homola and Dekker, 37) on the enzymic activity of GAD is therefore eliminated in the pH stat assay.

Manometric determination of CO₂

The number of moles of CO₂ produced in several experiments were determined by use of standard manometric tech-
niques (36) after calibration of the manometer and flasks with mercury. The enzyme solution was placed in the side arm and the α-MG-buffer solution (pH 4.6) in the main chamber. After fifteen minutes of thermal equilibration, the solutions were mixed and allowed to react for various times depending on the experiment.

Circular dichroic measurements

Circular dichroic measurements were made with a Jouan dichrograph modified by Foss and McCarville as reported by Johnson and Graves (38). The sensitivity of this instrument was very high, being limited by a noise level of approximately $2.5 \times 10^{-5}$ absorbance units (on good days) in the wavelength range used in this dissertation (300 to 500 μm). All CD curves shown were obtained by drawing smooth curves through the experimental traces.

Preparation of pyridine and picoline buffers

These buffers were prepared by dissolving the correct amount of pyridine or α-picoline in water and adjusting the pH with hydrochloric acid. Thus, these buffers contained various amounts of chloride ion, depending on the pH.

Thin layer chromatography (TLC)

Small TLC plates (25 mm x 75 mm) were coated with silica gel G by the method of Stahl (39). Three solvent systems were used: butanol (4): acetic acid (5): water (1), and ethanol and
water. The spots were detected by spraying with a solution of 2,4-dinitrophenylhydrazine in sulfuric acid, ethanol, and water (40).

**Paper chromatography of the αMG - GAD reaction mixture**

A 10^{-4} M solution of GAD was made 0.05 M in αMG and adjusted to pH 4.6 by cautious addition of 1 N KOH. After two hours of incubation (essentially complete conversion of enzymic PLP to PMP) the mixture was added to an equal volume of hot ethanol. The precipitated protein was removed by centrifugation and 25 μl of the supernatant was spotted on Whatman no. 1 paper. Known solution of PMP, αMG, GABA, and a solution of the partially purified reduction product of the oxime of 4-keto pentanoic acid were also spotted for comparison. The paper was then placed in a chromatographic tank containing butanol (4): acetic acid (5): water (1) until the solvent front had moved up six inches at which time the paper was dried, sprayed with a ninhydrin solution (40), and heated in a 110°C oven for thirty minutes.

**Determination of second order rate constants**

Guggenheim's method (41) for calculating 1st order rate constants was applied to the recombination reaction between apoGAD and a large excess of PLP. His method gives equal weight to all experimental points in contrast to the usual methods of plotting 1st order data which emphasize, unduly,
the initial and final values. Indeed, the Guggenheim method can be used when the initial and final values are not known. The method involves a series of measurements, \( A_1, \ldots, A_n \) (\( A \) in this case being the observed enzymic activity), at various times \( t_1, \ldots, t, \ldots, t_n \), without restriction as to intervals, and \( n \) more readings, \( A'_1, \ldots, A', \ldots, A'_n \), at times \( t_1 + \delta, \ldots, t + \delta, \ldots, t_n + \delta \), each a constant time interval, \( \delta \), after the corresponding measurement in the first series. In general \( \delta \) should be larger than the half time of the reaction. The difference between the measurements in the two sets of data \( (A'_t - A_t) \) are then plotted as the natural log versus the time. The slope of this line is equal to the apparent 1st order rate constant.

\[
\frac{k_{1st}}{2.303} = \frac{\log(A'_t - A_t)}{t - t_1}
\]  

(8)

The 2nd order rate constant is then obtained by dividing the pseudo 1st order constant by the PLP concentration.

\[
k_{2nd} = \frac{k_{1st}}{(PLP)}
\]  

(9)
RESULTS

Reaction with $\alpha$-methylglutamate

Roberts (7) found that $\alpha$MG acts as a weak competitive inhibitor of glutamate decarboxylase when added simultaneously with substrate, but that when the enzyme was preincubated with $\alpha$MG, the inhibition appeared to be noncompetitive, with the amount of inhibition dependent on the length of time of preincubation. He also showed that the inhibited enzyme could be reactivated by addition of PLP.

Sukhareva and Torchinsky (33) reported that addition of $\alpha$MG to GAD causes the optically active 420 m$\mu$ absorption peak of the native enzyme to shift to an optically inactive peak at 330 m$\mu$.

From this information, I suspected that in the presence of $\alpha$MG the enzyme was being resolved and the PLP was being converted to a 330 m$\mu$ absorbing form. In an attempt to repeat the work of Sukhareva and Torchinsky, I found that a large excess of $\alpha$MG was necessary to convert all the enzymic PLP to the 330 m$\mu$ form (Fig. 6). This material, which absorbed maximally at 325 m$\mu$, could be separated from the protein by passage of the $\alpha$MG - GAD reaction mixture through a column of Sephadex G-25 or by precipitation of the protein with either hot ethanol or trichloroacetic acid. In a typical experiment 0.3 ml of enzyme ($4 \times 10^{-4}$ M) was added to 0.7 ml of 0.1 M $\alpha$MG solution at pH 4.45. After 3½ hours, 2.0 ml of hot eth-
Fig. 6. Spectral changes of GAD in the presence of aMG

A. Absorption spectra of GAD in the presence of various concentrations of aMG after an eight hour incubation

B. Absorption spectra of GAD (10^{-4} M) at various times after addition of aMG (concentration at time zero was 10^{-2} M).

Curve 0 represents the spectrum before addition of aMG and has been corrected for the subsequent 10% dilution. All solutions were at 20° C and contained 0.1 M pyridine buffer at pH 4.6.
anol was added to precipitate the protein, which was then removed by centrifugation. The supernatant was then concentrated on a rotary evaporator (50° C) and diluted to one ml with water. The spectrum of this solution is shown in Fig. 7 and if it is assumed that all the absorption at 327 m\(\mu\) is due to PMP, the concentration of the cofactor was \(1 \times 10^{-4}\) M. Acidification of this sample or a sample of commercially prepared PMP causes the absorption maximum of each to shift from 327 to 293 m\(\mu\) (Fig. 7).

