Aspartate aminotransferase: interaction with pyridoxal phosphate analogs and analysis of electronic absorption spectra

In-yu Yang

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Aspartate aminotransferase: Interaction with pyridoxal phosphate analogs and analysis of electronic absorption spectra

by

In-yu Yang

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DEDICATION

To the people of Taiwan.
INTRODUCTION

As part of a continuing effort to elucidate the underlying mechanism of enzymatic transamination catalyzed by aspartate aminotransferase, this work was undertaken with the hope of contributing to the understanding of the events taking place at the enzyme active site during the course of catalysis.

To that end a number of PLP analogs were employed to probe the active center. These include the 4-vinyl analog of PLP (VPLP), cis- and trans-methyl VPLP (cis- and trans-Me-VPLP), the 4-acetylenic analog of PLP (APLP), pyridoxamine thiophosphate (PM thiophosphate) and pyridoxal sulfate (PL sulfate). In addition, band shape analysis of electronic absorption spectra has been applied to the protein band of the enzyme spectrum. Particular emphasis is placed on the examination of the vibronic fine structure. The procedure is relatively new and strictly empirical. Effort was made to demonstrate the usefulness of the method in detecting perturbation in aspartate aminotransferase.
REVIEW OF PERTINENT LITERATURE

Aspartate Aminotransferase

The enzyme L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1.) has been variously called glutamic-aspartic (or glutamate-aspartate) transaminase, glutamic-oxaloacetic (or glutamic-oxalacetic) transaminase, aspartate-glutamate transaminase, glutamate-aspartate aminotransferase, aspartic transaminase and aspartate aminotransferase. To be in line with current usage and for the sake of internal consistency, the name aspartate aminotransferase (AAT) will be used throughout this work.

History

Although the intermolecular transfer of an amino group from an α-amino acid to an α-keto acid was first observed by Herbst and Engel (1934), the history of biochemical transamination did not begin until 1937 with the announcement of its discovery in muscle tissues by Braunstein and Kritzmann (1937a, 1937b, 1937c, 1937d, 1937e). In the meantime, the activity of vitamin B₆ was defined (György, 1934), and its isolation (Lepkovsky, 1938), structure determination (Stiller et al., 1939; Harris et al., 1939; Kuhn and Wendt, 1939; Kuhn et al., 1939a, 1939b, 1939c) and synthesis (Harris and Folker, 1939; Kuhn et al., 1939c) soon followed. Subsequently, Snell (1945) demonstrated non-enzymatic, reversible interconversion of pyridoxal (PL) and pyridoxamine (PM) and postulated (Snell, 1944) their participation in biological transamination. While the mechanism of enzyme-catalyzed transamination involving pyridoxal phosphate...
(PLP) and pyridoxamine phosphate (PMP) was proposed as early as 1947 (Schlenk and Fisher, 1947), the first comprehensive theory on the general mechanism of pyridoxal catalysis was formulated in 1953 by Braunstein and Shemyakin (1953). Independently a very similar scheme, based on model studies was proposed by Metzler et al. (1954). The Braunstein-Snell hypothesis still encompasses all known reactions in which PLP plays a catalytic role. The extensive literature regarding PLP, PLP-dependent enzymes, especially aspartate aminotransferase, and pertinent model systems has been the subject of many reviews (Snell, 1958; Braunstein, 1960; Snell, 1962; Guirard and Snell, 1964; Fasella, 1967). In addition proceedings (Snell et al., 1963; Harris et al., 1964; Snell et al., 1968; Yamada et al., 1968) of conferences on these topics have been published.

More recently, the chemistry of PL and PLP analogs (Snell, 1970; Korytnyk and Ikawa, 1970; Florentiev et al., 1970; Johnson and Metzler, 1970). Schiff base intermediates (Snell and Di Mari, 1970), structural and dynamic aspects of transamination (Ivanov and Karpeisky, 1969; Braunstein, 1970) and stereochemistry of PL catalysis (Dunathan, 1971) have also been summarized.

**Primary structure of AAT**

Large quantities of highly purified pig heart cytoplasmic AAT were first made available by Jenkins et al. (1959). Further purification led to the separation of the enzyme into at least three starch-gel electrophoretically distinctive subforms (Martinez-Carrion et al., 1967). Studies on the primary structure were thus made possible.
The amino acid composition of the enzyme containing all the subforms (Turano et al., 1963) revealed no unusual features. Later studies (Martinez-Carrion et al., 1967; Banks et al., 1968), while confirming the previous results, established that the amino acid composition of the three major subforms is essentially identical. They further demonstrated that the least negatively charged subform, the α subform is the enzymatically most active species.

Work on the amino acid sequence of AAT has been carried out in Russia, Britain and Italy. Employing chymotryptic, restricted and complete tryptic digestion and cyanogen bromide cleavage of carboxymethylated and maleoylated-and-carboxymethylated AAT, Ovchinnikov et al. (1971a, 1971b, 1972a, 1972b) obtained a partial sequence containing 383 amino acid residues. Similar work (Doonan et al., 1972), using trypsin and pepsin on maleoylated-and-carboxymethylated and aminoethylated enzyme, identified twenty composite peptides containing 400 amino acid residues. Recently Ovchinnikov et al. (1972c, 1973) announced the elucidation of the complete sequence of AAT.

The sequence determination ended the controversy over the molecular weight of the enzyme (Jenkins et al., 1959; Polyanovsky and Shpikiter 1962; Martinez-Carrion et al., 1967; Banks et al., 1968; Feliss and Martinez-Carrion, 1970). Determination of AAT three-dimensional structure by X-ray crystallography now awaits the growth of suitable crystals.
Essential residues

A number of amino acid residues in AAT are of critical importance to the catalytic function of the enzyme. Selective modification of these residues leads to the inactivation of the enzyme. In many instances, the degree of inactivation is closely related to the extent of destruction of these residues.

