Organic synthesis of pyridoxal 5'-phosphate analogues and their interactions with apoaspartate aminotransferase

Chi-Neng Han

Iowa State University

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ORGANIC SYNTHESIS OF PYRIDOXAL 5'-PHOSPHATE
ANALOGUES AND THEIR INTERACTIONS WITH
APDASPARTATE AMINOTRANSFERASE.

IOWA STATE UNIVERSITY, PH.D., 1978
Organic synthesis of pyridoxal 5'-phosphate analogues and their interactions with apoaspartate aminotransferase

by

Chi-Neng Han

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

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Iowa State University
Ames, Iowa
1978
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DEDICATION

To my father, and to my mother who was unable to see her son grow up.
I. INTRODUCTION

An enzyme catalyzing the reversible transfer of an amino group between L-glutamic acid and pyruvic acid or other oxo acids was demonstrated by Braunstein and Kritzmann (1) in muscle and other animal tissues. Later, Gunsalus and co-workers (2) and Snell (3) established the role of pyridoxal 5'-phosphate (PLP) as the cofactor of aminotransferases. Since then an extensive literature has accumulated concerning various chemical, biological and medical aspects of PLP-dependent transamination. Besides its role in transamination, PLP is an essential coenzyme for a variety of other enzymes that are involved in all phases of nitrogen metabolism. The coenzyme acts as a reactive center in the active sites of the enzymes requiring it. Besides the fundamental importance of PLP to enzymology, there are diseases involving PLP-dependent enzymes. For examples, vitamin B₆ deficiency may be related to diabetes associated with abnormal tryptophan metabolism (4). Defective vitamin B₆-enzymes have been associated with the genetic diseases (5), xanthurenic aciduria, and cystathioninuria. It is clearly of importance to investigate how enzymes of this group function.

This study deals with two aspects of certain analogues of the coenzyme pyridoxal phosphate: (1) Inhibitory properties that might be of pharmacological significance. (2) A possible biochemical function for the 5'-phosphate group.
Vitamin B₆ is an essential nutrient for living organisms, and, as the coenzyme, is required by a variety of enzyme systems. This fact provides a good opportunity for rational design of inhibitors that might be used as drugs. Many pharmacological effects have been observed with analogues of vitamin B₆, such as inhibition of the growth of microorganisms (6, 7) and tumors (8, 9). The effects of these agents on the central nervous system (9, 10) and on sickle cell hemoglobin (11) have also been documented. Although numerous vitamin B₆ analogues have been synthesized (12, 13), they have not yet been studied enough, either from a pharmacological point of view or in investigations of enzyme mechanisms. Although systematic synthetic schemes have been developed (12), these analogues still remain difficult to make. Here the author will discuss a possible new scheme which can increase the yields and thus makes it easier to study vitamin B₆ analogues.

The coenzyme PLP not only acts as a reactive center of enzymes but it also provides us with a natural probe for the investigation of the functioning of enzymes. Therefore, a full understanding of the physical organic characteristics of the functional groups of this coenzyme should lead to a better understanding of PLP-dependent enzymes. It has been fully established that the 4-formyl, 3-hydroxyl, and pyridine nitrogen are essential for catalysis. However, the role of the 5'-phosphate of the native coenzyme in the holoenzyme is still
obscure. Therefore the analogues modified in the 5'-chain were made in this laboratory in order to explore the role of the 5'-phosphate in the catalytic action of L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1.)

The structures and abbreviations of these analogues are shown as follows.

pyridoxal 5'-phosphate (PLP)

pyridoxamine 5'-phosphate (PMP)

diethyl 5'-14-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate [(Et)_2 MP]

5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphoric acid (MPA)
ethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate (EtMP)

5-deoxypyridoxal 5'-sulfonate (PLSN)

5'-carboxymethyl-5-deoxypyridoxal (CMDPL)

5'-carboxymethyl-5-deoxy-pyridoxamine dihydrochloride (CMDPM)

ethyl pyridoxal 5'-phosphate (EtPLP)

methyl pyridoxal 5'-phosphate (MePLP)
pyridoxal (PL)

5-(4-formyl-3-hydroxy-2-methyl) pyridylethyl phosphonic acid (EPA)
II. LITERATURE REVIEW

A. Aspartate Aminotransferase

An impressive mass of information concerning the structure and function of pyridoxal phosphate-dependent enzymes, especially aspartate aminotransferase (AAT) has been obtained and reviewed by several authors (14-18, 13).

The general pattern of the chemical events occurring at the active sites has been established; the transamination proceeds through a number of discrete steps catalyzed by various well-disposed functional groups on the enzyme; conformational changes may contribute to placing the functional groups in the most appropriate position during the catalysis. However, the identity of some groups involved in catalysis is still ambiguous. The three-dimensional structure of the active site is not known although the amino acid sequence has been determined by two groups (19, 20). Well-ordered crystals of cytoplasmic aspartate aminotransferase have been first grown by vapor diffusion from solutions of polyethylene glycol in the laboratories of Arnone and Metzler (21a). The diffraction pattern extends beyond 2.8 Å, indicating that this crystal form is suitable for high resolution X-ray analysis. Three dimensional intensity data for native and heavy-atom derivative crystals are currently being collected. Recently, a cytoplasmic AAT from chicken heart was also crystallized and a 5 Å resolution
map was obtained (21b). Perhaps an accurate knowledge of the enzyme's spatial atomic topography will provide a reliable basis for elaboration of the correct reaction mechanism. In this respect, AAT could offer great opportunities because, a series of substrate analogs are available, each of which forms predominantly a few of the intermediates of the overall process.

AAT is particularly active in heart muscle, and in most procedures for purification of the enzyme, pig hearts are used as a readily available source. A highly purified cytoplasmic AAT was first obtained from pig heart by Jenkins et al. (22). AAT prepared by the usual method (22-24) contains only the anionic cytoplasmic isoenzyme. The mitochondrial isoenzyme, which is cationic, is largely destroyed by the heat treatment. Other methods (25-28) lead to the isolation of extensively purified and crystallized mitochondrial AAT. Martinez-Carrion et al. (27) has isolated no less than three main subforms from the cytosol AAT, \( \alpha, \beta \) and \( \gamma \) in the order of increasing anode mobility and decreasing catalytic activity. Marino et al. (29) was able to obtain five fractions by ampholine electro-focusing. All subforms possess the same amino acid composition. The enzyme is an apparently symmetric dimer. Each subunit, of molecular weight 46,344 is a single chain of 412 amino acids. Attempts have been made to establish the significance of this dimeric aggregation. Upon succinylation of the amino groups with succinic anhydride (30, 31) and amidation of the carboxylate groups with lysine methyl ester (32), lowered specific
activity was observed with the reversibly dissociated monomeric AAT. It is impossible to tell whether the decrease in specific activity is the result of monomerization in itself or of other chemical and conformational modifications. AAT can be dissociated at high dilutions (2.5-250 μg/ml, pH 8, 20°C) (33, 34, 35), with a moderate increase in specific activity. However, there is still controversy over the interpretation of the data concerning dimer-monomer equilibrium of AAT (36, 37, 38). Recently, Boettcher and Martinez-Carrion (39) prepared hybrid dimeric AAT containing one sodium borohydride-reduced monomer (pyridoxamine phosphate (PMP) form) and one native monomer and concluded that no interactions occur between active sites. No difference in the equilibrium constants or rates of reaction between the native and the hybrid enzymes was observed. The conclusion can not be overrated, since the recombination of native subunits could occur during the course of transamination.

1. Spectral properties

The chromophore PLP is sensitively dependent upon structural factors and environment and acts as a natural probe at the active sites of PLP-dependent enzymes. The protonated forms of AAT absorbing maximally at 23.2 kK (430 nm) (1 kK = 10³ cm⁻¹) have been shown to be internal Schiff bases with ε-amino groups of lysine residues of the protein. This protonated internal Schiff base dissociates to give an
absorption band at about 27.6 kK (362 nm). The possible structures are shown below.

In model systems, spectra of PLP-containing Schiff bases change little with changes in the state of dissociation of the ring nitrogen. The position of the absorption maximum is somewhat different from that of the corresponding model compounds (for example, the nonprotonated Schiff's base of L-leucine with 5-deoxypyridoxal absorbs at 29.2 kK (40)), probably because of the particular environment provided by the protein. An alternative structure for the internal Schiff's base came from Turchin's $^1$H NMR investigation (41) and the model study by Fisher and Metzler (42). Fisher's compound (shown as following) absorbs at 27.5 kK, a position similar to that observed for the native enzyme. Therefore, an azomethine double bond
turned away from the phenolate was proposed for the structure for the internal Schiff base. A peculiarity is the exceptionally low $pK_a$ of the imino nitrogen in the enzyme ($pK_a = 6.3$, $pK_a$ of model compounds = 11-12), which could be partly explained by a protonation of the ring nitrogen induced by a residue at the active site (43); an additional factor could be the existence of a hypothetical positively charged group of the protein in the proximity of the phenolate of the coenzyme (43).

2. Mechanism of transamination

The general theory of PLP-catalyzed transamination was developed in 1953-54 by Braunstein and Shemyakin (44) and independently by Metzler et al. (45). A possible mechanism for AAT which is consistent with this theory is summarized in Figure 1. Amino acids react only with the active nonprotonated aldimine form of AAT (46). As demonstrated by Cordes and Jencks (47) and Jencks and Cordes (48) in model experiments, an amino
acid (with nonprotonated amino group) will form a pyridoxylidene Schiff base much more rapidly by a reaction of carbonyl transfer from an imine of the coenzyme (transaldimination) than by condensation with the free aldehyde. Therefore, a tetrahedral intermediate (Figure 1, stage 2) was proposed for the transaldimination. Intermediates which absorb at 23.2 kK and 20.4 kK (Figure 1, stages 3-5) were observed with the reactions of quasi-substrates D,L-α-methylaspartate and D,L-β-hydroxyaspartate with AAT (49, 50, 46). Saturation of AAT with aspartate also produces peaks at 23.2 kK, 20.4 kK and 30.3 kK (51). The quinonoid intermediate (Figure 1, stage 4) absorbing at 490 nm was also seen in nonenzymatic systems (52-53). The successful isolation of a 1,4-dihydropyridine formed in the reaction mixture of pyridoxal and diethylaminomalonate (54) further supports the postulated existence of a quinonoid intermediate. Hydrolysis of the ketimine (Figure 1, stage 5) and dissociation of the keto acid product from the enzyme terminates the half-reaction and leaves the enzyme in a PMP form. A second substrate α-keto acid enters into the active site and reverses the course of the half-reaction, reconverting the enzyme to its PLP form.

Model systems play a decisive role in exploring the mechanism of PLP-dependent reactions. However, model system studies could not portray the tremendous catalytic ability of the enzyme. Extensive kinetic studies on the enzyme (55-62) and on model systems (63-65) indicate that, even though the
Figure 1. A possible mechanism for the "half-reaction" of enzymic transamination. Wave numbers designate absorption maxima.
general pattern is the same, the reaction rates are at least $10^6$ times greater in the enzymatic than in the model system. Moreover, the enzyme displays a marked substrate specificity, which is lacking in the model system. Perhaps a dynamic molecular model can better invoke the roles of the catalytically active protein in contributing to the rate enhancement, in addition to its specificity.

3. Dynamic aspects

As suggested by Koshland's induced-fit theory (65) enzymatic active sites assume their functioning conformations when substrates are bound to the enzyme. In addition, the enzyme may undergo further conformational changes during the catalysis. Syncatalytic modification provides a clue to such a possibility. Riordan and Christen (66) had shown that, in the presence of the substrate pair, glutamate and $\alpha$-ketoglutarate, one of totally five thiol groups of AAT became two orders of magnitude more reactive toward N-ethylmaleimide. Using $^{14}$C-labeled N-ethylmaleimide in similar experiments, Torchinsky et al. (67) identified this syncatalytically susceptible residue as a "semiburied" cysteine 390. Because of the nearly complete (~90%) inactivation of the enzyme and the decreased affinity for the substrates after the syncatalytic modification, it had been postulated that Cys 390 was catalytically essential. However, Birchmeier et al. (68) studied syncatalytic modification of Cys 390 with a variety of reagents and reported that 60%
enzymatic activity was found with cyanide-modified enzyme. Therefore, suspicion was aroused against Cys 390 being essential to catalysis.