A PMP solution with the same absorption at 327 m\(\mu\) (0.86 optical units or approximately \(1 \times 10^{-4}\) M PMP) as the unknown solution was prepared. A one ml aliquot of each was then brought to pH 8 by addition of 0.6 N NaOH and then incubated for one hour with 1.0 ml of a 5 mg/ml (\(10^{-4}\) M active sites prior to dilution) solution of glutamic oxalacetic acid apotransaminase prepared by the method of Wada and Snell (42). Each solution was dialyzed against 100 volumes of 0.065 M tris buffer (pH 8.3) to remove the \(\alpha\)MG and other low molecular weight materials and then assayed for transaminase activity with the results shown in Table 2. This experiment, in which apotransaminase was reactivated by the unknown compound from the \(\alpha\)MG - GAD reaction mixture was repeated with a different preparation of the unknown compound and a second preparation.

\[1^1 \text{I would like to thank Dr. P. S. Furbish for preparing the glutamic oxalacetic acid apotransaminase and kindly furnishing it to me and for helping with the transaminase assay.}\]
Fig. 7. Absorption spectra of PMP (---) and the deproteinized α-MG-GAD reaction mixture (----)
Sufficient PMP was dissolved in 0.1 M α-MG to give an absorption at 325 mμ equal to that of the reaction mixture. The spectrum of each solution was recorded before and after addition of HCl (final concentration 0.1 M).
of the apotransaminase with results similar to those shown in Table 2, i.e., the unknown gave 75% of the transaminase activity found in the solution reactivated with authentic PMP.

Table 2. Activation of apotransaminase by PMP and the unknown compound from the αKG - GAD reaction mixture

<table>
<thead>
<tr>
<th>Solution</th>
<th>Transaminase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>apotransaminase</td>
<td>0.1 unit</td>
</tr>
<tr>
<td>apotransaminase + PMP</td>
<td>0.90 &quot;</td>
</tr>
<tr>
<td>apotransaminase + unknown</td>
<td>0.71 &quot;</td>
</tr>
</tbody>
</table>

The absorption spectra of the two reactivated transaminase solutions, after dialysis against the tris buffers, are shown in Fig. 8. After dilution of each solution with an equal volume of 0.02 M α-ketoglutaric acid, the 330 mµ absorption peak of each solution shifted to 420 mµ (Fig. 8).

The twenty percent difference in the activities of the two reactivated transaminase solutions is not considered significant as the two solutions were not adjusted to equal protein concentrations after the dialysis. Indeed, the spectra shown in Fig. 8 indicate that the solution containing authentic PMP did have a somewhat higher concentration of 330 mµ absorbing material (enzyme bound PMP).

To summarize the above information: 1) both the unknown and the PMP solutions, at pH 4.6, have an absorbance peak centered at 327 mµ which shifts to 293 mµ upon acidification, 2) both activate apotransaminase to approximately the same extent,
Fig. 8. Absorption spectra of apotransaminase incubated with the deproteinized αMG-GAD reaction mixture (A) or with PMP (B).

The solid line represents the spectra obtained after dialysis against tris buffer and the dashed lines represent the spectra after the subsequent addition of α-ketoglutaric acid (see text).
and 3) apotransaminase activated by either compound demonstrates the same spectral shifts after addition of α-ketoglutaric acid. It therefore seems certain that one of the products of the αMG-GAD reaction is PMP.

The low molecular weight fraction obtained from the αMG-GAD reaction mixture also contained a compound which, after acidification of the fraction, could be extracted with ether. After thin layer chromatography on plates of silica gel G, the compound could be located by the bright yellow spot which appeared after spraying with 2,4-dinitrophenylhydrazine. Chromatography on the silica gel plates with three solvent systems (butanol: acetic acid: water, 4:5:1, ethanol, and water) showed that, in all cases, the 2,4-dinitrophenylhydrazine positive compound had Rf values identical with those of 4-ketopentanoic acid (Fig. 9).

Attempts to determine the structure of the ether-extractable compound by mass spectrometry were unsuccessful as both this compound and 4-ketopentanoic acid apparently decompose on the silica gel during the twenty four to forty eight hours between chromatography and injection into the mass spectrometer. Identification of this compound by other means was made difficult by the small amounts of it available. This compound would presumably be produced only in amounts equivalent to the number of enzymic active sites, i.e., approximately 10⁻⁶ M.

It appeared that αMG and GAD reacted to yield PMP, 4-
A.) TLC of the underivatized compounds

butanol:acetic acid:water

B.) TLC of the 2,4-dinitrophenylhydrazine derivatives

Butanol:acetic acid:water

Fig. 9. TLC of the ether extracted compound from the aMG - GAD reaction mixture

$\text{4keto} = \text{4-ketopentanoic acid}$

$R = \text{unreacted 2,4-D reagent}$
ketopentanoic acid, carbon dioxide, and apoenzyme.

\[
\text{Reaction 1} \quad \text{HOOC-CH}_2\text{CH}_2\text{COOH} + \text{Enzyme} \rightarrow \text{CH}_3\text{NH}_2 + \text{apoenzyme}
\]

As mentioned in the introduction Kalyankar and Snell (22) have observed this type of reaction (decarboxylation dependent transamination) in non-enzymic systems using pyridoxal and \(\alpha\)-dialkyl amino acids. Aaslestad and Larson (43) and Bailey and Dempsey (44) have found a similar reaction in soil bacteria in which \(\alpha\)-dialkyl amino acids are decarboxylated and the amino group transferred to a suitable \(\alpha\)-keto acid. The reaction reported here (Equation 10) is analogous to the presumed half-reaction occurring in the enzymic decarboxylation dependent transamination demonstrated with these bacteria.

I was disturbed, however, by the fact that a fiftyfold excess of \(\alpha\)MG converted only about one half of the 20 \(\mu\) absorption of the native enzyme to the 325 \(\mu\) absorption peak (PMP), as shown in Fig. 6. Since reaction 1, above, ought to be irreversible, it was difficult to understand why a fiftyfold excess of \(\alpha\)MG did not drive the reaction to completion. It appeared that the concentration of \(\alpha\)MG was changing by more than the small percentage predicted for a stoichiometric transamination with enzymically bound PLP.

Therefore, the moles of gas evolved (CO\(_2\) ?) from a solu-
tion of αMG and GAD were measured manometrically and compared with the moles of enzymic PLP lost. The PLP concentrations were determined by diluting the enzyme or the reaction mixture with an equal volume of 1 N NaOH. The precipitated protein was removed by centrifugation and the absorption at 388 μ was measured. A molar extinction coefficient of 6600 (34) was used to calculate the concentration of PLP. When 3 ml of 0.025 M D,L-αMG in 0.1 M pyridine (pH 4.6) was incubated with 1.53 x 10⁻⁷ moles of enzymic PLP, 2.25 x 10⁻⁵ moles of gas were evolved and 1.38 x 10⁻⁷ moles of PLP were reacted, that is, 163 times as much CO₂ was produced as PLP was lost.