Lysine  The cofactor PLP was first demonstrated to be linked to the \( \epsilon \)-amino group of a lysine residue by reduction of the imine bond to an acid-stable linkage with sodium borohydride, followed by proteolytic and acid hydrolysis and identification of the isolated \( \epsilon \)-pyridoxyllysine (Hughes et al., 1962). Subsequently a chymotryptic peptide was erroneously reported to have the sequence \((\epsilon\text{-pyridoxyl})\)-Lys-Ser-Asx-Phe (Polyanovsky and Keil, 1963). Morino and Watanabe (1969) redetermined the sequence of this active site chymotryptic peptide and showed it to be Ser-(\( \epsilon \)-phosphopyridoxyl)-Lys-Asn-Phe. The latter finding was confirmed by later studies (Ovchinnikov et al., 1971a; Doonan et al., 1972). Elucidation the enzyme amino acid sequence (Ovchinnikov et al., 1972c, 1973) places the lysine residue at position 258. Lysine-258 whose \( \epsilon \)-amino group forms an "internal" aldimine with the coenzyme PLP can perhaps be considered the most essential residue of all. Its function in keeping the bound coenzyme in an aldimine linkage is important in view of the observation that the rate of nucleophilic attack on an aldimine is several orders of magnitude greater than that of a similar attack on a corresponding free aldehyde (Cordes and Jencks, 1962). The \( \epsilon \)-amino group has also
been postulated to participate in the reversible protonation and deprotonation of $\alpha_C$ and $\alpha_C$ of AAT-substrate complexes (Avling et al., 1968; Ivanov and Karpeisky, 1969).

Histidine Aspartate aminotransferase contains eight histidine residues per monomer (Turano et al., 1963; Martinez-Carrion et al., 1967; Banks et al., 1968; Stankewicz et al., 1971; Ovchinnikov et al., 1973). The imidazole group of one of the histidine residues has been shown by various groups of researchers to be of critical importance to the function of the enzyme.

Martinez-Carrion et al. (1967) irradiated AAT in the presence of methylene blue or Rose bengal red and showed that AAT undergoes a loss of activity which follows first order kinetics down to 90% inactivation. Amino acid analysis of native and photo-oxidized enzyme showed that approximately two histidine residues were destroyed. The number of tyrosine and tryptophan residues remained essentially unchanged. The bound co-enzyme, too, was found to be unaffected by this treatment. Furthermore, the first-order rate constant for the destruction of the histidine residues is equal to that of the loss of enzymatic activity, suggesting that one of these histidine residues is critically connected with the function of the enzyme. Peptide maps, stained with diazotized sulfanilic acid, of chymotryptic digests of native and photo-oxidized AAT (Vorotnitskaya et al., 1968) showed a considerable decrease in the amounts of two histidine-containing peptides, consistent with irradiative destruction of imidazole groups. These latter authors also reported protection against photo-inactivation of the enzyme by PLP, quasi-substrate $\beta$-methylaspartate
and substrate pairs.

Fluorescence studies (Burridge and Churchich, 1970) of the photo-inactivation of the enzyme, employing lumichrome as the photo-sensitizer, suggest that the photo-dynamic action of lumichrome operates over large distances since low concentrations of the photo-sensitizer were sufficient to cause enzyme inactivation and the photo-sensitizer did not appear to bind to the enzyme in the process. However, it is not known whether methylene blue or Rose bengel red operates in the photo-oxidation of the enzyme in the same way. The mechanism underlying photo-oxidation and the consequent inactivation of AAT was studied in detail by Peterson and Martinez-Carrion (1970). Photo-inactivation was shown clearly not to result from alteration in substrate affinity since dissociation constants of various substrates and inhibitors were even lower with photo-oxidized than native enzyme. Photo-inactivated enzyme is capable of forming all of the visible enzyme-substrate intermediates characteristic of the half-transamination reaction, except for the semiquinoid intermediate. This inability correlates with reduced exchange of the \( \alpha \)-hydrogen of the amino acid with tritium from tritiated water and a decrease in the rate of the forward reaction. Thus the primary defect after photo-oxidation of the critical histidine residue occurs in the removal of the \( \alpha \)-hydrogen from the enzyme-substrate Schiff base.

Additional support for the involvement of a critical histidine residue in the functioning of the enzyme comes from a flourine-19 nuclear magnetic resonance study of the binding of trifluoroacetate anion (Cheng and Martinez-Carrion, 1972). Taking advantage of differences in chemical
shifts arising from free and bound trifluoroacetate, the authors reported an anion binding site on the enzyme with a pK of 6.2. Controlled oxidation of the enzyme, which presumably destroys one to two histidine residues, results in the elimination of the site. Since succinate and α-methylaspartate compete with trifluoroacetate for the site, a histidine seems to be at the enzyme active site.

Tyrosine Tetranitromethane (TNM), a reagent presumably rather specific for tyrosine residues was first used by Turano et al., (1968) to react with AAT. Cysteine as well as tyrosine residues were found to be readily modified. Concurrently, the enzyme was rapidly inactivated. By blocking and de-blocking presumably all the reactive cysteine residues before and after the nitration reaction, it was concluded that nitration of approximately one tyrosine residue alone can account for nearly all the inactivation observed, thus pointing out the importance of the tyrosine residue.

Christen and Riordan (1970) reported that one tyrosine residue in AAT becomes unusually reactive toward TNM in the presence of the substrate pair, glutamate and α-ketoglutarate. Concomitant with the nitration of the tyrosine residue, the enzymatic activity is abolished. However, in the absence of substrate, neither the aldimine nor the amine form of the enzyme is inactivated. In the presence of competitive inhibitors, or either substrate alone, only slight inactivation occurs. Thus, susceptibility of the functional tyrosine residue toward nitration is greatly enhanced during the catalytic process. The term "syncatalytic!"
was coined to characterize this chemical modification which occurs synchronously with the catalytic process. Peptides containing this tyrosine residue have been isolated (Polyanovsky et al., 1972) and the residue identified as Tyr-40 in the primary structure of AAT.