Tyrosine 40 is another residue which can be modified syncatalytically. Turano et al. (69) first observed marked inactivation of apoAAT on specific modification of one tyrosine (per monomer) with 1,5-difluoro-2,4-dinitrobenzene or tetrani-tromethane. Cysteine residues were also oxidized by the latter reagent.

Riordan and Christen (66) and Christen (70) found that 1.0-1.4 tyrosine residues (per dimer) were syncatalytically modified, and the enzyme was inactivated 90-95%. Under the same experimental conditions, tetrani­tromethane irreversibly oxidized cys 390, which sufficed to cause >90% inactivation of the enzyme. In contrast to syncatalytic modification by tetrani­tromethane, the modification of Cys 390 alone did not lead to irreversible amination of the coenzyme (71, 72). However, independent modification of the functional Cys 390 followed by treatment with tetrani­tromethane in the absence of substrates results in the same chemical changes of AAT as observed on syncatalytic nitration. The results cast some doubt on essentiality of Tyr 40 for catalysis.

In addition to the spatial rearrangements of the functional residues, changes in the environment of the active site and reorientation of the coenzyme may also occur. Several
lines of evidence support this idea. The first piece of evidence comes from the lack of circular dichroism (CD) in stage 3 (Figure 1) and a small negative CD in stage 4, but the rest of intermediates possess positive CD's. Further confirmation comes from the studies by Arigoni (73) and Besmer and Arigoni (74). Upon reduction of AAT with sodium borotritide he demonstrated that in the internal Schiff base, \( ^3H \) was added to the exposed re face of the imine bond, in contrast to the substrate aldimine, in which the opposite, si face was shown to be exposed. Karpeisky and Ivanov (75) and Ivanov and Karpeisky (76) have suggested a dynamic molecular model in which the coenzyme ring is revolving 40° about an axis between 2-methyl and 5-chain during the transamination. This model is subject to improvement as new informations accumulate, since conformational changes in the protein will suffice to count for all the experimental data, too. As to the changes in CD, Ivanov and Karpeisky (76) attributed these to the formation and breaking of a hydrogen bonding between the pyridine N and an unidentified residue (HZ) of the enzyme.

4. Stereochemical aspects

Dunathan has pointed out (77, 78) a most important factor controlling the direction of PLP-dependent reactions, namely, which bond at \( C_\alpha \) is to be broken to form the quinonoid intermediate. He postulated that the bond to be broken should lie in a plane perpendicular to that of the conjugated \( \pi \) system. This conformation should minimize the transition state energy
by gain in resonance energy, therefore facilitating the bond breaking. Furthermore, in the case of AAT, the prototropic aldime-ketimine rearrangement involves an acid-base catalysis. A cis proton transfer by a single acid-base group was also elucidated (79).

5. Analogue studies

Numerous PLP analogues modified in every position of the molecule have been synthesized (80, 12, 13), and used to elucidate the significance of structural features of the co-enzyme. Based on analogue studies, it is almost certain that, essential roles in catalysis can be assigned to the 4-formyl, 3-hydroxy and pyridine N. The 2-methyl group is not essential. The emphasis will be laid upon the 5-modified analogues.

Wada and Snell (81) observed that the interconversion of PM and PL was catalyzed rapidly by apoAAT. The authors concluded that the phosphate group played only a binding role and was not required for enzymic transamination. The ratio of concentrations required for half-saturation of the apoenzyme with PMP as the coenzyme (82) and with pyridoxamine as a co-substrate for transamination with oxalacetate (81) is

\[ \frac{K_{PMP}}{K_{m(PL)}} = \frac{4.4 \times 10^{-6}M}{\sim 2.3 \times 10^{-3}M} = \sim 2000 \]

This ratio provides a rough quantitative estimate of the contribution of the phosphate group to stabilization of the co-
enzyme-apoenzyme linkage. When saturated with pyridoxamine, AAT catalyzes the complete amino transfer reaction between aspartate and 2-oxoglutarate at $10^{-3}$ times the turnover rate of intact holoenzyme. This suggests that the phosphate group is needed not only for firm binding but also for effective catalysis. Moreover, Hullar (83) was able to show that 5-ethylenephosphonate analog (EPA) of PLP possessed only 2% activity of apoAAT-PLP. Furbish et al. (84) studied 5-modified analogues with apoAAT and found that they did not have coenzymatic activities with apoAAT. In contrast to the high spatial tolerance for 2- and 6-substituent in analogues, apoAAT can not tolerate analogues with even minor modification in the 5-position (see Table 1). It seems that a dianionic group in the 5-chain is important for catalysis. The importance of the bridge oxygen of PLP is also manifested in the low coenzymatic activity of EPA.

Two analogues, PLS (85) and VCA (86) turn out to be interesting active site labelling reagents for AAT. It was suggested that an unknown group X in the enzyme reacted with a Schiff base of PLS and the active site lysine, forming a thiazolidine ring. Subsequently, the loss of the 4' proton initiated the elimination of the sulfate and a methyl group was generated in the 5-position. Therefore, two residues in the active site were cross-linked through the coenzyme analogue. Presumably, VCA reacted in the same fashion except that the 5-vinyl double bond was saturated during the process.
Table 1. Coenzymatic activity of 5-modified analogues of PLP.

<table>
<thead>
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<th>Analogue</th>
<th>5-side chain</th>
<th>coenzymatic activity</th>
<th>reference</th>
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<tbody>
<tr>
<td>5-deoxy-Pl</td>
<td>-CH$_3$</td>
<td>none</td>
<td>binds weakly</td>
</tr>
<tr>
<td>MPA</td>
<td>-CH$_2$PO$_3^-$</td>
<td>none</td>
<td>binds rapidly</td>
</tr>
<tr>
<td>PL-5'-Me-phosphonate</td>
<td>-CH$_2$OP(CH$_3$)O$_2^-$</td>
<td>none</td>
<td>binds rapidly</td>
</tr>
<tr>
<td>5'-Me-PLP</td>
<td>-CH(CH$_3$)OPO$_3^-$</td>
<td>3%</td>
<td>84</td>
</tr>
<tr>
<td>CMDPL</td>
<td>-CH$_2$CH$_2$COO$^-$</td>
<td>none</td>
<td>binds rapidly</td>
</tr>
<tr>
<td>cyanoethyl-PLP</td>
<td>-CH$_2$OPO$_3$(CH$_2$CH$_2$CN)$^-$</td>
<td>variable</td>
<td>84</td>
</tr>
<tr>
<td>5'-homo-PLP</td>
<td>-CH$_2$CH$_2$OPO$_3^-$</td>
<td>1%</td>
<td>87</td>
</tr>
<tr>
<td>EPA</td>
<td>-CH$_2$CH$_2$PO$_3^-$</td>
<td>7%</td>
<td>83, 88</td>
</tr>
<tr>
<td>VPA</td>
<td>-CH=CHPO$_3^=$ (trans)</td>
<td>2%</td>
<td>83, 88</td>
</tr>
<tr>
<td>PLS</td>
<td>-CH$_2$-OSO$_3^-$</td>
<td>none</td>
<td>85</td>
</tr>
<tr>
<td>VCA</td>
<td>-CH=CH-CO$_2^-$(trans)</td>
<td>none</td>
<td>86</td>
</tr>
</tbody>
</table>
However, the coenzymatic activities of analogues vary from enzyme to enzyme. For instance, PLS has been tested with other apoenzymes. It was found to have 10 and 23% coenzymatic activity in tryptophanase and arginine decarboxylase (89), respectively. However, it failed to activate the apoenzymes of brain glutamic acid decarboxylase (90), phosphorylase (91), aspartate-β-decarboxylase (92) and D-serine dehydratase (89).

CMDPL was not found to have coenzymatic activity in tryptophanase (93), arginine decarboxylase (94), D-serine dehydratase (95) or AAT (84). However, one may consider its use as an antibacterial agent. It has been found that bacterial aposerine dehydratase binds CMDPL with a higher binding constant than is found with PLP itself.
III. EXPERIMENTAL

A. Materials

1. Analogues of pyridoxal 5'-phosphate

Diethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate [(Et)₂MP] was synthesized by Kevin Kelly, according to Dr. Chuzo Iwata's unpublished procedure of this laboratory.

5-(4-Formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonic acid (MPA) and its monoethyl ester derivative (EtMP) were obtained from acid hydrolysis of (Et)₂MP, followed Dowex 50W-X4 (ion exchange resin) column separation procedure developed by the author.

Ethyl pyridoxal 5'-phosphate (EtPLP) and methyl pyridoxal 5'-phosphate (MePLP) were made essentially according to the procedure of Murakami and Iwanami (96) and modified by the author.

5-Deoxypyridoxal 5'-sulfonate was synthesized by Bolden (97).

5'-Carboxymethyl pyridoxal-5-deoxypyradoxal (CMDPL) was prepared by the author according to the procedure of Iwata and Metzler (98).

5'-Carboxymethyl pyridoxamine-5-deoxypyradoxal dihydrochloride (CMDPM) was prepared by Iwata and Metzler (98), and recrystalized from methanal-ether before use.

5-(4-Formyl-3-hydroxy-2-methyl) pyridyl ether phosphonic acid (EPA) was first synthesized by Hullar (83) and was
prepared here by Dr. Tetsu Miura.

2. Other chemicals

All other chemicals were obtained from commercial sources and were reagent grade.

Triethanolamine hydrochloride was prepared by dissolving reagent grade triethanolamine in a minimal amount of water in the presence of an excess of HCl. The hydrochloride salt was precipitated after the addition of ethanol, and was recrystallized three times from water-ethanol and three times from water.

Ethanolamine was distilled before use.

3. The enzyme

Aspartate aminotransferase was prepared in this laboratory (see Methods), and only the α subform was used for studies.

B. Methods

1. Preparation of holo aspartate aminotransferase

The procedure is based on the method of Jenkins et al. (22), as modified by Martinez-Carrion et al. (Method A) (27), and is adopted from Yang's Ph.D. dissertation (99).

2. Enzyme concentration

Determination of the enzyme concentration was based upon molar absorptivities of $6.36 \times 10^4$ and $6.55 \times 10^4$ at 35.8 kK for the apoenzyme and holoenzyme, respectively (84), and a molecular weight of $4.63 \times 10^4$ (19) per subunit.
3. Activity assay

Routine assays were performed by the method of Jenkins et al. (22), except that triethanolamine hydrochloride was used for the buffer instead of Tris. Oxaloacetate produced was followed at 35.7 kK on the Cary 1501. The absorption coefficient of oxaloacetate as given by Velick and Vavra (100) \( (0.57 \times 10^3 \text{M}^{-1} \text{cm}^{-1}) \) was used to express specific activity as \( \mu \text{mole/min/mg} \) of enzyme. AAT prepared in this laboratory was found to have specific activity of 210-260 \( \mu \text{mole/min/mg} \).

4. AAT resolution

The native enzyme was resolved according to the method of Scardi et al. (101), with an adaptation by Furbish et al. (84). The apoAAT thus prepared had little detectable activity (0.2%-0.4% of that of the reconstituted AAT) and was reactivated to 85-99% of the activity of the native enzyme under the condition described below.

5. Activity measurement of apoAAT-analogue complexes

ApoAAT (\( \sim 10^{-4} \text{M} \)) was incubated with PLP analogue (\( \sim 10^{-3} \text{M} \)) for 20 min, then diluted to a final concentration of \( 10^{-7}-10^{-8} \text{M} \) in the assay solution. The reaction was allowed to proceed for 10 min.

6. Spectrophotometric measurement

Absorption spectra were obtained on a Cary Model 1501 recording spectrophotometer equipped with a Cary-Datex digital
output system and an IBM card punch. Spectra were corrected for turbidity and baseline errors.