It therefore appeared that a second reaction, a "normal" decarboxylation must also be occurring.

\[
\text{Reaction 2} \quad \text{HOOC-CH}_2\text{CH}_2\text{C-COOH} \rightarrow \text{HOOC-CH}_2\text{CH}_2\text{C-H} + \text{CO}_2
\]

(11)

In this case the enzyme would act only as a catalyst and each active site could convert many molecules of the α-amino acid to the product amine.

To determine if only one of the diastereoisomers of the D,L-αMG was reacting in this manner, 0.005 M D,L-αMG was incubated with an excess of enzyme and the gas evolved was determined manometrically. Under these conditions about ten percent of the enzyme was inactivated but the moles of gas produced approached only thirty five percent of the total moles of D,L-
\( \alpha MG \) added and not the expected value of fifty percent. A possible explanation for the low amount of carbon dioxide evolved could be that the reaction (reaction 2, above) is strongly inhibited by the product.

Paper chromatograms (butanol: acetic acid: water, 4:5:1) of the deproteinized \( \alpha MG \) - GAD reaction mixture show the presence of a new ninhydrin-positive compound which was not PMP. This compound would be expected to be \( \alpha \)-aminopentanoic acid. Since this compound is not commercially available, an attempt was made to prepare this amine by reduction of the oxime of \( \alpha \)-ketopentanoic acid on a palladium-charcoal catalyst:

\[
\begin{align*}
\text{CH}_3 \\
\text{HOOCC-CH}_2\text{CH}_2\text{C}=\text{O} \\
+ \\
\text{NH}_2\text{OH-HCl} \\
\text{NaAc}
\end{align*}
\]

\[
\begin{align*}
\text{H}_2 \xrightarrow{\text{Pd-C}} \text{HOOCC-CH}_2\text{CH}_2\text{C}=\text{H} \\
\text{OH}
\end{align*}
\]

Scheme D

This preparation was not carried through to the isolation of the final product, but paper chromatograms of the partially purified reduction mixture gave only one ninhydrin-positive spot and this had an \( R_f \) value identical to that of the compound from the \( \alpha MG \) - GAD reaction mixture (Fig. 10).

It has therefore been concluded that after addition of \( \alpha MG \) to the enzyme, two reaction are occurring simultaneously: reaction 1) a decarboxylation dependent transamination, and reaction 2) a "normal" decarboxylation which is 150 times
Fig. 10. Paper chromatography of the $\alpha$MG-GAD reaction mixture

<table>
<thead>
<tr>
<th></th>
<th>&quot;A&quot;</th>
<th>Enz-$\alpha$MG</th>
<th>$\alpha$MG</th>
<th>PMP</th>
<th>GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
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</tr>
<tr>
<td>C</td>
<td></td>
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<tr>
<td>D</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- A. partially purified reduction product of the oxime of $\beta$-ketopentanoic acid
- B. $\alpha$MG - GAD reaction mixture
- C. $\alpha$MG
- D. PMP
- E. $\gamma$-aminobutyric acid
faster than the transamination.

Absorption and circular dichroism of the reaction intermediates

Shukuya and Schwert found (9) that saturating amounts of substrate, glutamic acid, caused a temporary decrease in the 420 m\( \mu \) absorption of the native enzyme. When a 200 fold excess (0.003 M or approximately three times the \( K_m \)) of glutamate was added to the enzyme at 22° C, the absorption at 420 m\( \mu \) rapidly decreased by about ten percent and then, as the substrate was used up, returned to its initial value in approximately twenty seconds.

We found that after addition of \( \alpha \)MG to GAD, but prior to formation of PMP, two spectral intermediates were observed (Fig. 6). Immediately after mixing (10 sec), the 420 m\( \mu \) absorption of the native enzyme was reduced to one half its original magnitude and a new peak at 340 m\( \mu \) was present. In less than five minutes the 340 m\( \mu \) peak was converted back to one at 420 m\( \mu \). This second 420 m\( \mu \) absorption peak was then slowly converted to one at 325 m\( \mu \) (PMP).

To complete a study of the induced optical activity of the PLP moiety of GAD, I wished to measure the circular dichroism of the 420 m\( \mu \) intermediate found in the presence of \( \alpha \)MG and compare it with the 420 m\( \mu \) intermediate of the reaction between the enzyme and the normal substrate, glutamate. As the response time of the dichrograph used in these experiments
was approximately ten seconds, it was necessary to extend the steady-state time period observed by Shukuya and Schwert to several minutes. This was accomplished by increasing the glutamate concentration 3.3 fold and running the reaction at a colder temperature. Addition of glutamic acid (final concentration, 0.01 M) to GAD at 0°C, caused a rapid decrease of about 15 percent in the absorbance at 420 μm, which then remained constant for several minutes before returning to its original value as the substrate was used up (Fig. 11). Examination of the circular dichroism of an identical sample under the same conditions showed, in contrast to the slight change in absorption, that the optical activity at this wave length was completely abolished in the presence of saturating amounts of substrate (Fig. 11). As shown in this figure, the optical activity of the native enzyme was regenerated at longer times, parallel with the slight increase in absorbance.

Fig. 12 shows the time course of the optical density and circular dichroism at 418 μm after addition of αMG to GAD. In this case the αMG concentration (0.05 M) was large enough to convert all the enzymic PLP to PMF in ten minutes. It is apparent from Fig. 12 that the 420 μm intermediate at 25°C has approximately the same amount of optical activity associated with it as does the 420 μm peak of the native enzyme. This is in contrast to the 420 μm steady-state "intermediate" of the glutamate - GAD reaction (0°C), which shows no circu-
Changes occurring in the 420 m$\mu$ absorption (A) and circular dichroic (B) peaks of GAD after addition of glutamate

At time zero, 0.5 ml of 0.056 M glutamate, adjusted to pH 4.6 with KOH, was added to 2.3 ml of a pH 4.6 pyridine, 0.1 M, buffer containing the enzyme. The portions of curves A and B prior to zero time have been corrected for the subsequent dilution. All solutions were kept at 0°C.
Fig. 12. Changes occurring in the 420 m absorption (A) and circular dichroic (B) peaks of GAD after addition of \( \alpha \)-KG.