Although it seems beyond doubt that a tyrosine residue is syncatalytically modified by TNM, tyrosine is not the only kind of residue derivatized. Contradicting an earlier assertion (Christen and Riordan, 1970), Birchmeier and Christen (1971) reported that one cysteine residue is also syncatalytically modified by N-ethylmaleimide (NEM). And indeed TNM and a host of other reagents also modify the cysteine residue (Birchmeier et al., 1973). Furthermore, it is becoming increasingly clear that modification of the cysteine alone is sufficient to cause up to 95% inactivation of the enzyme, thus making the importance of the tyrosine residue ever more ambiguous.

However, according to Turano et al., (1971), TNM simultaneously modifies one cysteine and one tyrosine residues to yield greater than 90% loss of the enzymatic activity. Furthermore, it was maintained that the inactivation correlates better with the modification of the tyrosine rather than the cysteine residue, thereby upholding the critical function of the tyrosine residue.

Most recently, Bocharov et al., (1973) reported to have been able to modify each of the two residues singly and claimed that complete inactivation of the enzyme requires modification of both the tyrosine and the cysteine residues. However, it was acknowledged that derivatization of the cysteine alone leads to 95% loss of the enzymatic activity.
(not considered complete inactivation) compared to 50% when the tyrosine residue alone is modified.

Obviously, the controversy regarding the essentiality of a tyrosine residue in AAT comes from the lack of stringent specificity of the chemical modifiers employed.

Circular dichroism (CD) of AAT in the region of 33.3-33.9 kK has also been used as the basis for postulating the existence of an essential tyrosine residue.

According to Ivanov et al., (1967), the negative CD band with extremum at 33.3-33.9 kK does not originate from the coenzyme but is induced in some amino acid residue of the apoenzyme upon its association with the coenzyme. It was argued that a tyrosine residue with an ionized phenolic group can account for the position of the extremum and the shape of the dichroic band observed. It was maintained that tryptophan, the only other amino acid that absorbs in the same spectral range as the tyrosyl anion, has a two-fold broader absorption band and thus cannot be the source of the optical activity. Consequently, it was contended that there exists in the active site of AAT a tyrosine residue and the phenolic group was further postulated to play an essential role during the course of catalysis (Ivanov and Karpeisky, 1969; Braunstein, 1970).

Cysteine Early purification of AAT involved the use of maleate buffer (Jenkins et al., 1959) which was shown by Turano et al., (1964) to react with thiol groups of the enzyme forming S-(α, β-dicarboxyethyl) derivatives, thus reducing the number of titrable sulfhydryl groups.
This finding resolved the discrepancy (Polyanovsky and Torchinsky, 1963; Torchinsky, 1964; Turano et al., 1963) regarding the content of thiol groups in the enzyme. Nevertheless, the maleate-reacted enzyme showed no decrease in specific activity, indicating that the thiol groups derivatized are not essential for the enzyme activity. It has now been established that each subunit (M.W. 46,500) has five sulfhydryl groups (Martinez-Carrion et al., 1967; Torchinsky and Sinitsyna, 1970; Stankewicz et al., 1971). With the elucidation of the complete amino acid sequence (Ovchinnikov et al., 1973), the number of cysteine residues in the enzyme seems beyond doubt.

Various reagents, such as iodoacetic acid, iodoacetamide, β-bromopyruvate, maleic acid, NEM, 5,5-dithiobis (2-nitrobenzonate) (DTNB), silver nitrate and p-chloromercuri benzoate (p-CMB), have been employed to modify the thiol groups of AAT. Although minor incongruities exist among the numerous reports, the general finding points to the existence of several classes of thiols in the enzyme. Two (groups I and II) of the five thiol groups can be easily modified without any loss in enzyme activity. Two (groups IV and V) appeared to be fully buried and inaccessible to thiol reagents in the native enzyme. One (group III) seems relatively non-reactive and functionally important (Cournil and Arriodupont, 1971; Torchinsky et al., 1972; Birchmeier et al., 1973).

Thiol group III was shown to become two orders of magnitude more reactive toward NEM in the presence of the substrate pair, glutamate and α-ketoglutarate (Birchmeier and Christen, 1971). The modification of
this particular sulfhydryl group leads to 95% inactivation of the enzyme. The process of inactivation is accompanied by a considerable impairment of the enzyme's affinity for its substrate analogs and presumably the true substrates themselves (Torchinsky and Sinitsyna, 1970). Based on the close correspondence among the syncatalytic modification of this thiol group, the nearly complete inactivation of the enzyme and the decreased affinity for the substrates, it has been postulated that the sulfhydryl group is essential and is situated in the proximity of the active site of the enzyme. Independently, Birchmeier et al. (1972) and Torchinsky et al. (1972) each isolated a peptide containing this critical cysteine residue. After obtaining the amino acid composition and determining the amino and/or carboxyl terminals, it was possible to show that the cysteine residue is the 390th residue of the enzyme now that the complete primary structure is known.

More recently, Birchmeier et al. (1973) have compiled extensive data on syncatalytic modification of thiol group III. Although derivatization with NEM, DTNB, TNM, glutathione, sulfide, mercaptoethanol or methyl mercaptan markedly reduce the enzymatic activity, de-blocking of DTNB-modified thiol group III fully reactivated the enzyme. Moreover, the enzyme modified with cyanide was found to be 60% enzymatically active. Apparently it is the bulk or charge of the substituent on the sulfhydryl group that leads to AAT inactivation. Thus, contrary to its essentiality claimed previously, thiol group III, as a free sulfhydryl moiety, seems not to have an essential role in either catalysis or maintenance of an active enzyme conformation.
Mechanism of transamination

Many reviews covering the enzymatic mechanism of transamination have been published (Meister, 1962; Braunstein, 1960, 1964, 1970; Guirard and Snell, 1964; Ivanov and Karpeisky, 1969; Snell and Di Mari, 1970). It suffices to summarize the salient points.