A JASCO Model ORD/UV-5 equipped with a Scientific SS-20-2 Modification was used for the Circular dichroism (CD) measurement. Ellipticity recorded on charts was manually transcribed at 2 nm intervals onto IBM cards and was converted to $\Delta \varepsilon$ and was replotted vs. wave number by the computer.

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian A-60, Hitachi-Perkin Elmer R20B, or Varian HA-100. When samples were dissolved in organic solvents, tetramethylsilane (TMS) was used for internal standard. Otherwise TMS in a capillary was used for external standard, in order to be able to recover the material afterwards.

Mass spectra were obtained using an AEI MS-902 mass spectrometer.

7. Buffer systems

When enzyme experiments were performed at high pH, the buffer was made from 0.2 M triethanolamine hydrochloride adjusted to pH 8.3 with sodium hydroxide. However, 0.01 M triethanolamine hydrochloride was used in the pH titration experiments. When experiments were carried out at low pH, the buffer was made from 0.2 M acetic acid adjusted to pH 5.4 with sodium hydroxide. Double distilled water was used to prepare buffer solutions.
IV. RESULTS

A. Organic Synthesis of Pyridoxal 5'-Phosphate Analogues

1. Attempted synthesis of 5'-bromo-5-deoxypyridoxal from 5'-bromo-5-deoxypyridoxamine dihydrobromide by nonenzymatic transamination catalyzed by aluminum (III) ion. Isolation of 5'-0-methylpyridoxal

\[
\text{CH}_2\text{NH}_3^+\text{Br} \overset{\text{OHCCO}_2^-\text{Na}^+}{\text{Al}^{+++}, \text{MeOH}} \rightarrow \text{H}_3\text{C} \overset{\text{N}^+\text{Br}}{\text{H}_2\text{C} \overset{\text{O}}{\text{H}}} \overset{\text{C} = \text{O}}{\text{CH}_2\text{Br}}
\]

5'-Bromo-5-deoxypyridoxamine dihydrobromide was synthesized according to the procedure of Khomutov (Bolden (97)). To a two liter solution of 0.0005 M (0.3 g) of methanolic 5'-bromo-5-deoxypyridoxamine dihydrobromide, (0.0025 M, 0.6 g) of sodium glyoxylate was added. The reaction mixture was stirred slowly for 5 min after which 0.00025 M (0.2 g) of aluminum nitrate nonahydrate was added. After stirring for 10 min, the mixture was allowed to stand for 2 hours until the peak ratio \( A_{25.8kk}/A_{33.8kk} \) in 0.1 NaOH reached 3.5. After rotary evaporation the residue was dissolved in a minimal amount of water and applied to a column (1.5 cm X 25 cm) of Amberlite CG 50 (100-200
mesh) ion-exchange resin in the acid form. The column was eluted with water at a rate of 60 ml/hr. The fractions with \( A_{25.8 \text{nm}}/A_{33.8 \text{nm}} \) ratio greater than 5.3 were pooled with lyophilized. Yield: 8 mg. A reason for the low yield was that the product was volatile. Yellow materials were found in the condensing pot during the lyophilization. An attempt to sublime the reaction residue directly did not succeed because aluminum ion complexed with the product.

A high resolution mass spectrum showed a parent ion peak at 181.07445, which gave a formula of \( C_9H_{11}NO_3 \) (exact mass: 181.07389) with an error of 3 ppm. The product refluxed in an alkaline solution for a few hours did not change to pyridoxal. So a structure of the product could be proposed as following:

\[
\begin{array}{c}
\text{CHO} \\
\text{HO} \\
\text{CH}_2\text{-O-CH}_3 \\
\text{H}_3\text{C} \\
\text{N}
\end{array}
\]

A possible reaction mechanism will be discussed later on.

2. \( N-(2\text{-Methyl-3-hydroxy-5-hydroxymethyl-4-pyridylmethylene})\)-p-toluidine

\[
\begin{array}{c}
\text{CHO} \\
\text{HO} \\
\text{CH}_2\text{OH} \\
\text{H}_3\text{C} \\
\text{N}
\end{array} \quad \xrightarrow{\text{p-toluidine}} \quad 
\begin{array}{c}
\text{H} \\
\text{C} \equiv \text{N} \\
\text{CH}_3 \\
\text{HO} \\
\text{CH}_2\text{OH} \\
\text{H}_3\text{C} \\
\text{N}
\end{array}
\]
The commercial p-toluidine was recrystallized from water, and was thereafter sublimed. Pyridoxal hydrochloride was neutralized with sodium bicarbonate to give its free base. Pyridoxal (3 g, 0.018 mol) was added to 200 ml of ethanol. The solution was stirred at 40°C until most of pyridoxal was dissolved. p-Toluidine (2.1 g, 0.020 mol) was then added and was stirred for 10 min. A yellow precipitate formed immediately. It was filtered and washed with three portions of ether and three portions of 100 ml each of water, giving 3.8 g of product.

The filtrate was evaporated under vacuum, and washed with ether and water the same way but with 20 ml for each portion. 0.25 g of product was recovered. Yield: 88%. The product can be recrystallized from ethanol or chloroform. Mass spectrum (m/e): 256.3 (M+). NMR: In DMSO-d6, δ 7.32 s, δ 7.92 s, δ 9.12 s. Two methyl peaks were buried by a DMSO peak and the 5-methylene peak was broad. However they can be seen in CDCl3 solvent.

In CDCl3, δ 2.42 s, 2.58 s, δ 4.89 s.
The Schiff base of pyridoxal and p-toluidine (0.1 g) was added to 40 ml of anhydrous benzene. The solution was stirred and heated until the Schiff base was dissolved. 0.04 ml of SOBr$_2$ was added. The precipitate was filtered and washed with anhydrous benzene three times. 0.12 g of product was obtained.

Mass spectrum (m/e): 320 (M + 2$^+$) and 318 (M$^+$) with equal intensity (an indication of the existence of one bromine), 256 (M$^+$ of starting material). Judging from the peak ratio of (320 + 318)/256 in the mass spectrum, a 54% yield was obtained. When the product dissolved in the water, the bromo Schiff base was proven to cyclize to the following compound. Both the 320 (M + 2$^+$) and 318 (M$^+$) peaks disappeared to give rise to a peak at 238 in the mass spectrum.
Two spots could be separated from the reaction product on TLC (solvent: ethyl acetate), with $R_f$'s 0.85 and 0.40. The spot of $R_f$ 0.4 was demonstrated by its mass spectrum to be the starting material. The starting material was also obtained by a crystallization from acetone-skelly A. Attempts were made to purify the bromo Schiff base on a silica gel column (eluted with ethyl acetate).

However, the bromo Schiff base appeared sensitive to moisture (and/or air). A black yellow material remained after concentration with a rotary evaporator. A major peak at 238 in the mass spectrum was found for this black yellow residue, which suggested that the bromo Schiff base was cyclized.

Likos (102) studied the reaction of glutamic acid decarboxylase with a substrate analogue serine-o-sulfate and proposed structure A for its dephosphorylated product as following:
Since glutamic acid decarboxylase can be utilized only once to produce stoichiometric amount of the product, it's difficult to prepare enough compound for characterization. It would be desirable to synthesize compound A so that we could compare its properties with those of the enzymic product side by side.

4. Organic synthesis of the product of glutamic acid decarboxylase and serine-o-sulfate (compound A)

To a one ml solution of 0.025 ml \((4.1 \times 10^{-4} \text{ mol})\) of ethanolic ethanolamine, 5 mg \((1.3 \times 10^{-5} \text{ mol})\) of the bromo Schiff base was added. The mixture was stirred for a few minutes. A bright yellow band was separated on TLC (Eastman Chromagram Sheet, 13181 silica gel, solvent:acetone) and was eluted by ethanol. After rotary evaporation a small amount
of water was added to dissolve the residue. The water was then evaporated under reduced pressure. 2.6 mg of product was obtained. Much of the product probably was trapped by the viscous ethanolamine which was present in excess on TLC.

A high resolution mass spectrum showed a parent ion peak at 210.10089, which gave a formula of \( C_{10}H_{14}N_2O_3 \) (exact mass: 210.10044) with an error of 2.2 ppm. The possible structures are as following:

The structure B is impossible, because a mixture of pyridoxal and ethanolamine does not give the same spectral change with pH as is observed for the product.

Structures C and D are essentially the same when dissolved in aqueous solutions. Their spectral change with pH should be the same way as that of compound A. Structure C actually is the hydrate form of compound A.
5. 5'-Bromo-5-deoxypyridoxal

The hydrobromic salt of the bromo Schiff base (10 mg) was added to 4 ml of ethanol. The solution was stirred and 0.15 ml of 48% hydrobromic acid was added. After 30 min, the reaction solution was evaporated under vacuum to almost dryness. The resulting solution was applied on an Amberlite CG 50 (100-200 mesh) column (1.2 cm X 0.6 cm), and eluted with water. After rotary evaporation, 7 mg of residue was obtained. Mass spectrum (m/e): 231 (M + 2^+) and 229 (M^+) with equal intensity, 150 (M^+ - Br), 107 (p-toluidine).

6. Diethyl 5-(3-hydroxy-4-hydroxymethyl-2-methyl) pyridyl-methyl phosphonate (Iwata's unpublished procedure)

![Chemical structure](image)

This compound can be made in either of two ways

1) To a suspension of 18.4 g (81 mmol) of α²,β₂-isopropylidene-α⁵-pyridoxylchloride (103) (prepared from the corresponding hydrochloride by neutralization with saturated NaHCO₃ and extraction with methylene chloride) and 12.0 g (87 mmol) of diethyl phosphite in 240 ml of anhydrous ether was added 2 g (87 mmol) of sodium metal (one piece: 1 cm X 0.5 cm X 0.3 cm).
After refluxing 50 hr the mixture was filtered and the filtrate was evaporated.

2) \(\alpha^4,3\)-0-isopropylidene-\(\alpha^5\)-pyridoxyl-chloride (21.8 g, 97 mmol) was combined with triethylphosphite (25 g, 150 mmol) and refluxed for one hour at 150°C. After cooling excess triethylphosphite is removed under vacuum.

Either residue may be purified by vacuum distillation, but a good vacuum (<0.3 mm) is necessary to prevent excessive decomposition from high pot temperature (185-190°C @ 0.3 mm). Method "l" gave 52% diethyl \(\alpha^4,3\)-0-isopropylidene-\(\alpha^5\)-pyridoxyl-phosphonate (I) after distillation. The residue can also be purified to a lesser extent by chromatography on alumina with benzene or cyclohexane. Mass spectrum of I (m/e): 329 (M⁺), 271 (M⁺ - CH₃COCH₃), 243 (m/e 271-CO), 215 (M/e) 243-C₂H₄, 106 [m/e 271-P(O)(OEt)₂]. NMR of I in CCl₄: 1.18t (6H), δ1.47s (6H), δ2.24d (3H), δ2.81d (2H), δ3.94 (4H) sextet, δ4.82d (2H), δ7.77d (1H). Values are given for the center of each set. The 5'-CH₂ protons have a large 3¹P-¹H splitting (22 Hz). Smaller 3¹-¹H splittings (~3Hz) were observed for the 2-CH₃, 4'-CH₂, and 6-H signals.

A solution of 1.6 g (4.9 mmol) I in 20 ml of 1 N HCl was heated on a steam bath for 15 min, the cooled solution was neutralized with NaHCO₃ and extracted with CHCl₃. The CHCl₃ solution was dried (Na₂SO₄) and evaporated. Recrystallization from benzene gave 0.9 g (60%) of II, mp 128-129°C. Mass spectrum of II (m/e): 289 (M⁺), 271 (M⁺ - H₂O), 243 (m/e 271-
CO\), 215 \text{ (m/e 243-C}_2\text{H}_4\text{)}, 106 \text{ (m/e 271-P(O)(OEt))}.