At time zero, 0.5 ml of 0.1 M \( L\)-\( \alpha \)-KG, adjusted to pH 4.6 with KOH, was added to 0.5 ml of 0.1 M pyridine buffer (pH 4.6) containing the enzyme. The portions of the curves prior to addition of \( \alpha \)-KG have corrected for the subsequent dilution. All solutions were kept at 20°C.
lar dichroism at this wave length.

Spectra and circular dichroism of the enzymic oximes

The inhibitory action of hydroxylamine and of other carbonyl reagents in general on glutamic acid decarboxylase and other pyridoxal dependent enzymes has long been known, c.f., the work of Roberts (45). Shukuya and Schwert (9) have shown that hydroxylamine reacts with GAD at pH 6.3 to form a complex with two absorbance maxima (330 and 380 μm) of approximately equal height. Huntley and Metzler (32) have shown that at pH 5.0 this reaction with hydroxylamine leads to a spectrum identical with that previously obtained at pH 6.3 (Fig. 13) but that the reaction is complete in less than ten seconds, in contrast to the twenty minutes required for completion at the higher pH.

Roberts and Simonsen (46) have reported that methoxyamine (NH₂-O-CH₃), and aminooxyacetic acid (NH₂-O-CH₂-COOH) are potent inhibitors of the glutamate decarboxylase found in mouse brain. I have found that both these hydroxylamine analogs react rapidly (<10 seconds) at pH 5.0 with the GAD from E. coli to form complexes with absorption maxima in the 370 to 390 μm range (Fig. 14). At pH 6.3 the reaction is much slower with the aminooxyacetate (Fig. 15) and does not appear to go to completion with methoxyamine.

In contrast to the enzymic oximes, the spectra of free PLP in the presence of hydroxylamine (47) or in the presence
Fig. 13. Absorption and circular dichroic spectra of GAD in the presence of NH$_2$OH.

- GAD-pH 5 is the native enzyme in pyridine buffer (0.1 M at pH 5.0).
- Oxime-pH 5 is the above solution plus $1 \times 10^{-3}$ M NH$_2$OH.
- Oxime-pH 6.2 is the native enzyme in 0.1 M $\alpha$-picoline buffer (pH 6.2) + $10^{-3}$ M NH$_2$OH.
Fig. 14. Absorption and circular dichroic spectra of GAD in the presence of \( \text{NH}_2\text{O-CH}_3 \) and \( \text{COOH-CH}_2\text{O-NH}_2 \) at pH 5.0
A. GAD in a 0.1 M pyridine buffer (pH 5.0)
B. 1.2 ml of solution A plus 0.1 ml of \( 3 \times 10^{-2} \) M \( \text{NH}_2\text{O-CH}_2\text{COOH} \)
B'. Solution B plus 0.1 ml of 1 M acetate
C. 1.2 ml of solution A plus 0.1 ml of \( 3 \times 10^{-2} \) M \( \text{NH}_2\text{O-CH}_3 \)
C'. Solution C plus 0.1 ml of 1 M acetate
ABSORPTION

CIRCULAR DICHLROISM

$\Delta A \times (1.09 \times 10^4)$
Fig. 15. Absorption and circular dichroic changes during the reaction of GAD with NH$_2$O-CH$_2$COOH at pH 6.3 (0°C).

After dialysis against α-picoline buffer (pH 6.2), 1.0 ml of GAD was diluted with 0.1 ml of 3 x 10$^{-2}$ M NH$_2$O-CH$_2$COOH and the spectra recorded at the times shown.
Fig. 16. Absorption spectra of PLP in the presence of methoxyamine and aminooxyacetic acid.

The spectra above were taken before and after addition of 0.1 ml of $3 \times 10^{-2}$ M methoxyamine or aminooxyacetic acid to 3 ml of a $1 \times 10^{-4}$ M solution of PLP in 0.1 M acetate buffer (pH 4.6).
Fig. 17. Effect of several anions on the circular dichroic spectra of the enzymic oxime
A. GAD + 1 x 10^{-3} M NH_{4}OH in 0.1 M pyridine (pH 5.0)
B. Solution A diluted 70% with 1 M acetate (pH 5.0)
C. Solution A diluted 10% with 2.2 M KCl
of methoxyamine or aminooxyacetic acid (Fig. 16) has an absorption maximum at 330 m\(\mu\) with only a small shoulder at higher wavelengths (350 m\(\mu\) in the case of hydroxylamine and 360-380 m\(\mu\) for the two hydroxylamine analogs).

The circular dichroic spectra of the various oximes of GAD in the presence of 0.1 M pyridine (pH 5.0) or 0.1 M 2-methylpyridine (pH 6.3) buffers are shown in Figs. 13, 14, and 15. At pH 5.0 the enzyme-hydroxylamine and methoxyamine complexes show very little circular dichroism in their 380 m\(\mu\) absorption bands while the aminooxyacetic acid complex has a positive CD peak at this wave length. Since Huntley and Metzler (32) had previously reported a negative CD at 380 m\(\mu\) for the enzyme in the presence of hydroxylamine and acetate buffer, the effect of acetate on the optical activity of these oximes at pH 5.0 was determined. In each case the enzymic oxime was diluted ten percent with 1.0 M acetate (pH 5.0) to give a final concentration of 0.1 M acetate (Figs. 14 and 17). Spectra corrected for this dilution (not shown) demonstrate that acetate causes a slight (10 percent) diminution of the circular dichroism of the aminooxyacetate oxime absorption peak at 380 m\(\mu\), a reversal in the sign of the small CD peak of the methoxyamine oxime, and a rather large negative CD peak at 380 m\(\mu\) in the case of the hydroxylamine addition product.

A ten percent dilution of the hydroxylamine-enzyme oxime with 2.2 M KCl, caused only slight changes in the circular
dichroism. However, since the pyridine buffers used also con-
tained chloride ion, the optical activity of a chloride free
solution is not known, but it can be said that chloride does
not cause a negative CD peak at 380 m\(\mu\). At pH 6.3, acetate had
no effect on the positive CD peaks at 380 m\(\mu\) of the hydroxyl-
amine and aminooxyacetate-enzyme oximes.

**Properties of the apoenzyme**

After incubation of GAD with 0.1 M \(\alpha\)MG (pH 4.6) for six
hours, 3 ml of reaction mixture was run through a column (10 x
1.5 cm) of Sephadex G-25 previously equilibrated with 0.1 M
pyridine (pH 4.6) and the protein containing fractions were
pooled. No decarboxylase activity was found in the protein
fractions. The cloudy solution was clarified by centrifuga-
tion and the spectrum of the supernatant determined (Fig. 18).
Addition of 0.1 ml of 1 N NaOH to one ml of the supernatant
caused the small absorption shoulder of the apoenzyme at 330
m\(\mu\) to shift to 390 m\(\mu\) indicating that this peak was due to
enzymically bound PLP and not PMP. A similar phenomenon in
which a small part of the PLP of glutamic oxalacetic acid
transaminase was converted to an inactive 340 m\(\mu\) absorbing
form during aging of the enzyme preparations has been reported
by Martinez-Carrion et al (46).