The first step (reaction 1 in Figure 1) in the mechanism is the transfer of a proton from the amino acid to the enzyme-coenzyme aldimine; this is necessary before the Schiff base between the coenzyme and the substrate can be formed. This protonated aldimine (I) then undergoes transaldimination (reactions 2 and 3 in Figure 1) to form a protonated Schiff base (III) presumably via a tetrahedral intermediate (II). This is followed by the removal of a proton to yield the deprotonated enzyme-substrate aldimine (IV). That only the basic form of the enzyme interacts with amino acids has been demonstrated by pH dependence studies (Fasella et al., 1966). The existence of the intermediates III and IV largely hinges on the formation of 23.0-2 and 27.6-8 \( \text{kK} \) absorbing species first observed in the interaction of \( \alpha \)-methylaspartate and the enzyme by Jenkins et al., (1959) spectrophotometrically. Equilibrium studies of AAT-\( \alpha \)-methylaspartate complex (Fasella et al., 1966) and temperature jump experiments on the enzyme in the presence of each substrate pair (Fasella and Hammes, 1964) support the formation of such complexes on the catalytic pathway of AAT. The following step in the course of transamination is the removal of the substrate \( \alpha \)-proton leading to the formation of a semiquinoid structure (V) (reaction 5 in Figure 1). Conversion to the ketimine (VI) which is another tetrahedral intermediate
Figure 1. Mechanism of transamination. Wavenumbers designate absorption maxima. Square box symbolizes apoAAT and is used only once for clarity.
(reaction 6 in Figure 1), occurs by protonation of the $4^\prime$-carbon. Hydrolysis of the ketimine (VI) and dissociation of the keto acid product from the enzyme (reaction 7 in Figure 1) terminates the half-reaction and leaves the enzyme in the amine form. Addition of a keto acid reverses the course of the reaction and reconvert the enzyme to its aldimine form thereby completing a cycle of transamination. Jenkins (1961) first observed the striking spectrum of the complex of AAT and enythro-β-hydroxyaspartate with its absorption maximum at 20.4 kK and postulated it to possess the structure of V. Since the pseudo-substrate can undergo loss of its α-proton, Jenkins' observation was a major contribution to the elucidation of the mechanism of enzymatic transamination past the stage of extraction of the α-proton. Other lines of evidence supporting the remainder of the reaction scheme came from fast reaction kinetics (Fasella and Hammes, 1964) and equilibrium studies (Jenkins, 1964). The recent demonstration of a spectrum possessing a high-intensity, fine-structured absorption maximum at 20.5 kK (Matsumoto and Matsushima, 1972) in a non-enzymic system and the first successful isolation of a 1,4-dihydropyridine formed in the reaction mixture of pyridoxal and diethyl aminomalonate (Abbott and Bobrik, 1973) lend additional credence to the postulated existence of intermediate V.

Although the main scheme of enzymatic transamination as summarized above has been widely accepted, various aspects of the reaction are still subjects of speculation. Among them is the function of the 5'-phosphate moiety. Furbish et al. (1969) consider the possibility of the group's direct participation as a proton donor and acceptor in the catalytic
pathway. However, Snell (1970) dismisses the 5'-phosphate group as a general requirement for enzymatic transamination in view of the following observations. First of all, pyridoxamine-pyruvate transaminase (Ayling and Snell, 1968a, 1968b) catalyzes the reversible transamination of pyridoxal and pyridoxamine at an overall rate similar to that of pyridoxal 5'-phosphate-dependent AAT. Secondly, the apoenzyme of AAT catalyzes transamination reactions with free pyridoxal or pyridoxamine (Wada and Snell, 1962a, 1962b). However, the overriding importance of the 5'-phosphate group cannot be overlooked. The apoAAT-catalyzed transamination reactions are of markedly lower rates and have a higher $K_M$ value for pyridoxamine compared to holoAAT, illustrating that the 5'-phosphate group, in the coenzyme-apoenzyme interaction, permits the optimal alignment of catalytic groups at the enzyme active site.

Another postulation on enzymatic transamination represents an attempt to correlate the overall, three-dimensional spatial relationship to the functional groups on the apoenzyme, the coenzyme and the substrate molecules. In the so-called "stabilization-orientation-change of conditions by positioned change" hypothesis, Ivanov and Karpeisky (1969) and Braunstein (1970) contend that the state of protonation of the groups at the active site varies for different stages of the reaction; at each stage, the groups may be protonated in such a way as to facilitate the next stage. A feature of this hypothesis is a 40-degree rotation of the coenzyme in initial tetrahedral intermediate (II)
(Figure 1) along the C_2-C_5 axis, and another such rotation, now in the opposite direction, in the second tetrahedral intermediate (VI). However, 2-substituted analogs (Morino and Snell, 1967; Bocharov et al., 1968), even as bulky as 2'-propylPLP, show considerable coenzyme activity. In view of this wide latitude in spatial tolerance at the 2-position, Snell (1970) questions the validity of the pivotal role of the 2-methyl group.

**Stereochemical aspects of transamination**

The stereochemistry of transamination and other reactions catalyzed by PLP-dependent enzymes has been extensively reviewed by Dunathan (1971). Only those aspects bearing directly on AAT are discussed here.

Explaining the labilization of the sigma bonds around the α-carbon of an amino acid substrate in PLP-catalyzed reactions, Dunathan (1966) first postulated that the bond to be broken should lie in a plane perpendicular to that of the cofactor-imine pi system. This conformation should minimize the energy of the transition state for bond breaking by allowing maximum sigma-pi overlap between the breaking bond and the ring-imine pi system. It is also the conformation which is closest in its geometry to the planar semiquinoid intermediate (V), and thus molecular motion in approaching the transition state is minimized.