\text{Anal. Calcd. for C}_{12}\text{H}_{20}\text{NO}_{5}\text{P: C, 49.82; H, 6.97; N, 4.84.}
\text{Found: C, 49.85; H, 6.83; N, 4.84.}

7. Diethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate [(Et)\text{2MP}] (Iwata's unpublished procedure)

\begin{align*}
\text{II} & \quad \text{(Et)\text{2MP}} \\
\text{2CH}_2\text{OH} & \quad \text{OEt} \\
\text{H}_3\text{C} & \quad \text{OEt} \\
\text{N} & \quad \text{OEt} \\
\text{CH}_2\text{P=O} & \quad \text{CHO} \\
\text{H}_3\text{C} & \quad \text{OEt}
\end{align*}

To 3.0 g (10.3 mmol) of II in CHCl\text{3} (200 ml) was added 10 g of activated MnO\text{2} (104). The mixture was stirred in the dark at room temperature. When TLC (in acetone) indicated complete conversion (12 hrs) the mixture was filtered and the residue washed with CHCl\text{3} until washings were colorless. After rotary evaporation the residue can be purified by recrystallization (CH\text{2Cl}_2-Skelly A, 1:6) or chromatography on alumina (elution with chloroform). Yield 53\%, mp 70-73\text{o}. Mass spectrum of [(Et)\text{2MP}] \text{(m/e)}: 287 (M\text{+}), 259 (M\text{+} - \text{CO}), 231 (m/e 259 - \text{CO or C}_2\text{H}_4\text{)}, 203 (m/e 231 - \text{CO or C}_2\text{H}_4\text{)}, 123 (m/e 231 - P(O)\text{2(OEt))}, 122 (m/e 231 - P(O)(OH)(OEt)). NMR in CC\text{14}: resembles that of I. The 5'-CH\text{2} peaks disappeared when dissolved in alkaline D\text{2O}.

\text{Anal. Calcd. for C}_{12}\text{H}_{18}\text{NO}_{5}\text{P: C, 50.17; H, 6.32; N, 4.88.}
\text{Found: C, 50.07; H, 6.34; N, 4.92.}
UV data: See Tables 2 and 3, and Figure 2.

Oxime of (Et)$_2$MP: To a solution of 0.4 g (1.4 mmol) of (Et)$_2$ in 50 ml of ethanol were added 0.35 g (5 mmol) of NH$_2$OH·HCl and 0.41 g (5 mmol) of NaOAC. The mixture was refluxed for 3 hr and evaporated under reduced pressure. The residue was extracted with hot ethanol several times. After removing ethanol, the residue was recrystallized from ethanol, giving 0.25 g (59%) of oxime of VIII, mp 227-228°.

Anal. Calcd. for C$_{12}$H$_{19}$N$_2$O$_5$P: C, 47.68; H, 6.34. Found: C, 47.48; H, 6.09.

8. 5-(4-Formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonic acid and monoethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate (MPA and EtMP)

A solution of 1 g (3.45 mmol) of Et$_2$MP in 50 ml of 20% HCl was refluxed. The progress of hydrolysis was monitored by removing aliquots into 0.1 HCl. The uv-visible absorption spectrum of the hydrolysate was recorded. The absorbance ratio ($A_{33.8kk}/A_{29.5kk}$) was noted. The reaction was carried out until the ratio attained a minimal value of 1.8. Approximately 5 hours were required to attain this value. The solution was
Table 2. Band parameters for resolved absorption spectra (105). The symbols $H_3P$, $H_2P$ etc. refer to the state of protonation of a compound; (a) and (h) refer to aldehyde and covalent hydrate respectively. The position $\nu$, height $\varepsilon$, width $W$ and skewness $\rho$ are those of the fitted lognormal curves. The molar area $a^0$ is the measured area ($a$) of a band divided by the mol fraction of a given form present. A number of values for molar areas were assumed by comparison with other compounds. These are designated by asterisks.

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III. EtMP

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Table 2. (Continued)

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<td>( \varepsilon_o ) (mol(^{-1}) ( \cdot ) cm(^{-1} \times 10^{-3} ))</td>
<td>( W ) (KK)</td>
<td>( \rho )</td>
<td>( a )</td>
<td>( a^0 )</td>
<td>( f )</td>
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Table 3. Apparent $pK_a$ values

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<th>Analogues</th>
<th>$pK_a$ Values</th>
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<td>(Et)$_2$MP</td>
<td>3.03, 7.00</td>
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<tr>
<td>MPA</td>
<td>4.07, 6.76, 9.20</td>
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<tr>
<td>EtMP</td>
<td>3.91, 8.21</td>
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<tr>
<td>EtPLP</td>
<td>3.56, 7.63</td>
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</table>
Figure 2. Ionic forms of $(\text{Et})_2^\text{MP}$
concentrated under reduced pressure. The concentrate was applied to an 1.2 X 50 cm column of Dowex 50W-X4 (100-200 mesh) in H\(^+\) form previously equilibrated with water. The column was eluted with water under nitrogen at a flow rate of 36 ml hr\(^{-1}\). The elution profile was recorded by a uv monitor at 35.7 kk. EtMP appeared in the second peak and MPA appeared in the third peak of the elution profile. The effluent was concentrated in vacuo, and lyophilized. Yield: 51 mg or 40% for MPA, 26 mg or 20% for EtMP. Both can be recrystallized from water-acetone. NMR of MPA in D\(_2\)O: \(\delta\) 2.38 s (3H), \(\delta\) 3.29 d (2H), \(\delta\) 7.5 s (1H, broad), \(\delta\) 10.13 (1H, broad). NMR of EtMP in D\(_2\)O: \(\delta\) 1.09 t (3H), \(\delta\) 2.31 d (3H), \(\delta\) 3.22 d (2H), \(\delta\) 3.71 (2H) sextet, \(\delta\) 7.41 s (1H0, broad), \(\delta\) 10.10 s (1H, broad). Electrophoreasis: MPA and EtMP migrated toward the anode in about the same distances at pH 6.4, but a little less than that of pyridoxal 5'-phosphate. One trace impurity in each sample of MPA and EtMP moved ahead and was detectable by fluorescence.

UV data: See Tables 2 and 3, and Figures 3 and 4.

9. Monoethyl pyridoxal 5'-phosphate (EtPLP)

Triethylamine (0.04 g) was added to 2 ml of an ethanolic suspension of 0.1 g pyridoxal 5'-phosphate. The mixture was stirred with 0.08 g of dicyclohexylcarbodiimide for 5 hrs. The solution was then filtered. After evaporation to dryness on a rotary concentrator the residue was dissolved in 1 ml of water and washed with ether several times. The solution was concentrated and applied on a column (1 cm X 40 cm) of Dowex
Figure 3. Ionic forms of MPA
Figure 4. Ionic forms of EtMP
50W-X4 (100-200 mesh) ion exchange resin in the acid form. The column was eluted with oxygen-free water at a rate of 50 ml/hr. 10 ml fractions were collected. An 0.1 ml aliquot of each fraction was added to 2 ml of 0.1 N NaOH and the spectrum was recorded (for elution profile, see Figure 5). The fractions with absorbance ratios $A_{25.8kK}/A_{32.6kK}$ greater than 10 were pooled and lyophilized. Yield: 45 mg or 41%. The product was recrystallized from water-acetone. NMR in D$_2$O: $\delta$1.17t (3H), $\delta$2.62s (3H), $\delta$3.93g (2H), $\delta$5.15d (2H), $\delta$6.57s (1H), $\delta$8.28s (1H). Values are given for the center of each set.

Electrophoresis: The sample moved as a single spot toward the anode at pH 6.4 with a mobility less than that of PLP. One trace impurity, moving ahead of EtPLP were detectable by fluorescence.

UV data: See Tables 2 and 3, and Figure 6.

10. Monomethyl pyridoxal 5'-phosphate (MePLP)

The compound was first synthesized by Pfeuffer et al. (106) by a 6-7 step procedure. However, the author was able to obtain the compound in one step using the same procedure in (9) except that methanol was substituted for ethanol. Yield: 42%. The product was recrystallized from water-acetone. NMR in D$_2$O: $\delta$3.1s (3H), $\delta$4.03d (3H), $\delta$5.55d (2H), $\delta$6.95s (1H). The chemical shifts are 0.5 ppm more downfield, because helmreich et al. used DSS as the internal standard.
Figure 5. Elution profile for the purification of EtPLP

-X-X- absorbance at 25.8kK
-0-0- absorbance at 32.6kK
ELUTION PROFILE

ABSORBANCE

FRACTION NUMBER
Figure 6. Ionic forms of EtPLP
B. Interactions of the Analogues with Apoaspartate Aminotransferase

1. Binding to the apoenzyme

When apoAAT was mixed with individual analogues in a ratio of about 1 to 0.75 at pH 8.3, the absorption band of the free analogue at about 25.6 kK was shifted to the 26.4-28.0 kK region. This fact suggested binding of the analogue to apoAAT (Figures 7-11). An exception was (Et)2MP whose absorption remained at 25.6 kK. A small amount of absorption in the 23-24 kK region which decreased with time was observed with the analogues MPA, EtMP, MePLP and EtPLP. In contrast to this behavior, the 23 kK shoulder increased with time with PLSN. There is always a positive circular dichroism (CD) corresponding to the major absorption band and sometimes a negative CD corresponding to the shoulder (Table 4).

2. Activity measurement

None of these analogues has coenzymatic activity at pH 8.3 except for EPA which possesses 7-10% of activity of the native enzyme (83,88). Activity measurement was done also at pH 7.5 and 6.5 with MPA. The latter was not active at those pH's either. ApoAAT was incubated with EtPLP for four hours before assay, in order to test the idea that EtPLP might be hydrolyzed to PLP as was cyanoethyl-PLP (85). No increase in activity was observed.
Figure 7. Reconstitution of apoAAT with EtMP, (apoAAT was subtracted), apoAAT, $5.51 \times 10^{-5}$M, EtMP, $4.14 \times 10^{-5}$M

1) 2.5 min after mixing
2) 40 min
Figure 8. Reconstitution of apoAAT (5.55 x 10^{-5} M) with PLSN (4.18 x 10^{-5} M) at pH 8.3. (ApoAAT was subtracted)

1) 2 min after mixing
2) 25 min
Figure 9. Reconstitution of apoAAT with PL (apoAAT was subtracted)

1) 6 min after PL ($4.81 \times 10^{-5}$M) was added to apoAAT ($5.44 \times 10^{-5}$M)

2) 12 min
Figure 10. Reconstitution of apoAAT (5.53 x 10^{-5} M) with EtPLP (3.44 x 10^{-5} M)

1) 2 min 45 sec after mixing
2) 7 min
3) 30 min
Figure 11. Reconstitution of apoAAT (5.6 x 10^{-5}M) with MePLP (3.55 x 10^{-5}M) at pH 8.3 (apoAAT was subtracted)
Table 4. Summary of data for binding to apoAAT at pH 8.3

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration of apoenzyme x 10^5(M)</th>
<th>Concentration of analogues x 10^5(M)</th>
<th>Binding to apoenzyme (time)</th>
<th>Absorption bands a</th>
<th>Circular dichroism a,b</th>
<th>Δ A/A x 10^3</th>
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<td>&lt; 2 min</td>
<td>363 nm</td>
<td>362 nm</td>
<td>2.0</td>
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<tr>
<td>(Et)2MP</td>
<td>4.92</td>
<td>3.77</td>
<td>-</td>
<td>25.6kK (391 nm)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MPA</td>
<td>5.50</td>
<td>3.60</td>
<td>3-5 min</td>
<td>23.6kK (424 nm) (∼420 nm) 28kK (357 nm) 356 nm</td>
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<td>∼40 min</td>
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<sup>a</sup> Indicates a partially "buried peak" on a shoulder.

<sup>b</sup> Positions of small negative CD, if any, are given in the parentheses.

<sup>c</sup> Data were taken from Furbish et al. (84).

<sup>d</sup> An average value of three experimental data.