Incubation of the apoenzyme, in pyridine buffer, pH 4.6,
with 1.5 x 10\(^{-4}\) M PLP leads to a shift in the PLP absorbance
maximum from 388 m\(\mu\) towards 420 m\(\mu\) (Fig. 18). Spectral mea-
Fig. 15. The effect of acetate on the absorption and circular
dichroic spectra of apoGAD in the presence of PLP
A. Apoenzyme in pyridine buffer (0.1 M, pH 4.6)
B. Solution A plus $1.5 \times 10^{-4}$ M PLP
C. Solution A plus 0.1 M acetate followed by
   $1.5 \times 10^{-4}$ M PLP
D. Solution A plus 0.1 M NaOH
surements were made difficult by increasing turbidity of the solution during the reaction. Indeed, all studies with the apoenzyme were complicated by the instability of the protein.

The circular dichroic spectrum of the apoenzyme before and after addition of PLP shown in Fig. 18 demonstrates that the optically active absorbance band of the native enzyme at 420 m\(\mu\) can be regenerated.

The regain in decarboxylase activity as a function of time when the apoenzyme is incubated with a large excess of PLP is shown in Fig. 19. Approximately 1 \(\times\) \(10^{-5}\) M enzymic active sites were incubated with a twenty fold excess (2 \(\times\) \(10^{-4}\) M) of PLP at 25\(^\circ\)C in the presence of a 0.1 M pyridine - 0.1 M KCl buffer at pH 4.6. At various times aliquots of this solution were removed and diluted (80 fold) into the decarboxylase assay media. This dilution was sufficient to prevent any significant recombination between apoenzyme and PLP during the ten minute course of the assay.

A Guggenheim plot (41) of this data is shown in Fig. 20. Although the data are a bit rough, a pseudo first order rate constant was calculated from the slope and divided by the PLP concentration (2 \(\times\) \(10^{-4}\) M) to give an apparent second order rate constant of 8 sec\(^{-1}\)M\(^{-1}\) for the regain in enzymic activity when apoglutamic acid decarboxylase is incubated with PLP. This recombination rate constant is considerably slower than the 1 \(\times\) \(10^{15}\) sec\(^{-1}\)M\(^{-1}\) reported for glutamic oxalacetic acid
Fig. 19. Reactivation of apoGAD (1 x 10^{-5} M) by PLP (2 x 10^{-4} M) at 20° C

The enzyme, in a 0.1 M pyridine - 0.1 M KCl (pH 4.6) buffer was incubated with PLP for various times and then assayed for enzymic activity (see text).
Fig. 20. First order plot (Guggenheim) of the reactivation of apoGAD by PLP
transaminase (49) or the 100-200 sec\(^{-1} M^{-1}\) reported for phosphorylase (50).

During the course of several experiments in which attempts were made to reactivate the apoenzyme with PLP, it was found that no reactivation occurred in the presence of 0.1 M acetate buffer at pH 4.6. Therefore, the effect of several anions on the apoenzyme - PLP recombination was studied. PLP (10\(^{-4}\) M) was incubated with apoenzyme (approximately 10\(^{-4}\) M active sites) in the presence of various buffers for 30 minutes at pH 4.6. An aliquot of each reaction mixture was then diluted (120 fold) into the assay media and the decarboxylase activity followed (Table 3).

Table 3. Effect of anions on the recombination between PLP and apoGAD

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Enzymic activity after 30 minute incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M pyridine (pH 4.6)</td>
<td>100 %</td>
</tr>
<tr>
<td>&quot; + 0.01 M phosphate</td>
<td>84.2%</td>
</tr>
<tr>
<td>&quot; + 0.01 M glutarate</td>
<td>29.3%</td>
</tr>
<tr>
<td>&quot; + 0.01 M formate</td>
<td>14.4%</td>
</tr>
<tr>
<td>&quot; + 0.01 M acetate</td>
<td>10.4%</td>
</tr>
<tr>
<td>&quot; + 0.001 M acetate</td>
<td>49.9%</td>
</tr>
<tr>
<td>&quot; + 0.1 M acetate</td>
<td>3.4%</td>
</tr>
<tr>
<td>&quot; [ + 0.1 M acetate ]</td>
<td>3.9%</td>
</tr>
<tr>
<td>&quot; + 0.1 M KCl</td>
<td>15.4%</td>
</tr>
</tbody>
</table>

Due to the 120 fold dilution, the maximum concentration of anion inhibitor in the assay medium was at most 0.00083 M.
As the concentration of glutamate in the assay was 0.01 M, the inhibitors could not have significantly affected the assay by competitive inhibition and the decrease in activity can only be attributed to their effect on the recombination of PLP and apoenzyme prior to dilution into the assay solution.

Incubation of PLP with apoenzyme in the presence of 0.1 M acetate caused no increase in the absorption or CD at 420 μM (Fig. 18). However, there was noted a small negative CD peak at 385 μM indicating that PLP was binding under these conditions but that no Schiffs' base was formed.

The binding of several analogs of PLP to apoGAD has also been studied. Incubation of the apoenzyme with 5-deoxypyridoxal (DPL), 5'-carboxymethyl-5-deoxypyridoxal (CMDPL), or pyridoxine-5-phosphate (POP), (structures shown in Fig. 1) caused no increase in the absorbance and no circular dichroism at 420 μM. The DPL and CMDPL apoenzyme solutions did exhibit small negative CD peaks (only slightly greater than the noise level of the dichrograph) in the 380-390 μM region similar to but of lower magnitude than obtained when the apoenzyme was incubated with PLP in the presence of acetate.
Further investigation of the αMG - GAD reaction should lead to interesting deductions about the active site of GAD. Kalyankar and Snell (22), have found that pyridoxal and α-di-alkyl amino acids react in the same two ways that αMG and GAD react, i.e., "normal" decarboxylation and decarboxylation dependent transamination. They proposed a mechanism for both these reaction involving a common carbanion intermediate (Fig. 3). It should be noted that structures IX, X and XI in this figure are resonance forms of the same bidendate carbanion. Protonation of the formyl carbon of the pyridoxal moiety leads to reaction path B, while the "normal" decarboxylation (path A) follows protonation of the α-carbon of the amino acid moiety. In the case of GAD, the "normal" reaction with αMG is 150 times faster than the transamination.