Thus, in the key step of enzymatic transamination, the aldimine (IV)-ketimine (VI) tautomerization (reactions 5 and 6 in Figure 1), the conformation about the single bonds C_α-N in IV and C_4'-N in VI must be such that the C-H bond to be broken lies perpendicular to the plane of
the double bond pi system as shown in Figure 1. For each intermediate (IV or VI), two such conformations exist, one of which has the C-H bond above the pi system and the other below. In addition to these two conformations, the other variables in the stereochemistry of enzymatic transamination are the configuration of the proton added to C'\textsubscript{4} of PLP (or removed from C'\textsubscript{4} of PMP) and the stereochemistry of proton transfer.

The discovery that apoAAT catalyzes the reversible transamination of non-phosphorylated PL and PM (Wada and Snell, 1962a, 1962b) paved the way for answering the question on the configuration. It was shown (Dunathan, et al., 1968a) that, in the course of transamination, C'\textsubscript{4}-d\textsubscript{2}-PM in H\textsubscript{2}O loses only one deuteron and C'\textsubscript{4}-h\textsubscript{2}-PM incorporates only one deuteron in D\textsubscript{2}O. Kinetic isotope effect measurements showed that these two products are enantiomeric, disymmetric C'\textsubscript{4}-d\textsubscript{1}-PM's (Dunathan, et al., 1968b). These results demonstrated the stereospecificity of protonation at C'\textsubscript{4}. The absolute symmetry of protonation at C'\textsubscript{4} was established by observing the rates of transamination of partially asymmetrically synthesized samples of C'\textsubscript{4}-d\textsubscript{1}-PM of known absolute configuration. The sample containing mostly (S)-C'\textsubscript{4}-d\textsubscript{1}-PM consistently showed a much larger isotope effect in transamination than those containing excess (R)-C'\textsubscript{4}-d\textsubscript{1}-PM, thus it is the pro-S proton of PM which is enzyme labile. This assignment of absolute symmetry was confirmed more decidedly by Besmer and Arigoni (1969). Identical configuration of protonation and deprotonation at C'\textsubscript{4} has also been observed in other transamination reactions catalyzed by pyridoxamine-pyruvate transaminase (Ayling et al., 1968), α-dialkyl
aminotransferase (Bailey, et al., 1970) and serine hydroxymethylase (Voet et al., 1973). Since the imine nitrogen hydrog en-bonds with the 3-phenolic group of the ring (Figure 1), the pro-S configuration dictates that the enzyme-labile proton at C'$_4$ occupies the si (rather than re) face of the cofactor plane (VI, toward the reader).

The stereochemistry of proton transfer was studied, with pyridoxamine-pyruvate transaminase (Ayling et al., 1968) and presumably applies to AAT. By conducting the transamination reaction in D$_2$O, L-2d$_1$-alanine was prepared. When this compound was used with PL as the substrate for the transamination reaction in H$_2$O, significant amounts (4%) of deuterium were transferred to the PM formed, demonstrating the transfer of the C$_\alpha$ proton of the amino acid to C'$_4$ of the product PM. Subsequently it was found that transfer of a proton from L-2h$_1$-alanine to PM in D$_2$O gave a much higher efficiency (50%) (Dunathan, 1971), apparently due to reduced exchange with the medium. These results provide strong evidence for the existence of an intramolecular i,3-prototropic shift (IV $\leftrightarrow$ V $\leftrightarrow$ VI) on the same side (si face) rather than on different sides of the cofactor plane. The establishment of this cis transfer thus defines the conformation around C$_\alpha$-N bond in IV. The stereochemistry thus far described confines the configurations and conformations of the aldimine (IV) and the ketimine (VI) to those shown in Figure 1. Spatial arrangements of other intermediates can be inferred from those of IV and VI.

The high efficiency of proton transfer and the low efficiency of deuteron transfer as described above implies that the proton transferred
becomes equivalent to several other protons during transfer, suggesting the possibility that some apoenzyme side chain, e.g. ε-amino group of a lysine residue, acts as the carrier for the transfer.

**PLP analog studies**

Analogs of PLP with modification at every position of the molecule have been synthesized and many tested with PLP-dependent enzymes, including AAT. Comprehensive reviews have been compiled by Snell (1970) and Korytnyk and Ikawa (1970). The analogs most pertinent to this work are those modified at the 4- and 5-position (Table 1).

The binding of PNP and 4-deoxy-PNP demonstrates that Schiff base formation is not a requirement for coenzyme binding.

It appears that some negative charge at the 5-position is required for the binding of the analogs to apoAAT. Although the binding of 5-deoxy-PL seems to be real, it occurs only slowly (Furbish et al., 1969).

A number of analogs with modifications at the 5-position possess significant coenzyme activity. Examination of these substituents suggests spatial tolerance in the vicinity of C\(^5\) and in the length of the side chain. For AAT at least, two dissociable protons seem important at the end of the 5-substituent. The activity of cyanoethyl-PLP apparently comes from free PLP derived from the hydrolysis of the analog (Furbish et al., 1969). Coenzyme activity of PL-5'-methylene-phosphonate and the 5-ethenylphosphonate analog of PLP was reported as preliminary results (Hullar, 1969). It was the trans isomer of the latter analog that was used to activate apoAAT. Whether the cis analog is active as coenzyme remains to be seen.
Table 1. Coenzyme activity of 4- and 5-analogs of PLP