<sup>e</sup> An average value of two experimental data.
3. Reduction of apoenzyme-analogue complexes by sodium borohydride

Reduction of E-MPA and E-EtPLP at pH 8.3 caused an immediate loss of the major absorption band (28 kK and 27.4 kk, respectively) and replacement with an absorption band at 30.8 kK. Solid trichloroacetic acid (just enough to precipitate the protein) was added. After centrifugation the supernatant and the precipitate were separated. The precipitate was dissolved in 6 M guanidine hydrochloride in order to take the spectrum. The chromophore was found in the protein solution, implying that it was bound to the protein. The mixture of apoAAT plus (Et)₂MP was treated with sodium borohydride in the same way. The chromophore was found in the supernatant and was not bound with the protein (Table 5).

4. Attempted displacement of analogues by PLP

Two control experiments were done to determine whether apoAAT can still bind PLP after sodium borohydride treatment:

(i) ApoAAT (5.61 x 10⁻⁵ M) was treated with sodium borohydride. The subsequent addition of PLP (5.56 x 10⁻⁵ M) at pH 5.4 gave rise to a 23.6 kK peak (Table 6).

(ii) Equal amounts of apoAAT (5.34 x 10⁻⁵ M) and PLSN (5.51 x 10⁻⁵ M) were mixed at pH 8.3. About 56% of the active sites were saturated, as judged by the fact that the positive CD at 366 nm reached 56% of its maximum. A positive CD at 326 nm resulted from the addition of sodium borohydride to the
Table 5. Summary of data for reduction of apoenzyme-analogue complexes by sodium borohydride

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration of apoenzyme x 10^5(M)</th>
<th>Concentration of analogue x 10^5(M)</th>
<th>Chromophore found with</th>
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<tr>
<td>(Et)_2MP</td>
<td>4.92</td>
<td>3.77</td>
<td>supernatant</td>
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<td>MPA</td>
<td>5.60</td>
<td>3.60</td>
<td>apoAAT</td>
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<tr>
<td>EtPLP</td>
<td>5.47</td>
<td>3.70</td>
<td>apoAAT</td>
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Table 6. Summary of data for displacement of analogues by PLP

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<tr>
<th>Analogue</th>
<th>Concentration of enzyme (M) x 10^5</th>
<th>Concentration of analogue (M) x 10^5</th>
<th>Absorption band λ max</th>
<th>CD λ max</th>
<th>Concentration (M)</th>
<th>Absorption λ max</th>
<th>CD λ max</th>
<th>Δε</th>
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<td>(424 nm)</td>
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<td>MPA</td>
<td>5.43 5.30 30.8 kK (325 nm)</td>
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<td>5.46</td>
<td>25.6 kK</td>
<td>(391 nm)</td>
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<tr>
<td>EtMP</td>
<td>5.16 11.9 &lt;325 nm</td>
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<td>5.73</td>
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<td>360 nm 8.3</td>
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<td>5.45</td>
<td>25.7 kK</td>
<td>(389 nm)</td>
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a Concentrations were calculated after the mixing of three components (apoAAT, analogue and PLP).

b The experiment was done at pH 5.4.
above solution. After the residual sodium borohydride was allowed to react for another two hours, PLP \((5.28 \times 10^{-5} \text{M})\) was added. A positive CD at 360 nm, developed with \(\Delta \varepsilon = 8.3\) (compared to \(\Delta \varepsilon\) of the holoenzyme = 16, PLP occupied about 52% of the active sites). ApoAAT \((5.26 \times 10^{-5} \text{M})\) was saturated with PLSN \((2.03 \times 10^{-4} \text{M})\). After sodium borohydride reduction, the addition of PLP \((5.75 \times 10^{-5} \text{M})\) resulted in a positive CD at 360 nm \((\Delta \varepsilon = 2.5)\), and a negative CD at 415 nm \((\Delta \varepsilon = -2.6)\) (Table 6). The above two control experiments indicate that reduced apoAAT can still bind PLP, and the more PLSN the apoAAT binds, the less binding sites are left available for PLP. The reason for this is that PLSN actually binds at the active site and inhibits the binding of PLP. E-MPA was reduced by sodium borohydride at pH 8.3. After 3 hours of reaction, PLP was added to the solution. A peak appeared at 25.6 kK, indicating that the PLP remained free and unbound (Figure 12, Table 6).

EtMP and PL were individually added, gradually to apoAAT at pH 8.3 until there was no more increase in the positive CD corresponding to the original major absorption band. Sodium borohydride was then added and, after three hours, PLP was used in the attempt to displace the analogue. However, the results suggested that EtMP and PL were bound at the active site (Table 6). If the analogue were not fixed in the active site by reduction, displacement of the analogue, for example, EtPLP, could be observed by direct circular dichroism at pH 5.4.
Figure 12. Attempted displacement of MPA by PLP after reduction

1) ApoAAT, $5.59 \times 10^{-5}$ M, MPA, $5.46 \times 10^{-5}$ M, pH 8.3
2) Sodium borohydride was added, pH 8.5
3) PLP ($5.38 \times 10^{-5}$ M) was added
Neither bound EtPLP nor free PLP absorb at 23.2 kK, but bound PLP does at pH 5.4. PLP was added to E-EtPLP at pH 5.4. An increasing 23.2 kK CD in company with a decreasing 27.6 kK CD indicated that PLP displaced EtPLP (Figure 13, Table 6).

5. Low pH titration of apoenzyme-analogue complexes

Portions (2-5 µl) of 2 M sodium acetate buffer (pH 5.4) were added to E-MPA, E-PLSN and E-ETPLP, individually. The pH was brought down from 8.3 to 6.0-5.5. No lower pH titration could be achieved, because the apoenzyme was denatured at acidic pH. The weak absorption band in the 23-24 kK range decreased with decreasing pH, while the major internal Schiff base band increased (for example, see Figure 14). No drastic absorption change was observed. Since there was no distinct pKa found for E-EtPLP, glutarate was complexed with E-EtPLP in an attempt to raise the pK of E-Et PLP (Jenkins and D'Ari, (107)). Portions (5-10 µl) of 2 M sodium acetate (pH 5.4) were added to the above solution, but there was no drastic spectral change even when the pH was brought down to 4.7 (Table 7, and Figure 15). An interesting fact is, the absorption spectrum of E-MPA changed with pH the same way as other analogues initially, then the peak at 23.1 kK did increase with decreasing pH with a concomitant decrease of the peak at 28 kK (Figures 16a, 16b, and 17). Two pKa's 8.2 and 7.0 were estimated (ionic strength, 0.02-0.04). The same phenomenon would be expected for EPA, whose 5'-side chain is dianionic at pH 8.3, similar to MPA.
Figure 13. Displacement of EtPLP by PLP at pH 5.4

1) Apo, 5.57 x 10^{-5} M, EtPLP, 5.59 x 10^{-5} M
2) 8 min after the addition of PLP (5.5 x 10^{-5} M
    Apo, 5.49 x 10^{-5} M, EtPLP, 5.50 x 10^{-5} M
3) 46 min
4) 11 hr
Figure 14. Low pH titration of E-EtPLP

1) Apo, $5.50 \times 10^{-5}$M, EtPLP, $5.60 \times 10^{-5}$M, pH 8.25
2) pH 7.61
3) pH 6.14
Table 7. Summary of data for low pH titration of apoenzyme-analogue complexes

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration$^a$ of apoenzyme x 10$^5$ (M)</th>
<th>Concentration$^a$ of analogue x 10$^5$ (M)</th>
<th>Low pH limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>5.10</td>
<td>3.30</td>
<td>6.0</td>
</tr>
<tr>
<td>EtMP</td>
<td>5.20</td>
<td>3.80</td>
<td>6.0</td>
</tr>
<tr>
<td>PLSN</td>
<td>5.20</td>
<td>3.66</td>
<td>6.2</td>
</tr>
<tr>
<td>EtPLP</td>
<td>5.47</td>
<td>3.70</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>5.47$^b$</td>
<td>5.58</td>
<td>4.7</td>
</tr>
<tr>
<td>EPA</td>
<td>5.56</td>
<td>3.81</td>
<td>7.3</td>
</tr>
</tbody>
</table>

$^a$ Concentrations were calculated before sodium acetate buffer was added.
$^b$ Glutarate (0.015 M) was added before titration.

However, the apoenzyme seemed to denature more easily with EPA, and the pH could not be lowered to less than 7.3. The spectrum did not change with pH above pH 7.3 (Table 7).

6. High pH titration of E-EtPLP

ApoAAT (5.47 x 10$^{-5}$M) was reconstituted with EtPLP (3.7 x 10$^{-5}$M) at pH 8.3. Portions (2-5 μl) of saturated Na$_2$CO$_3$ solution were added to the above solution. The 27.4 kK peak underwent a bathochromic shift to 26.6 kK as the pH was brought up to 10. Apparently, the coenzyme was released from the apoenzyme when the solution became basic.
Figure 15. Low pH titration of E-EtPLP-glutarate

1) ApoAAT, $5.47 \times 10^{-5}$ M, EtPLP, $5.58 \times 10^{-5}$ M glutarate, $0.015$ M, pH 8.3
2) pH 5.0
3) pH 4.74
Figure 16a. Low pH titration of E-MPA

1) Apo, $5.10 \times 10^{-5}$ M, MPA, $3.30 \times 10^{-5}$ M
   pH 8.40
2) pH 7.90
3) pH 7.55
Figure 16b. pH titration of apoAAT-MPA

3) pH 7.55
4) pH 7.10
5) pH 6.10
Figure 17. Ionic forms of E-MPA
7. Addition of L-glutamate to apoenzyme-analogue complexes

L-glutamate was added to individual E-MPA, E-EtMP, E-PLSN, E-PL, E-EtPLP and E-MePLP solutions at pH 8.3. The PLP form (~27 kK) was transformed into the PMP form (~31 kK). With EtMP and PLSN, a 24 kK absorption band was also observed (Figures 18-23 and Table 8). Various turn-over rates were seen. The transamination followed biphasic pseudo first order kinetics with E-EtMP, E-PLSN, E-EtPLP and E-MePLP as shown in Figures 24-27. The author tentatively interprets this as following: The first step is a fast reaction representing the real rate of transamination, the second step is a slow reaction in which an equilibrium is reached, following a slow release of the PMP form of the analogue from the apoenzyme. This point will be further explained in the discussion section.

\[ A_t = \varepsilon_x[X] + \varepsilon_y[Y] + C \]

where \( \varepsilon_x \) and \( \varepsilon_y \) are molar absorptivities, and \( A_t \) is the absorbance at time \( t \). The solvent or background contribution, if any, is represented by \( C \). Values of initial and final readings (for the absorption band of the PLP analogue) are

\[ A_0 = \varepsilon_x[X]_0 + C \]
\[ A_\infty = \varepsilon_y[X]_0 + C, \text{ since } [X]_0 = [Y]_\infty \]
Figure 18. Reaction of apoAAT-MPA with L-glutamate (apoAAT was subtracted)

1) ApoATT, $5.51 \times 10^{-5}$ M, MPA, $3.62 \times 10^{-5}$ M

2) 2 min after the addition of L-glutamate (17 mM)

3) 6.5 min
Figure 19. Reaction of apoAAT-EtMP with L-glutamate (apoATT was subtracted)

1) ApoAAT, $5.53 \times 10^{-5}$M, EtMP, $4.14 \times 10^{-5}$M
2) 2 min after the addition of L-glutamate (17 mM)
3) 24 min 20 sec
4) 43.5 min
5) 130 min
Figure 20. Reaction of E-PLSN with L-glutamate (apoAAT was subtracted)

1) ApoAAT, 5.55 x 10^{-5}M, PLSN, 4.18 x 10^{-5}M
2) 2 min after the addition of L-glutamate (17 mM)
3) 6 min
4) 9.5 min
MOLAR ABSORPTIVITY

MOLAR ABSORPTIVITY ($\times 10^3$)
Figure 21. Reaction of E-PL with L-glutamate (the apoAAT was subtracted)