Using the schematic representations of Dunathan (27) it is apparent that as the decarboxylation step occurs, the α-methyl group (or α-H, in the case of glutamate) must swing into the plane of the pyridine ring as shown in scheme E. If the natural substrate fits the active site with little extra space, this movement of the α-methyl group, in the case of αMG, into the planar conformation may distort the active site in such a manner as to push the proton donating group (group :B) down slightly, i.e., away from the α-carbon of the
Scheme E

amino acid and closer to the formyl carbon of the PLP, thus explaining why the proton is occasionally placed on the "wrong" carbon atom.

Alpha methylDOPA (α-MDOPA, structure XIV) was shown by Sourkes (51) to be an extremely potent inhibitor of mammalian DOPA decarboxylase. Oates, et al (52) observed a decrease in the formation of serotonin, tyramine, and tryptamine from the precursor amino acids when 1.06 - 6.0 gm of α-MDOPA were administered to hypertensive patients. Associated with the decrease in amino acid products was the development of transient
sedation and a lowering of blood pressure. Weissbach, et al (19), reported that αMDOPA was decarboxylated by DOPA decarboxylase and that the enzyme was inactivated in the process and that this inactivation could be prevented by addition of PLP (53). Their kinetic data bore a striking qualitative similarity to that reported earlier by Roberts (7) for the inactivation of GAD by αMG. It is probable that αMDOPA in the presence of DOPA decarboxylase also undergoes the two types of reactions (normal decarboxylation and decarboxylation dependent transamination).

Although the observation on the spectral intermediates of the αMG - GAD reaction are of a preliminary nature, it is possible to speculate, with some fear of chastisement, on the nature of the molecular species responsible for these absorption changes. The sequence of absorption peaks observed (Fig. 6), i.e., 420 m\(\mu\) (native enzyme) to 340 m\(\mu\) (first intermediate) to 420 m\(\mu\) (second intermediate) to 325 m\(\mu\) (products), could be most simply explained by the reaction mechanism shown in Scheme F.

This mechanism is, of course, identical with the first few steps of the general mechanism of pyridoxal catalysis proposed by Metzler, Ikawa, and Snell (17) and Braunstein and Shemyakin (16). I have included the expected absorption maximum of each intermediate.
The most interesting feature of these absorption changes was the appearance of the 340 μm intermediate. The author knows of no other case in which any spectral evidence has been found for the tetrahedral, disubstituted addition product of enzymatically bound pyridoxal (structure XV). Although pyridoxal enzymes must form such an intermediate prior to Schiff's base formation with substrate (structure XVI), the rate of formation has been apparently too fast for even stopped flow measurements (54, 55).

Shukuya and Schwert (9) first showed that the 420 μm absorption peak of GAD was only slightly diminished in the presence of saturating amounts of the natural substrate, glutamic acid. I confirmed this result (Fig. 11) and also found, in contrast to this slight change in absorption, that the sum of the circular dichroism of the enzyme - substrate complexes at this wave length was zero (Fig. 11). Nakazawa, et al (56),
have reported similar data for threonine deaminase, a pyridoxal dependent enzyme which has an optically active absorption peak at 420 mµ, that in the presence of saturating amounts of substrate becomes optically inactive with no change in the magnitude of the absorption at this wave length. Ivanov, et al (57) have shown that glutamic oxalacetic acid transaminase, in the presence of saturating amounts of the natural substrate, has no CD in the 420 mµ absorption band.

In contrast to these results with natural substrates, I found that the 420 mµ absorbing intermediate of the GAD - αMG reaction has approximately the same optical activity as the native enzyme. This suggests that the intermediate in the αMG - GAD reaction may not be on the direct reaction pathway, but is in equilibrium with a third 420 mµ species which is present only in small amounts and, by analogy to the observations with normal substrates, would have no CD at 420 mµ (see the following reaction scheme).

![Reaction Scheme](attachment:image.png)

Scheme G
Ivanov, et al also found that the transaminase showed a significant CD at this wave length in the presence of the substrate analog, \( \alpha \)-methyl aspartate.

This conclusion may not be warranted, however, since what was observed, in the case of the GAD, threonine deaminase, and the transaminase reactions with natural substrates, was the total absorption and CD at 420 m\( \mu \) of all the enzyme-substrate intermediates present in the saturated steady state. One would expect at least two of these intermediates, the PLP-substrate Schiff's base and the PLP-product Schiff's base to absorb maximally at 420 m\( \mu \). It is conceivable that these intermediates are optically active but that they have CD peaks of opposite sign and therefore the total CD is close to zero. Consequently, it is not possible to state, unequivocally, that the coenzyme-substrate complex is bound to the enzyme in a symmetric electrical environment merely because the saturated enzyme shows no optical activity at 420 m\( \mu \). However, for the purpose of preliminary speculation, it is best to choose the simplest explanation, that the 420 m\( \mu \) absorbing intermediate of the GAD - \( \alpha \)MG reaction is not on the direct pathway. This would of course, fit in well with the idea that the \( \alpha \)-methyl group, because of its bulky nature, distorts the active complex.

Before the absorption and circular dichroic spectra of the enzymic oximes can be understood, a thorough study of the
model systems involving pyridoxal and hydroxylamine and its analogs must be undertaken. At this time it is not known why the oximes in the non-enzymic systems absorb at 330 μm while Schiffs' bases between pyridoxal and amino acids absorb at 420 μm.

Pyridoxal derivatives with a tetrahedral carbon at the 4' position have an absorption peak in the 325 - 340 μm region (58). In order to have an absorption band at a higher wavelength a minimal requirement is that there be a double bond on the 4' carbon which is in conjugation with the pyridine ring. In order for this conjugation to occur the imine bond must be coplanar with the ring system. An examination of CPK molecular models shows that the anti oxime of pyridoxal or the anti form of any pyridoxal Schiffs' base cannot be arranged so that the -C=N- bond is in the plane of the ring.

\[
\begin{align*}
\text{XVII} & \quad \text{Anti oxime of PL} \\
\text{XVIII} & \quad \text{Syn oxime of PL}
\end{align*}
\]

Steric hindrance, forces this imine bond into a position more or less perpendicular to the ring and therefore the anti oximes cannot have an absorption maximum at wavelengths greater than 340 μm. Since the imine bond cannot rotate from one side of the ring to the other, the anti oxime of pyridoxal
should actually exist as a pair of diastereoisomers. On the other hand, the \textit{syn} form of the oxime or of a pyridoxal Schiff's base can easily rotate 360° around the $\text{C}-\text{C}^*$ bond and the $\text{-C=N-}$ bond can readily assume a position which is coplanar with the pyridine ring. It is therefore apparent, that if a pyridoxal oxime (enzymic or non-enzymic) has an absorption maximum at 380 m\textmu, then this oxime must be of the \textit{syn} configuration.