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Side chain(^a)</th>
<th>Coenzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNP(^b)</td>
<td>4-CH(_2)OH</td>
<td>None. Binding observed</td>
</tr>
<tr>
<td>4-deoxy-PNP(^b)</td>
<td>4-CH(_3)</td>
<td>None. Binding observed</td>
</tr>
<tr>
<td>5-deoxy-PL(^c)</td>
<td>5-CH(_3)</td>
<td>None. Binds weakly</td>
</tr>
<tr>
<td>5-deoxy-PL-5(^{\prime})-phosphonate(^c)</td>
<td>5-CH(_2)PO(_3)(^=)</td>
<td>None. Binds rapidly</td>
</tr>
<tr>
<td>PL-5(^{\prime})-Me-phosphonate(^c)</td>
<td>5-CH(_2)OP(CH(_3))(_2)O(^-)</td>
<td>None. Binds rapidly</td>
</tr>
<tr>
<td>5(^{\prime})-Me-PLP(^c)</td>
<td>5-CH(CH(_3))OPO(_3)(^=)</td>
<td>3%</td>
</tr>
<tr>
<td>5(^{\prime})-CM-5(^{\prime})-deoxy PLP(^d)</td>
<td>5-CH(_2)CH(_2)COO(^-)</td>
<td>None. Binds rapidly</td>
</tr>
<tr>
<td>Cyanoethyl-PLP(^c)</td>
<td>5-CH(_2)OPO(_3)(CH(_2)CH(_2)CN)(^-)</td>
<td>Variable</td>
</tr>
<tr>
<td>5(^{\prime})-homo-PLP(^d)</td>
<td>5-CH(_2)CH(_2)OPO(_3)(^=)</td>
<td>1%</td>
</tr>
<tr>
<td>PL-5(^{\prime})-methylenephosphonate(^e)</td>
<td>5-CH(_2)CH(_2)PO(_3)(^=)</td>
<td>7%</td>
</tr>
<tr>
<td>5-ethenylphosphonate analog of PLP</td>
<td>5-CH=CHPO(_3)(^=)</td>
<td>7%</td>
</tr>
</tbody>
</table>

\(^a\)Only those differing from PLP are shown.

\(^b\)Meister et al., (1954).

\(^c\)Furbish et al., (1969).

\(^d\)Fonda (1971).

\(^e\)Hullar (1969).
Near-ultraviolet absorption of protein molecules originates from tryptophan, tyrosine, phenylalanine and cystine residues. In fact, absorption spectra of many proteins can be closely approximated by those of model compounds containing equal residue concentrations of the four residues (Edelhoch, 1967). Since disulfide absorption possesses no vibronic fine structure (Metzler et al., 1972), the analysis of fine structure of protein spectra is concerned with the three aromatic residues. A conventional method in such analysis is the solvent perturbation method of difference spectroscopy (Herskovits and Sorensen, 1968a, 1968b). However, comparison of difference spectra and fine structure plots obtained by lognormal fitting (Metzler et al., 1972) is difficult. Thus, this review is mostly confined to vibronic fine structure directly measurable from electronic absorption spectra and circular dichroic (CD) recordings.

Phenylalanine

Horwitz et al., (1969) cooled phenylalanine and its derivatives to 77°K for examination of the fine structure in both CD and absorption spectra of these compounds. The 0-0 and 0 + 520 cm⁻¹ transitions occurred in both CD and absorption spectra, whereas a 0 + 180 cm⁻¹ band appeared only in CD. These three transitions start progressions with 930 cm⁻¹ spacing to higher energies. Thus, vibronic fine structure of N-acetyl-L-phenylalanine amide located at 37,38, 37.59, 37.92, 38.31, 38.53 and 38.83 kk can be assigned to the transitions 0-0, 0 + 180, 0 + 520,
0 + 930, 0 + 180 + 930 and 0 + 520 + 930 cm\(^{-1}\) respectively. The 930-cm\(^{-1}\) vibration does not affect either the sign or intensity of CD. In contrast, the 180- and 520-cm\(^{-1}\) vibrations always altered the CD intensity relative to that of the 0-0 band. In many cases, these vibrations also reverse the CD sign.

The advantage of liquid nitrogen cooling is the sharpening of the fine structure bands, thus making resolution far better than at 297\(^0\)K. Cooling also shifts the bands to slightly higher energies. The total rotatory strength increases three- to eight-fold upon cooling, suggesting that a conformational equilibrium exists at 298\(^0\)K.

**Tryptophan**

Near-ultraviolet CD and absorption bands of tryptophan residues arise from two overlapping electronic transitions known as the \(1L_a\) and \(1L_b\) transitions (Weber, 1960).

Many model compounds including N-acetyl-L-tryptophanamide, 3-methylindole, N-stearyl-L-tryptophan n-hexyl ester and L-tryptophan have been studied in various solvents (Strickland \textit{et al.}, 1969; Strickland \textit{et al.}, 1970; Strickland \textit{et al.}, 1971).

Low-temperature techniques were also applied here. In CD studies, the effect of temperature upon the rotatory strength at 77\(^0\)K is 18 times greater than that at 298\(^0\)K, which appears to indicate a high degree of conformational mobility at 298\(^0\)K. In contrast, chymotrypsinogen shows only a 25\% intensification, suggesting the rigidity of tryptophan residues in the protein (Strickland \textit{et al.}, 1969).
The extensive overlapping of the $^1L_a$ and $^1L_b$ transitions makes the assignment of fine structure peaks in vapor phase quite difficult. However, taking advantage of the general observation that the position of the $^1L_a$ transition is more sensitive to perturbation than that of the $^1L_b$ transition (Platt, 1951), Strickland et al. (1970) were able to distinguish the two electronic transitions, by differentially shifting them with solvent perturbation. Thus, studies of indole and 3-methylindole in perfluorinated hexane permitted the identification of the 0-0 and 0 + 850 cm$^{-1}$ $^1L_a$ transitions. Major $^1L_b$ bands are easily recognized as the 0-0, 0 + 730 and 0 + 980 cm$^{-1}$ transitions. In other solvents, the latter two transitions appear as a single unresolved band at 0 + 850 cm$^{-1}$. In these solvents, too, the 0-0 bands of both $^1L_a$ and $^1L_b$ transitions overlap.