1) ApoAAT, $5.04 \times 10^{-5}$M, PL, $6.64 \times 10^{-4}$M
2) 30 sec after the addition of L-glutamate ($6 \times 10^{-3}$M)
3) 3 min
4) 6 min 10 sec
5) 9 min 45 sec
Figure 22. Reaction of apoAAT-EtPLP with L-glutamate

1) ApoAAT, $5.53 \times 10^{-5}$ M, EtPLP, $3.44 \times 10^{-5}$ M
2) 2.5 min after the addition of L-glutamate (0.091 M)
3) 16 min
4) 34.5 min
5) 1 hr
Figure 23. Reaction of E-MePLP with L-glutamate

1) ApoAAT, 5.6 x 10^{-5} M, MePLP, 3.55 x 10^{-5} M
2) 2 min after the addition of L-glutamate (0.017 M)
3) 11 min
4) 29.5 min
Table 8. Reaction with L-glutamate at pH 8.3

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration of apoenzyme $x 10^5$ (M)</th>
<th>Concentration of analogue $x 10^5$ (M)</th>
<th>Concentration of L-glutamate (M)</th>
<th>$\lambda_{\text{max}}$</th>
<th>$k_{\text{obs}}$</th>
<th>$A_o - A_{t+o}$</th>
<th>$A_{t+o}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>5.06</td>
<td>3.32</td>
<td>0.017</td>
<td>30.8 kK (325 nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EtMP</td>
<td>5.07</td>
<td>3.80</td>
<td>0.017</td>
<td>23.8 kK (420 nm)</td>
<td>6.7 s$^{-1}$</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.4 kK (318 nm)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PLSN</td>
<td>5.10</td>
<td>3.83</td>
<td>0.017</td>
<td>24.2 kK (413 nm)</td>
<td>36.7 s$^{-1}$</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.8 kK (314 nm)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CMDPL</td>
<td>2.43</td>
<td>2.21</td>
<td>0.012</td>
<td>31.3 kK (319 nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.29$^d$</td>
<td>2.22</td>
<td>0.003</td>
<td>31.3 kK (319 nm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PL</td>
<td>4.90</td>
<td>64.6</td>
<td>0.006</td>
<td>-$^e$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EtPLP</td>
<td>5.53</td>
<td>3.44</td>
<td>0.019</td>
<td>31.8 kK (314 nm)</td>
<td>10 s$^{-1}$</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>MePLP</td>
<td>5.12</td>
<td>3.25</td>
<td>0.017</td>
<td>31.8 kK (314 nm)</td>
<td>25 s$^{-1}$</td>
<td>0.37</td>
<td></td>
</tr>
</tbody>
</table>

*a* Concentrations were calculated after the addition of L-glutamate.

*b* New absorption band(s) appeared after the addition of L-glutamate.

*ck_{obs} refers to the slow reaction, see the text.

*d* The reaction was run in the presence of 0.03 M of phosphite.

*e* The peak was too high to measure.
Figure 24. Pseudo first order plot for the reaction of apoAAT-EtMP with L-glutamate
Figure 25: Pseudo first order plot for the reaction of E-PLSN with L-glutamate
Figure 26. Pseudo first order plot for the reaction of E-EtPLP with L-glutamate
Figure 27. Pseudo first order plot for the reaction of E-MePLP with L-glutamate
Substituting into the rate expression \( X = [X]_o e^{-kt} \) we obtain

\[
A_t = \varepsilon_x [X]_o e^{-kt} + \varepsilon_y [X]_o (1 - e^{-kt}) + C
\]

or

\[
A_t - A_\infty = (\varepsilon_x - \varepsilon_y) [X]_o e^{-kt}
\]

\[
\frac{A_t - A_\infty}{A_0 - A_\infty} = e^{-kt}
\]

Hence, a plot of \( \ln \left( \frac{A_t - A_\infty}{A_0 - A_\infty} \right) \) vs. \( t \) will be linear with slope \(-k\), permitting evaluation of the rate constant without values for \( [X]_o \), \( \varepsilon_x \) and \( \varepsilon_y \). Values of \( k_{obs} \) for the slow reaction were determined and are shown in Table 8. When E-PL \((4.40 \times 10^{-5} M + 9.43 \times 10^{-4} M)\) and L-glutamate \((0.019 M)\) were mixed at pH 8.3, \(3.4 \times 10^{-4} M\) of PL was consumed in 20 sec according to the decreasing absorbance at 25.6 kK, and the active sites of the apoenzyme were still saturated with pyridoxal (similar to Figure 21, except the concentration of PL is higher). Therefore a \( k_{obs} \) for the decay of E-PL can be calculated as following, assuming that the binding of PL to apoAAT and the release of PM from apoAAT were not the rate limiting steps:

\[
PL + E \xrightleftharpoons{k_{obs}}^{} E-PL \xrightleftharpoons{k_{obs}}^{} E-PM \xrightleftharpoons{} E + PM
\]

\[
k_{obs} [E-PL] = 3.4 \times 10^{-4} M/20 \text{ s}
\]

\[
k_{obs} (4.40 \times 10^{-5} M) = 3.4 \times 10^{-4} M/20 \text{ s}
\]

\[
\therefore k_{obs} = 0.4 \text{ s}^{-1}
\]
E-CMDPL was converted to E-CMDPM in 2 min, so fast that no spectrum could be recorded. In the presence of phosphite anion (0.03 M), the binding of CMDPL to apoAAT was inhibited and slow. Immediately after the addition of L-glutamate, the peak at 27.4 kK dropped rapidly, resulting in a broad band centered at 25.6 kK. The transamination was also inhibited and lasted for 30 min. Again, this is a biphasic reaction (Figure 28). However, the slow reaction followed a curve in the pseudo first order plot. The broad band appeared at 25.6 kK upon the addition of L-glutamate implied a mixture of free CMDPL and protonated external Schiff base. Therefore, the inhibition of binding of CMDPL to apoenzyme by phosphite may also contribute to the slow reaction as well as to the slow release of CMDPM.

The slow reaction in Figures 24-27 was extrapolated to intercept the \((A_t - A_\infty)\) coordinate. The value of \((A_t - A_\infty)\) at \(t = 0\) was obtained, representing the value at which an equilibrium of \([\text{reacted E-PLP form}]/[\text{unreacted PLP form}]\) was developed in the fast reaction. The reacted E-PLP form includes E-PMP form and other intermediates. Hence, the equilibrium ratio \([\text{reacted E-PLP form}]/[\text{unreacted PLP form}]\) can be calculated from \((A_\infty - A_{t+\infty})/A_{t+\infty}\) (Table 8).

\(\alpha\)-ketoglutarate (0.013 M) was added to the E-EtPMP solution at pH 8.3 after the transamination was complete, the peak at 31.8 kK decreased with time. The peak at 27.4 kK was buried under the absorption band of \(\alpha\)-ketoglutarate, but an increasing
Figure 28. Pseudo first order plot for the reaction of E-CMDPL with L-glutamate (in the presence of 0.03 M of phosphite)
shoulder still could be seen around 27.4 kK. A positive circular dichroism at 364 nm (27.5 kK) was recovered 57% of that of E-EtPLP. E-MePMP was converted back to E-MePLP by α-ketoglutarate (0.014 M) in a similar way, and 74% of the positive CD at 363 nm was recovered. A striking fact is that, except E-EPA, none of above E-PMP forms possess a positive CD as native E-PMP does. Excess CMDPM (0.11 M) was combined with apoAAT (2.80 x 10^{-5} M) at pH 8.3 and still no positive CD corresponding to 31.3 kK absorption can be seen. Although the concentration of CMDPM needed to combine apoAAT has not yet been established, it seems unlikely that 0.1 M of CMDPM is still not high enough to bind apoAAT. Another sample of E-MePLP was allowed to react with L-glutamate in order to take CD with time at 314 nm immediately after the mixing. There was no obvious increase in CD in the period of 13rd s – 2nd min.

8. Addition of D,L-α-methylaspartate to apoenzyme-analogue complexes

D,L-α-methylaspartate was mixed with E-MPA, E-EtMP, E-PLSN, E-CMDPL, E-PL, E-EtPLP and E-EPA individually at pH 8.3 (Table 9). There were no significant changes in the spectra observed with E-EtMP, PLSN and EtPLP. In the case of E-CMDPL, a small shoulder rose in 23-24 kK region (Figure 29) accompanying a very small negative CD at 415-425 nm. The absorption of E-PL underwent a bathochromic shift (Figure 30) and a very small CD
Figure 29. Reaction of E-CMDPL with D,L-α-methylaspartate at pH 8.3 (apoAAT was subtracted)

1) ApoAAT, 5.11 $\times 10^{-5}$ M, CMDPL, 3.8 $\times 10^{-5}$ M
2) D,L-α-methylaspartate (7.8 $\times 10^{-3}$ M) was added
   apoAAT, 5.0 $\times 10^{-5}$ M, CMDPL, 3.68 $\times 10^{-5}$ M
3) D,L-α-methylaspartate (3.2 $\times 10^{-2}$ M) was added
   apoAAT, 4.48 $\times 10^{-5}$ M, CMDPL, 3.33 $\times 10^{-5}$ M
Figure 30. Reaction of E-PL (5.44 x 10^{-5} M + 9.56 x 10^{-5} M) with D,L-\(\alpha\)-methylaspartate at pH 8.3 (apoAAT was subtracted)

1) D,L-\(\alpha\)-methylaspartate (7.7 x 10^{-3} M) was added, apoAAT, 5.28 x 10^{-5} M, PL, 9.28 x 10^{-5} M

2) D,L-\(\alpha\)-methylaspartate (2.2 x 10^{-2} M) was added, apoAAT, 4.98 x 10^{-5} M, PL, 8.75 x 10^{-5} M

3) D,L-\(\alpha\)-methylaspartate (7.8 x 10^{-2} M) was added, apoAAT, 3.8 x 10^{-5} M, PL, 6.69 x 10^{-5} M
developed at about 430 nm. However, E-MPA and E-EPA underwent a distinct transformation in the 23.0 kK absorption band (Table 9 and Figures 31 and 32). The band increased in height and a negative CD was also induced.

9. Addition of D,L-erythro-β-hydroxyaspartate to apoenzyme-analogue complexes

A 20.4 kK absorption band was observed with E-MPA, E-EtMP, E-PLSN, E-CMDPL and E-PL immediately after D,L-erythro-β-hydroxyaspartate was added. E-EtPLP was the only exception (Table 10, Figures 33-37). The circular dichroism was examined with E-MPA, E-CMDPL, E-PL and E-EtPLP. A negative CD corresponding to the 20.4 kK absorption band was seen with E-CMDPL. The absorption was too low (Figure 37) to relate any negative CD to the 20.4 kK absorption band with E-PL. In all three cases, in contrast to the native enzyme, there was still no positive CD corresponding to the E-PMP forms.