Turchin, \textit{et al} (59) have reported on an investigation of the NMR spectra of oximes and azomethines of pyridoxal and PLP. By studying the chemical proton shifts and spin-spin interaction constants they found that in the pH range 0.1 to 5 "there exists only one geometrical isomer of the compounds investigated, namely the \textit{syn} isomer with the phenolic hydroxyl in position 3 of the pyridine ring situated in close proximity to the $\text{-C=N-}$ group. Ionization of the phenolic hydroxyl leads to lowering of the energy barrier between the possible conformers."

Since these oximes in water absorb at 330 m\textmu (47) it is apparent that something more than just the \textit{syn} conformation is required for absorption at the longer wave lengths. Indeed Heinert and Martell (31) have shown that even the Schiff's bases between pyridoxal and amino acids may absorb maximally at 330 m\textmu. They found an equilibrium between the two tautomeric forms represented by structures XIX and XX.
The quinoid form (XX) absorbs maximally at 420 μ while the enol-imine tautomer (XIX) has an absorption peak at 330 μ. Dudek and Dudek (60), in an investigation of the proton magnetic resonance of 15N-substituted Schiff bases of various phenol derivatives (structures XXI and XXII), found that the quinoid form predominates in hydrogen-bonding solvents (methanol) whereas the enol-imine form predominates in chloroform or methylene chloride solutions.

Pyridoxal's activity as a catalyst depends on its ability to extend the pi-electron system of the pyridine ring to the α-carbon of the amino acid. It seems logical then, that the active sites of these enzymes would be arranged in such a manner as to impose planarity on the entire system: pyridine ring, -C=N-, and α-carbon of the amino acid. Thus, if hydroxylamine and its analogs are good models for the substrate amino acids, pyridoxal enzymes would be expected to form only the syn oximes and these oximes should be forced into a conformation (planar) favoring conjugation with the pyridine
That this is the case, is supported by the spectra of the oximes of several PLP enzymes. The oximes of glutamic oxalacetic acid transaminase (61), D-alanine transaminase (62), L-alanine transaminase (63), and GAD (32, and Fig. 14), all have an absorption peak centered in the 370 to 390 m\(\mu\) region. In addition, GAD and D-alanine transaminase in the presence of hydroxylamine, but not methoxyamine or aminooxycetic acid, have a second absorption peak at 330 m\(\mu\). The 370 m\(\mu\) to 390 m\(\mu\) peaks must be due to the syn oximes in a planar conformation. The second peak at 330 m\(\mu\), found with only two enzymes in the presence of hydroxylamine may be due to the formation of a syn oxime which is not in a planar conformation or, alternatively, to the anti oxime in which planarity (and conjugation) is impossible. It is interesting that only hydroxylamine gives these second peaks at 330 m\(\mu\), while enzymic oximes of the analogs with more bulky substituents on the oximic oxygen absorb only at the higher wave lengths. Possibly the PLP fits into a crevice in the protein and the oximes of hydroxylamine are the only oximes which are small enough to assume a non-planer conformation in this crevice (structures XXIII and XXIV).

It is also not understood why these oximes absorb at 380 m\(\mu\) and not at 420 m\(\mu\) as do the PLP-amino acid Schiff's bases or the PLP-epsilon amino group Schiff's bases of the enzymes. Recently Segal, et al (64) have found that L-proline
reacts with alanine transaminase to form a species absorbing at 380 mμ. They have proposed the following structure for this species:

\[
\text{HOOC} \quad \text{N}^+ \quad \text{H} \\
\text{C} \quad \text{O} \quad \text{CH}_2 \quad \text{OH} \\
\text{P} \cdot \text{O} \cdot \text{CH}_2 \\
\text{CH}_3
\]

and suggested that the reason this compound, as well as oximes and hydrazine derivatives absorb at 380 mμ and not at 420 mμ is due to the difference in nucleophilicity of the Schiff's base nitrogen. They pointed out that the more nucleophilic the nitrogen, the more chance it would have of capturing the phenolic hydrogen (structures XXVI and XXVII) promoting quinoid formation (structure XXVIII) which Heinert and Martel have shown is responsible for the absorbance at 420 mμ. In any case it seems logical that the electron withdrawing power of the oximic oxygen might change the spectra from that of a Schiff's base, perhaps by changing the pK of one of the ion-
The effect of acetate on the circular dichroism of the GAD oximes, along with its effect on the recombination of PLP with apoenzyme, suggests that GAD has a specific site for binding carboxylate ions, which is near enough to the PLP moiety to affect its CD, possibly by neutralizing a positively charged group. It is possible, of course, that acetate may be binding at some distance from the active site in a manner which causes a conformation change in the protein which in turn affects the environment at the active site.

It should be pointed out that GAD may have four different carboxyl binding sites: 1) the site on the apoenzyme which binds the acetate which prevents PLP binding, 2) the holoenzyme site which binds the acetate which acts as a competitive inhibitor, 3) the holoenzyme sites which bind the carboxylate groups of the substrate, glutamic acid, and 4) the site of the enzymic oximes which binds acetate. It seems likely that sites 2 and 3 , above (competitive inhibitor site and sub-
strate site), are one and the same, but sites 1 and 4 may or may not be identical with 2 and 3.

As mentioned in the introduction, the site (presumably positively charged) which binds the \( \alpha \)-carboxylate group of the substrate must be in approximately the same location as the group which donates the proton to the substrate after it is decarboxylated (Fig. 4). It is probable that the positively charged carboxylate binding site is also the proton donor, perhaps an epsilon amino group.

In Table 3, above, it was shown that chloride ion increases the rate at which PLP combines with apoenzyme but that it had no effect on the acetate inhibition of PLP binding. It therefore seems reasonable that acetate and chloride bind at different sites. If the carboxylate inhibitors of PLP binding act at the site which normally binds the negatively charged phosphate group of PLP, one would expect that phosphate ion would be an even better inhibitor. Since phosphate is a relatively poor inhibitor it appears that the carboxylate ions are not acting at the PLP phosphate binding site.