Thus, the absorption spectrum of 3-methylindole in methylcyclohexane displays vibronic bands at 34.42 and 35.33 kK and that of N-stearyl-L-tryptophan n-hexyl ester at 34.54 and 35.46 kK. However, addition of a small amount of butanol to the hydrocarbon solvent greatly red-shifts and broadens the $^1L_a$ bands while leaving the $^1L_b$ bands largely unaltered. These effects presumably arise from the formation of hydrogen bonding between the indole $\equiv$NH group and the butanol oxygen atom. Thus, in these spectra, considerable absorption is seen in the region of 33.5-34.5 kK. The high absorbance of tryptophan spectra in water-glycerol in this region can be attributed to similar red shifts. Other tryptophan derivatives such as the amide and the ethyl ester of N-acetyl-tryptophan display this phenomenon clearly at 77$^0$K.
Low-temperature absorption spectra of several proteins containing a single tryptophan residue were examined by Strickland et al., (1971). The 0-0 $^1L_a$ and 0-0 $^1L_b$ tryptophan bands are resolved. The 0-0 $^1L_b$ band is located between 34.5 and 34.7 kK. In contrast, the 0-0 $^1L_a$ band ranges from 33.1 to 34.2 kK, presumably because the indole ring is hydrogen bonded, in view of the model studies described above.

Tyrosine

Low-temperature CD and absorption spectra of tyrosine model compounds (Horwitz et al., 1970) reveal considerable vibronic fine structure. The 0-0 transition starts an intense progression with 800 cm$^{-1}$ spacing to higher energies. A much weaker progression involves a 1250 cm$^{-1}$ spacing. Thus, for N-acetyl-L-tyrosine ethyl ester, vibrational bands corresponding to 0-0, 0 + 800, 0 + 1250, 0 + 2 x 800, 0 + 1250 + 800 and 0 + 3 x 800 cm$^{-1}$ transitions are found at 34.89, 35.70, 36.14, 36.48, 36.92 and 37.30 kK.

The vibronic CD bands have the same sign and occur at the same wave-numbers as the corresponding absorption bands. The peak positions vary according to the solvent, but the vibrational spacing remains fixed.

Applying model studies to tryptophan-free ribonuclease (RNase)-A, Horwitz et al., (1970) reported the finding of three types of tyrosine residues in the enzyme, each type displays its own characteristic 0-0 transition. RNase-S was shown to be different from RNase-A in that a single tyrosine residue has its 0-0 transition shifted to higher energies in RNase-S (Horwitz and Strickland, 1971).
Effects of hydrogen bonding on the CD and absorption spectra of tyrosine were investigated by Strickland (1972). The results suggest that a hydrogen bond between a tyrosine hydroxyl group and a carbonyl oxygen of the peptide backbone may be one mechanism for producing a large (0.1-0.5 kK) red shift in proteins.
EXPERIMENTAL

Materials

**Enzymes**

Aspartate aminotransferase was prepared in this laboratory (see Methods).

Aspartate transcarbamylase catalytic subunit was prepared by Glen Nagel according to the method of Sheperdson and Pardee (1960), and was a generous gift of Dr. Howard K. Schachman, Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720.

**Pyridoxal phosphate**

VPLP, cis-Me-VPLP, trans-Me-VPLP, APLP and their non-phosphorylated analogs were synthesized in the laboratory of Dr. Walter Korytnyk, Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14203.

PM thiophosphate was synthesized by Dr. Radii M. Khomutov, Institute of Molecular Biology of the Academy of Sciences of the U.S.S.R., Moscow B-312, U.S.S.R.

PL sulfate synthesis (see Methods) was carried out in cooperation with Dr. Radii M. Khomutov. A sample synthesized by Dr. Toshio Kuroda of the Technical Department of Wakamoto Pharmaceutical Co., Ltd., Tokyo was donated by Dr. Esmond E. Snell of the Department of Biochemistry, University of California, Berkeley, California 94720.
Ultra-pure chemicals

Urea, guanidine hydrochloride and tris hydrochloride were ultra-pure and were obtained from Schwarz/Mann.

Other chemicals

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

Methods

Preparation of holoAAT


Heat denaturation

Twenty-five pounds of fresh, unwashed pig hearts from the slaughter house (Swift & Co. Packing Plant, N. Ninth Avenue, Marshalltown, Iowa 50158) were trimmed free of auricles, tendons and fat with scissors. The ventricles were then minced in an electric meat grinder (General Slicing Machine Co., Inc., Model D) through 3 mm holes. The resulting mince (6 liters) was then diluted with one-half volume (3 liters) of glutarate buffer (0.05 M, pH 6.0) containing 0.005 M EDTA, in a 24-quart stainless steel pot (Bloomfield Industries, Inc.). The pot was then placed in a water bath (Groen Division Corporation steam jacketed kettle, D-20 Model) maintained at 85°. Water circulation was sustained with a stirrer (Lightnin Mixing Equipment Co., Model 10) clamped to one side of the kettle.

The thick suspension was stirred with a wooden paddle to avoid local overheating. Denaturation began at about 60°, so 1.5 g of α-ketoglutaric
acid were added at this temperature to convert any amine form of the enzyme to the aldimine form. When the temperature reached $75^\circ$ (approximately 20 min.), that of the bath was reduced to $77^\circ$ by the addition of ice and denaturation was allowed to proceed at $75^\circ$, with only intermittent stirring for 20 minutes.

The pot was then removed from the water bath to the cold room where the heavy protein precipitate was removed by straining the suspension through four layers of cheese cloth sewn into a bag 90 cm long by 35 cm in circumference. The brown filtrate was allowed to cool to $5^\circ$.

**Ammonium sulfate fractionation** For each liter of filtrate 310 g (50% saturation) of ammonium sulfate (National Biochemicals Corp., Enzyme Grade) were added and dissolved by stirring with the aid of a magnetic stirrer. The rate of addition was not critical. The precipitate was allowed to flocculate for 30 minutes and then removed by centrifugation (Beckman Model J-21 centrifuge with 3-liter rotor JA-10 and Sorval Model RC2-B with 3-liter rotor Type GS3) for 10 minutes at 8000 RPM.