10. Curve fitting

Experimental absorption spectra can be fitted with log-normal distribution curves (105) and can be resolved into individual components. Individual spectra of E-MPA, E-EtMP, E-CMDPL, E-EtPLP, E-MePLP and E-EPA were fitted in order to analyze band position ($\tilde{\nu}_0$), peak height ($\epsilon_0$), band width (W) and skewness (\(\rho\)) (see Figure 38-43).
Table 9. Summary of data for reaction with D,L-α-methylaspartate

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration of apoenzyme x 10^5 (M)</th>
<th>Concentration of analogue x 10^5 (M)</th>
<th>Concentration of D,L-α-methylaspartate (M)</th>
<th>Absorption band λ_max</th>
<th>Negative CD λ_max</th>
<th>ΔA/A x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>4.62</td>
<td>3.12</td>
<td>0.034</td>
<td>23.2 kK (431 nm)</td>
<td>~420 nm</td>
<td>-0.75</td>
</tr>
<tr>
<td>EtMP</td>
<td>2.60</td>
<td>1.98</td>
<td>0.034</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PLSN</td>
<td>5.33</td>
<td>4.12</td>
<td>0.012</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CMDPL</td>
<td>4.48</td>
<td>3.33</td>
<td>0.032</td>
<td>~23.5 kK (426 nm)</td>
<td>~420 nm</td>
<td>-0.38</td>
</tr>
<tr>
<td>PL</td>
<td>3.80</td>
<td>6.69</td>
<td>0.078</td>
<td>Bathochromic shift</td>
<td>~430 nm</td>
<td>too small</td>
</tr>
<tr>
<td>EtPLP</td>
<td>5.31</td>
<td>3.29</td>
<td>0.035</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EPA</td>
<td>3.82</td>
<td>2.95</td>
<td>0.073</td>
<td>23.0 kK (435 nm)</td>
<td>~420 nm</td>
<td>-0.76</td>
</tr>
</tbody>
</table>

a New absorption band appeared after the addition of D,L-α-methylaspartate.

b indicates a partially "buried" peak, or a shoulder.
Figure 31. Reaction of apoAAT-MPA with D,L-α-methylaspartate at pH 8.3

1) ApoAAT, 5.31 x 10^{-5} M, MPA, 3.58 x 10^{-5} M
2) D,L-α-methylaspartate (7.5 x 10^{-3} M), apoAAT, 5.16 x 10^{-5} M, MPA, 3.47 x 10^{-5} M
3) D,L-α-methylaspartate (3.4 x 10^{-2} M), apoAAT, 4.62 x 10^{-5} M, MPA, 3.12 x 10^{-5} M
Figure 32. Reaction of apoAAT-EPA with D,L-α-methylaspartate at pH 8.3

1) ApoAAT, $5.30 \times 10^{-5}$M, EPA, $4.09 \times 10^{-5}$M

2) D,L-α-methylaspartate ($7.6 \times 10^{-3}$M) was added, apo, $5.29 \times 10^{-5}$M, EPA, $4.08 \times 10^{-5}$M

3) D,L-α-methylaspartate ($7.3 \times 10^{-2}$M) was added, apo, $3.82 \times 10^{-5}$M, EPA, $2.95 \times 10^{-5}$M
Figure 33. Reaction of E-MPA with D,D-erythro-β-hydroxy-aspartate at pH 8.3 (apoAAT was substracted) 2 min after the addition of D,L-erythro-β-hydroxyaspartate (0.004 M) apoAAT, 5.42 x 10^{-5} M, MPA, 5.02 x 10^{-5} M
Table 10. Summary of data for reaction with D,L-erythro-β-hydroxyaspartate

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration of apoenzyme x 10^5 (M)</th>
<th>Concentration of analogue x 10^5 (M)</th>
<th>Concentration of D,L-erythro-β-hydroxyaspartate (M)</th>
<th>λ_max b,c</th>
<th>Half time d (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>5.42</td>
<td>5.02</td>
<td>0.004</td>
<td>~20.4 kK(490 nm)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23.2 kK(431 nm)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30.4 kK(319 nm)</td>
<td></td>
</tr>
<tr>
<td>EtMP</td>
<td>3.05</td>
<td>3.54</td>
<td>0.005</td>
<td>~20.4 kK(490 nm)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>~24.0 kK(417 nm)</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>31.4 kK(318 nm)</td>
<td></td>
</tr>
<tr>
<td>PLSN</td>
<td>5.46</td>
<td>4.38</td>
<td>0.004</td>
<td>20.4 kK(490 nm)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24.8 kK(403 nm)</td>
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<td></td>
<td></td>
<td></td>
<td>31.6 kK(316 nm)</td>
<td></td>
</tr>
<tr>
<td>CMDPL</td>
<td>5.38</td>
<td>3.72</td>
<td>0.004</td>
<td>20.4 kK(490 nm)</td>
<td>4</td>
</tr>
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<td></td>
<td></td>
<td>31.2 kK(321 nm)</td>
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<tr>
<td>PL</td>
<td>5.28</td>
<td>9.28</td>
<td>0.009</td>
<td>20.4 kK(490 nm)</td>
<td>2.5</td>
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<td></td>
<td></td>
<td></td>
<td>31.6 kK(316 nm)</td>
<td></td>
</tr>
<tr>
<td>EtPLP</td>
<td>5.31</td>
<td>3.29</td>
<td>0.004</td>
<td>31.8 kK(314 nm)</td>
<td>-</td>
</tr>
</tbody>
</table>

a Concentrations were calculated after the addition of D,L-erythro-β-hydroxyaspartate.

b ~ indicates a partially "buried" peak, or a shoulder.

c New absorption band(s) appeared after the addition of D,L-β-hydroxyaspartate.

d Half time of the absorption band at 20.4 kK, approximately.
Figure 34. Reaction of E-PLSN with D,L-erythro-β-hydroxyaspartate at pH 8.3 (apoAAT was subtracted)

1) ApoAAT, $5.55 \times 10^{-5}$ M, PLSN, $4.45 \times 10^{-5}$ M
2) 2 min after the addition of D,L-erythro-β-hydroxyaspartate (0.004 M)
3) 57 min
4) 2 hours
Figure 35. Reaction with E-EtMP with D,L-erythro-β-hydroxyaspartate at pH 8.3 (apoAAT was subtracted)

1) ApoAAT, $3.10 \times 10^{-5}$ M, EtMP, $3.64 \times 10^{-5}$ M
2) 2.5 min after mixed with D,L-erythro-β-hydroxyaspartate (0.005 mM). ApoAAT, $3.05 \times 10^{-5}$ M, EtMP, $3.54 \times 10^{-5}$ M
3) 2 hrs and 10 min
Figure 36. Reaction of E-CMDPL with D,L-erythro-β-hydroxyaspartate at pH 8.3 (apoAAT was subtracted)

1) ApoAAT, \(5.47 \times 10^{-5} \text{M}\), CMDPL, \(3.78 \times 10^{-5} \text{M}\)

2) 6 min after the addition of D,L-erythro-β-hydroxyaspartate (0.004 M) apoAAT, \(5.38 \times 10^{-5} \text{M}\), CMPDL, \(3.72 \times 10^{-5} \text{M}\)
Figure 37. Reaction of E-PL with D,L-erythro-β-hydroxyaspartate at pH 8.3 (apoAAT was subtracted)

1) ApoAAT, $5.51 \times 10^{-5}$M, PL, $9.56 \times 10^{-5}$M

2) 2.5 min after the addition of D,L-erythro-β-hydroxyaspartate (0.009 M), apoAAT, $5.28 \times 10^{-5}$M, PL, $9.28 \times 10^{-5}$M
Table 11. Band parameters for resolved absorption spectra of E-analogue complexes

<table>
<thead>
<tr>
<th>Analogue</th>
<th>$\nu_0$ (kK)</th>
<th>$\varepsilon_0$ (mol⁻¹ cm⁻¹ x 10⁻³)</th>
<th>W (kK)</th>
<th>$\rho$</th>
<th>$a^\circ$ (km·mol⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>E-MPA</td>
<td>23.43</td>
<td>1.067</td>
<td>4.070</td>
<td>1.269</td>
<td>46.725</td>
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<tr>
<td></td>
<td>27.98</td>
<td>6.128</td>
<td>4.781</td>
<td>1.671</td>
<td>328.301</td>
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<td>E-EtMP</td>
<td>24.20</td>
<td>1.391</td>
<td>3.749</td>
<td>1.275</td>
<td>56.157</td>
</tr>
<tr>
<td></td>
<td>27.48</td>
<td>5.810</td>
<td>4.019</td>
<td>1.475</td>
<td>256.017</td>
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<tr>
<td>E-CMDPL</td>
<td>27.25</td>
<td>8.628</td>
<td>3.940</td>
<td>1.614</td>
<td>378.388</td>
</tr>
<tr>
<td>E-EtPLP</td>
<td>27.41</td>
<td>7.297</td>
<td>3.830</td>
<td>1.460</td>
<td>305.878</td>
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<tr>
<td>E-MePLP</td>
<td>27.41</td>
<td>6.651</td>
<td>3.972</td>
<td>1.480</td>
<td>289.742</td>
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<tr>
<td>E-EPA</td>
<td>27.63</td>
<td>7.441</td>
<td>4.293</td>
<td>1.508</td>
<td>351.413</td>
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</tbody>
</table>
Figure 39. Resolved absorption spectra of E-MPA

X X X observed spectrum

--- resolved individual spectra and fitted sum of the individual lognormals
Figure 40. Resolved absorption spectra of E-EtMP
Figure 41. Resolved absorption spectrum of E-CMPLP

Only the major absorption band was fitted with a lognormal curve.
Figure 42. Resolved absorption spectrum of E-EtPLP

Only the major absorption band was fitted with a lognormal curve.
Figure 43. Resolved absorption spectrum of E-MePLP

Only the major absorption band was fitted with a lognormal curve.
Figure 44. Resolved absorption spectrum of E-EPA

Only the major absorption band was fitted with a lognormal curve.
V. DISCUSSION

A. Organic Synthesis of Pyridoxyl 5'-Phosphate

In general, vitamin B\textsubscript{6} derivatives (especially those modified in the 5'-chain) may be prepared from readily available vitamin B\textsubscript{6} compounds. Several key intermediates have been developed for synthetic schemes, for example, 2\textsuperscript{4},3-0-isopropylidene-2\textsuperscript{5}-pyridoxyl chloride hydrochloride, isopropylideneisopyridoxal and 5'-bromo-5-deoxypyridoxamine. These compounds have found extensive application in the modification of the 5'-positions (108, 103, 98, 109, 97).

The 4-hydroxymethyl group can be re-generated by acid hydrolysis of the isopropylidene. A 4-formyl group can be obtained by oxidation of a 4-hydroxymethyl or a 4-aminomethyl group. Among various oxidants, \textit{MnO\textsubscript{2}} appears to be the most satisfactory. However, in some cases, the oxidation step is difficult and low yields were obtained. The idea of using 5'-
bromo-5-deoxypyridoxamine as a synthetic intermediate was adopted from the well-known model reaction, in which the amino group was transferred from pyridoxamine to glyoxylate catalyzed by aluminum ion (III) (45). Pyridoxamine 5'-thiophosphate was formed from 5'-bromo-5-deoxypyridoxamine and trilithium thiophosphate. Efforts were made to oxidize this compound through non-enzymatic transamination by Khomutov (110) and by the author independently. None of us has succeeded. An attempt to make 5'-bromo-5-deoxypyridoxal via the same scheme led to the isolation of 5'-O-methylpyridoxal (see IV. Results (1)). A possible mechanism is shown below:
This turns out to be analogous to the reaction of pyridoxal-5'-sulfate with apoAAT (85), in which the sulfate was eliminated and a methyl group was generated in the 5'-position.

In many cases, PLP analogues have been isolated as Schiff bases. Schiff bases had been utilized as intermediates for the synthesis of PLP analogues modified in the positions other than 5'-position. It appeared that a pyridoxal Schiff base with a reactive group in the 5'-position would serve as an ideal intermediate for modifying the 5'-position. Therefore, the bromo Schiff base was synthesized (IV. Results (3)). The bromide is so reactive that it can react with many other reagents under mild conditions. p-Toluidine can be hydrolyzed off by acid or base to give back the 4-formyl group. This avoids the problem of oxidation. 5'-Bromo-5-deoxypyridoxal was made from the bromo Schiff base, which can also be a synthetic intermediate if mild conditions are employed.

B. Interactions of Analogues with Apoaspartate Aminotransferase

1. Binding characteristics

Several lines of evidence support the view that the analogues under study, except for (Et)$_2$MP, are bound to apoAAT at the active site, (I). The absorption band of the free analogue at about 25.6 kK was shifted to the 26.4 - 28.0 kK region upon mixing with apoAAT. There was always a positive CD corresponding to this major absorption band, a characteristic of bound PLP. However, the position of the major
absorption band was always shifted to an energy (Table 3) lower than that of the CD maximum. The amount of the shift varies from 13 nm for E-PLSN to 1 nm for E-MPA. This suggests that small amounts of free analogues were still present. A small amount of absorption in the 23 - 24 kK region which decreased with time, may be attributed to a "non-specific binding" (Furbish et al. (84)). The 23 kK shoulder increased with time with PLSN and also with E-CMDPL when CMDPL was in excess. This observation suggests that PLSN was poorly bound to the active site and that some free PLSN was moving into the nonspecific binding site immediately after mixing (2). Experiments in which PLP was utilized in an attempt to displace apoAAT-analogue (IV. Results, (3) and (4)) indicate that the analogues occupied the PLP binding site.