Interpretation of the rate constant calculated from the Guggenheim plot presents some difficulty; primarily the ambiguity as to what reaction was actually measured. Two of the possibilities are:

\[
\text{PLP + apoenzyme} \xleftrightarrow{k_+} \text{PLP Enzyme} \xrightarrow{k_-} \text{Schiff's base} \quad (12)
\]
\[
\text{PLP} + \text{apoenzyme} \overset{\text{non-covalent}}{\longrightarrow} \text{apoenzyme} + \text{PLP Enzyme} \quad (13)
\]

Since a twelve hour dialysis (purification procedure) does not cause large losses in activity, the reverse reactions of the type

\[
\text{Enzyme PLP} \longrightarrow \text{apoenzyme} + \text{PLP} \quad (14)
\]

(Schiffs' base)

can be neglected as the maximum time in the recombination experiments was less than sixty minutes.

Both reactions 12 and 13, above, would give straight lines when plotted by the Guggenheim method and therefore it is not possible at this time to distinguish between these two mechanisms.

The phosphate group on the 5' position of pyridoxal seems to be necessary for Schiffs' base formation with the enzyme. Although CMDPL and DPL are bound, as shown by the small negative OD peaks at 380 m\(_\mu\), the phosphate group could possibly change the conformation of the active site in such a manner as to favor formation of the covalent bond between the formyl carbon of pyridoxal and an epsilon amino group of a lysine residue.

The dissociation constant \((K_1)\) for acetate and GAD, calculated by Shukuya and Schwert (8) from the competitive inhibition data is 0.2 M but the data in Table 3 shows that 0.1 M
acetate completely prevented the recombination of PLP with apoenzyme. The dissociation constant of acetate and apoenzyme must, therefore, be at least an order of magnitude less than the $K_i$ for acetate and holoenzyme. The simplest and most attractive explanation is that the acetate binds to the apoenzyme at the positively charged epsilon amino group of the lysine residue to which PLP normally would form the Schiffs' base. PLP could be bound non-covalently to the apoenzyme and show the circular dichroism at 380 mμ, but it could not form the covalent linkage.
An attempt has been made to obtain information about the active site of glutamic acid decarboxylase by examining the enzymic reaction with the substrate analog α-methylglutamic acid, and by applying several physicochemical techniques to the enzyme, primarily measurement of the absorption and the induced circular dichroism of the tightly bound pyridoxal-5-phosphate in the presence of the substrate or various substrate analogs. All the results lead to the conclusion that there is a tight fit between the protein, cofactor, and substrate with little room for added groups, even those as small as a methyl group.

Treatment of the enzyme with αMG lead to formation of a compound which absorbed at 325 mp and could be separated from the protein by passage through a short Sephadex G-25 column. In addition to this material, which proved to be pyridoxamine-5-phosphate, the low molecular weight fraction also contained two additional compounds which from their chemical reactivities and Rf values on paper and silica gel chromatography seem to be 4-ketopentanoic acid and 4-aminopentanoic acid. During the reaction between αMG and GAD, 150 times more CO₂ was released than PMP was formed. It therefore appears that two reactions are taking place simultaneously: 1) a "normal" decarboxylation to yield 4-aminopentanoic acid and CO₂ and 2) a decarboxylation dependent transamination to yield 4-ketopenta-
noic acid, CO$_2$, PMP, and apoenzyme. The first reaction is 150 times faster than the second.

Immediately after addition of α-methyl glutamate to the enzyme, the 420 m\(\mu\) absorption of the decarboxylase is reduced to one half its original magnitude and a new peak at 340 m\(\mu\) is present. In less than five minutes the 340 m\(\mu\) peak is converted back to one at 420 m\(\mu\) which is then slowly converted to 325 m\(\mu\) (PMP).

In the presence of saturating amounts of glutamic acid, the large circular dichroic (CD) peak of the native enzyme at 420 m\(\mu\) is completely eliminated despite only a slight change in the absorbance at this wave length. In contrast, the 420 m\(\mu\) absorbing intermediate formed by the enzyme when saturated with αMG, was found to have CD at this wave length nearly identical to that of the native enzyme, suggesting that this intermediate may not be on the direct reaction pathway.

Combining the theory of Dunathan concerning the stereochemical geometry of the active sites of B$_6$ enzymes with the fact that the enzymic decarboxylation reaction proceeds with retention of configuration, it seems likely that the group which serves as the substrates α-carboxylate binding site is also the group which donates a proton to the product amine.

Examination of CPK space filling molecular models of pyridoxal oximes demonstrates that the imine bond and the pi system of the pyridine ring may only assume a coplanar con-
formation when the oxime is of the syn configuration. The compounds formed between the enzyme and hydroxylamine, methoxyamine, and aminoxyacetic acid show absorbance peaks near 380 \text{m\ensuremath{\mu}} indicating the presence of conjugation between the imine bond and the ring. Consequently all these enzymic oximes must necessarily be in the syn configuration. NMR data in the literature however, demonstrates that PLP oximes in solution, which we found absorb maximally at 330 \text{m\ensuremath{\mu}}, exist only as the syn isomer, implying that some factors, in addition to the correct isomer (syn), are necessary for absorption at 380 \text{m\ensuremath{\mu}}. In addition to the 380 \text{m\ensuremath{\mu}} absorption peak, the GAD-\text{NH}_2\text{OH} oxime, but not its oximes with the bulkier analogs of hydroxylamine, has a second absorption band at 330 \text{m\ensuremath{\mu}}. The active site of the enzyme is apparently arranged in such a manner as to favor the formation of the syn form of the oximes of PLP and to hold them in a planar conformation. There is, however, a loose enough fit at the active site so that the \text{-C=N-OH} group of hydroxylamine, but not its analogs, can rotate out of the plane of the pyridine ring.

Addition of PLP to the apoenzyme fraction obtained from the GAD-\alpha\text{MG} reaction mixture, restored the enzymic activity and the spectral and circular dichroic properties of the native enzyme. The recombination was prevented by 0.1 M acetate. It was also strongly inhibited by formate and glutarate.
while phosphate had little inhibitory effect and chloride increased the recombination rate. Addition of apoenzyme to pyridoxine-5-phosphate, 5-deoxypyridoxal, or 5-carboxymethylpyridoxal caused no significant spectral changes or circular dichroism.
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... Anyone who imagines that all fruits ripen at the same time as the strawberries knows nothing about grapes.

Paracelsus