2. Activity

Analогues seemed to bind apoAAT in a nearly normal way, but except for EPA they gave no measurable activity. Although cyanoethyl-PLP activated apoAAT in a prolonged incubation (84), EtPLP did not activate apoAAT. Apparently, the former analogue may be hydrolyzed in its specific way to PLP:

\[
\begin{align*}
\text{CHO} & \quad \text{CH}_2\text{-O-P-O-CH}_2\text{-C-CN} \\
\text{H}_3\text{C} & \quad \text{H}_3\text{C} \\
\text{N}^+ & \quad \text{N}^+ \\
\end{align*}
\]

\[
\begin{align*}
\text{CHO} & \quad \text{CH}_2\text{-O-P-O}^- \\
\text{H}_3\text{C} & \quad \text{H}_3\text{C} \\
\text{N}^+ & \quad \text{N}^+ \\
\end{align*}
\]
The presence of a dissociable α-proton adjacent to CN makes cyanoethyl-PLP a special case compared to EtPLP.

3. Low pH titration

A remarkable feature of the bound analogues (monoanionic), except MPA is that they do not undergo a spectral shift to 23.2 kK at low pH as does native enzyme. The same phenomenon was reported by Furbish et al. (84) with other analogues. They attributed this phenomenon as a result of a shift in the pKå of the protonated imine to below 6.3. Charge effects on this pKå have been reported elsewhere by Jenkins and L. D'Ari (107), Marino et al. (111), Fasella (112), and Furbish et al. (84). For example, glutarate was able to raise the pKå of E-CMDPL to 4.7. Therefore, it is reasonable to assume charge effects of these analogues on the pKå. In fact, the dianionic phosphonate group of MPA is in a close proximity to the imine nitrogen. The spectral change above pH of apo-MPA may be referred to the nonspecific binding characteristic, whereas the pKå of approximately 7.0 is attributed to the protonated imine nitrogen.

4. Transamination

Although none of the analogues (except EPA) displays any activity in the assay, the apoAAT-analogue complexes do turn over with L-glutamate. That is, they undergo a half-transamination reaction.

The reactions of MPA and CMDPL were too fast to follow spectrophotometrically. However, for the other analogues
the reaction was clearly biphasic. Part of the reaction occurred very rapidly and part more slowly. The most striking feature is that, during and after the transamination, none of the amine (PMP) form possesses a positive CD. The circular dichroism is an indicator of an asymmetric environment surrounding the chromophase. The lack of a positive CD is indicative of a different environment from that of the native enzyme. A difference in the orientation of the PMP form of the analogue and/or in the conformation of the protein could account for the CD result. Moreover, PL and PM were found (IV. Results, 7) (81) to come off and on to the apoAAT rapidly during the reaction with amino acid or α-ketoacid. Therefore, a defect in binding ability of the analogue and a consequent inappropriate orientation of the analogue in the active site may well lead to the complete release of its PMP form from the active site. The biphasic kinetics may be interpreted as follows. The first step is a fast reaction representing the real rate of transamination, the second step is a slow reaction in which an equilibrium is reached, following a slow release of the PMP form of the analogue from the apoenzyme.

Fasella and Turano (32) have estimated that the binding free energy ($-\Delta G^\circ$) of the 5'-phosphate group is 60% (7 - 8 kcal/mole) of that (11 - 14 kcal/mole) of PLP molecule and 75% of that (9 - 11 kcal/mole) of PMP molecule. Consequently, a defective binding group in the 5'-position of the analogue should contribute to abortive binding and the release of the PMP form.
Furthermore, apoAAT-CMDPL is seven times more active in the presence of a high concentration of CMDPL than in its absence (84), inferring that the inactivity by CMDPL is partly a result of the weak binding of the PMP forms. Anions have been reported to be effectors for transamination (13). From IV. Results, 7 and Figure 28, the inhibition of binding of CMDPL to apoenzyme by phosphite may also contribute to the slow reaction as well as to the slow release of CMDPM. This fact is another indirect evidence that the PMP form comes off during the reaction. Compatible with this is another observation that phosphate inhibits the transamination between PM and ketoacids catalyzed by apoAAT (82).

Particularly intriguing is the fact that the magnitudes of the values for $k_{\text{obs}}$ are parallel to those of $(A_o - A_{t \to o})/A_{t \to o}$ in the cases of E-EtMP, E-ELSN, E-EtPLP and E-MePLP. The explanation follows below. For the half transamination reaction:

$$E + \text{PLP} \rightleftharpoons \text{E-PLP} \stackrel{k_{\text{obs}}}{\rightleftharpoons} \text{E-PMP} \rightleftharpoons \text{E} + \text{PMP}$$

PLP and PMP represent the aldehyde form and the amine form of the analogue, respectively. For simplicity, intermediates between E-PLP and E-PMP, and the substrate L-glutamate are omitted in the scheme. The value of $(A_o - A_{t \to o})/A_{t \to o}$ reflects the equilibrium ratio between E-PMP (including intermediates) and E-PLP developed in the fast reaction. The fact that the fast reaction can't proceed all the way through and finally
the reaction rate is limited by a slow release of the PMP form, indicates that there are factor(s) which control the thermodynamic stability between E-PMP and E-PLP. These factor(s) are unfavorable to the formation of E-PMP form for E-EtMP, E-EtPLP and E-MePLP (see \( (A_o-A_{t+o})/A_{t+o} \) values in Table 8). It is assumed that the values for k of the analogues are identical since none of their PMP forms can bind properly in the active site on the basis of lacking in CD. Therefore, in the equilibrial state, a higher value of \( (A_o-A_{t+o})/A_{t+o} \) will be associated with a faster drop in the concentration of E-PLP, resulting in a higher value of \( k_{obs} \) for the slow reaction.

It is of interest to note that the size of the 5-chain plays a certain role in the slow reaction. Of the same length of the chain, MePLP is faster than EtPLP, and PLSN is faster than EtMP.

An alternative explanation for the biphasic kinetics is the theory of half of the sites reactivity. Since apoAAT was only 65-75% saturated with analogues, it is possible that two different conformers exist, one is a dimer combined with two molecules of the analogue, another one is a dimer combined with one molecule of the analogue. These two different conformers displayed different reactivities.

It is true that the overall reaction rates of E-EtMP, E-PLSN, E-EtPLP and E-MePLP are slower than that of E-PL. However, whether the 5'-phosphate group has a role in catalysis
is not yet settled on the ground, unless the true transamination rate (fast reaction rate) is measured.

Wada and Snell (81) estimated that the turnover number of apoAAT saturated with pyridoxamine was $25, 10^{-3}$ that of native holoenzyme. It is possible that its true turnover number is higher than what they estimated, because PL which was formed during the course of the reaction, might possibly come off the active site (actually PL was loosely bound to apoAAT), affecting the overall rate of transamination. It would be expected that the turnover number for the half transamination should not be smaller than that for the overall transamination. By contrast, they obtained a turnover number of 10 for the transamination between PM (utilized as a substrate) and α-ketoglutarate catalyzed by apoAAT. It appeared that the rate limiting step for the transamination between PM and α-ketoacid must be either the release of PL from apoAAT or the binding of PM to apoAAT. A turnover number of 24 (0.4 s$^{-1}$) for the transamination between PL and L-glutamate was obtained by the author. Compared to the result of Wada and Snell (81), this indicates a fact that the release of PM from apoAAT is faster than the release of PL from apoAAT.

Another interesting fact is, that D,L-α-methylaspartate caused no significant changes in the spectra observed with E-EtMP, E-PLSN and E-EtPLP, which are monoanionic analogue complexes. This is parallel to the fact that their $pK_a$'s are low and their Schiff base forms are hardly protonated. The only
exception among the monoanionic analogue complexes is E-CMDP, which had a small shoulder in the 23 - 24 kK region. E-PL underwent a bathochromic shift when reacted with D,L-α-methylaspartate. Possibly it is due to the formation of Schiff base of free PL and substrate. However, a very small negative CD was observed with E-PL in the 23 - 24 kK region. E-MPA and E-EPA gave rise to a 23.2 kK peak, similar to that of the native enzyme, when D,L-α-methylaspartate was added. However, all the 23.2 kK absorption bands induced negative CD's while the CD of native enzyme is zero.

E-EtPLP possesses the unique characteristic of not having a 20.4 kK intermediate with D,L-erythro-β-hydroxyaspartate. However, other analogues showed a 20.4 kK absorption band of a small to medium size similar to that of the native enzyme. E-MePLP was not tested with D,L-erythro-β-hydroxyaspartate.

The bridge oxygen of the phosphate ester of PLP seems important for catalysis since E-EPA has only 7% - 10% activity of apo-PLP (83,88). The phosphate ester may interact with the protein residue(s). Because: (1) It has been known that the 5'-phosphate group can be split off by sodium borohydride only when PLP is bound to the enzyme (113). (2) Khurs and Khomutov (114) reported a partial splitting of the S-P bond when E-pyridoxamine-5'-thiophosphate reacted with α-ketoglutarate or a substrate pair. The charge effects of 5'-chain on the activity are even more pronounced. None of two ester analogues
of PLP (Et-PLP and MePLP) displayed detectable coenzymatic activity. The length of the 5'-chain seems also critical for activity when compare MPA and EPA.

In conclusion, the charges on the 5'-chain of analogues seem to affect the physical chemical properties of the active site and the equilibrium among the bound reaction intermediates. The half-transamination reactions of E-EtMP, E-PLSN, E-EtPLP and E-MePLP follow biphasic kinetics, which may be a result of a slow release of the PMP forms of the analogues.
VI. SUMMARY

A possible new intermediate for preparation of 5'-modified pyridoxal 5'-phosphate analogues, N-(2-methyl-3-hydroxy-5-bromomethyl-4-pyridylmethylene) p-toluidine hydrobromide, was synthesized. Other 5'-modified analogues, diethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate, 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonic acid, monoethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate, 5-deoxypyridoxal 5'-sulfonate, 5'-carboxymethyl-5-deoxypyridoxal, monoethyl pyridoxal 5'-phosphate, monomethyl pyridoxal 5'-phosphate, and 5-(4-formyl-3'-hydroxy-2-methyl) pyridylethyl phosphonic acid were also made in our laboratory in order to test with apoaspartate aminotransferase from pig heart muscle.

With the exception of diethyl 5-(4-formyl-3-hydroxy-2-methyl) pyridylmethyl phosphonate, all the analogues bond to apoaspartate aminotransferase. None of them, except the dianionic side-chain analogue, 5-(4-formyl-3-hydroxy-2-methyl) pyridylethyl phosphonic acid displayed detectable coenzymatic activity. There was no positive circular dichroism corresponding to the amine forms of those analogues that had no coenzymatic activity. Apoenzyme-5-(4-formyl-3-hydroxy-2-methyl)pyridylmethyl phosphonic acid has a $pK_a$ value around 7, other apoenzyme complexes with the monoanionic side-chain analogues apparently have $pK_a$ values below the 6.3 of the native holoenzyme.
The charges on the 5'-chain of the analogues seem to affect the physical chemical properties of the active site and the equilibrium among the bound reaction intermediates. The half-transamination reaction of the apoenzyme complexes with monoethyl 5-\((4\text{-}\text{formyl}\text{-}3\text{-}\text{hydroxy}\text{-}2\text{-}\text{methyl})\) pyridylmethyl phosphonate, 5-deoxypyridoxal-5'-sulfonate, monoethyl pyridoxal 5'-phosphonate and monomethyl pyridoxal 5'-phosphate follow biphasic kinetics, which may be a result of a slow release of the amine forms of the analogues.
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