The supernatant was poured through four layers of cheese cloth sewn into a cone 30 cm in top circumference by 20 cm in depth and strung over a large funnel to remove floating lipid. For each liter of this solution 150 g (72% saturation) of ammonium sulfate were added with stirring. Again the rate of addition did not appear to be critical but vigorous stirring was avoided to prevent excessive foaming. The light precipitate was collected by centrifugation as before. The precipitate was taken up in a minimum amount of the glutarate buffer and a trace of $\alpha$-ketoglutarate (0.5 ml of a 0.2 M stock solution) and dialyzed against water ($2^\circ$).
overnight to remove ammonium sulfate.

**Chromatography on hydroxyapatite** The solution after dialysis against water was brought to 0.02 M in phosphate, pH 6.8 by the addition of 2 M phosphate buffer, pH 6.8. The further addition of 0.5 ml of \(\alpha\)-ketoglutarate appeared to increase the yield. The brown protein solution was applied to a hydroxyapatite column (2.6 x 50 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.8.

The aminotransferase appeared as a bright lemon yellow band below the adsorbed heme proteins. This band was eluted with 0.08 M phosphate buffer, pH 6.8. There sometimes was a pale yellow band eluted before the aminotransferase - this appeared to be a flavoprotein. The fractions which contained the aminotransferase were readily identified by its yellow color and were pooled, treated with a further addition of \(\alpha\)-ketoglutarate and precipitated by the addition of 500 g of ammonium sulfate per liter. The precipitate was taken up in a minimum amount of 0.04 M sodium acetate buffer, pH 5.1. After addition of several drops of \(\alpha\)-ketoglutarate, the dissolved precipitate was thoroughly (15 hours) dialyzed against 0.04 M acetate buffer, pH 5.1.

**Chromatography on CM-Sephadex** The extensively dialyzed protein solution was carefully added to the top of a 2.6 x 50 cm column of CM-Sephadex C-50 which had been equilibrated, according to the manufacturer's instructions, in 0.04 M acetate buffer, pH 5.1 (higher pH values would cause all subforms of the enzyme to wash off the column indiscriminately). The column was then washed with at least 300 ml of 0.04 M acetate buffer, pH 5.1 followed by an equal quantity of 0.06 M acetate buffer, pH 5.1.
This lengthy wash was essential for the complete removal of the γ-subform. The β- and α-subforms were eluted from the column, in that order, with a shallow gradient made by 350 ml of 0.06 M acetate buffer, pH 5.4, in the mixer and 350 ml of 0.12 M acetate buffer, pH 5.4, in the reservoir. Alternatively, the β-subform might be removed first by eluting the column with 0.06 M acetate buffer, pH 5.35 (this pH critical) followed by 0.12 M acetate buffer, pH 5.4 to elute the α-subform off the column. The fractions which contained the α-subform were again visually identified and were pooled. No α-ketoglutarate was added in order to avoid possible enzyme-substrate abortive complex in the final product. The combined fractions were treated with 500 g of ammonium sulfate per liter. The precipitate was taken up in a minimum amount of 0.04 M acetate buffer, pH 5.4 and dialyzed overnight against the same buffer.

Storage The dialyzed aminotransferase solution was centrifuged to remove the small amount of denatured protein. Complete removal of particulate matter was achieved by pouring the supernatant through 10 µ nylon net (as equipped on Pharmacia column sample applicators). To avoid deterioration of enzyme quality arising from repeated thawing and freezing, the clear enzyme solution was divided into 1 ml portions in capped vials and then stored in the freezer.

Preparation of hydroxyapatite

The hydroxyapatite used in the first column chromatography for the purification of aspartate aminotransferase was prepared according to the original method of Tiselius et al., as modified by Jenkins (1970).
Principle Brushite (CaHPO₄·2H₂O), an acidic, granular form of calcium phosphate was first prepared and then converted largely into hydroxyapatite (Ca₁₀(PO₄)₆(OH₂) by treatment with alkali. An extensive series of washes were employed to yield a granular and uniform product suitable for column chromatography.

Procedure Preparation of brushite and conversion to hydroxy apatite by alkali treatment Fifteen liters of 0.5 M calcium chloride and dibasic sodium phosphate were placed in two carboys fitted with siphons which were equipped with medicine droppers at the ends for proper dripping. The solutions were allowed to drip into 20 liters of water in a stream jacketed kettle, to one side of which was clamped an electric stirrer. The rate of addition of the two solutions was adjusted to 120 drops per minute and the mixture was stirred vigorously. When both solutions were consumed (approximately twenty hours), the precipitate of brushite should be granular and settle rapidly. The precipitate was washed four times with about forty liters of water to remove sodium chloride. After the fourth wash the brushite was treated with one equivalent of sodium hydroxide (300 g in 1 liter of water) in 35 liters of water. After a ten-minute equilibration period, the solution was brought to a gentle boil which was maintained for one hour. The brushite must not be heated in the absence of alkali as this would cause rapid conversion to a gelatinous form. After boiling, the precipitate settled to give a clear solution within ten minutes of the cessation of heating and
stirring. This precipitate was again washed four times by decantation. It was essential that only a ten-minute settling period be allowed between these washes as the supernatant solution was initially very turbid.

Equilibration with phosphate buffer The hydroxyapatite was equilibrated at pH 6.8 by a series of washes with boiling phosphate buffer.

1. Heat just to boiling with 35 liters of 0.01 M phosphate buffer (use 175 ml of 2 M phosphate stock buffer, pH 6.8).
2. Repeat the previous step but boil for five minutes.
3. Repeat the previous step twice but with fifteen-minute boiling periods.
4. Boil the suspension twice for fifteen minutes with 0.001 M phosphate buffer.
5. Store the hydroxyapatite in 0.001 M phosphate buffer at 2°C.

Operation of column It was necessary to take the following precaution to insure a good flow rate.

1. Before packing a column, wash the hydroxyapatite with a large volume of water to remove all fines.
2. Pour the column in as thick a slurry as possible to avoid having the fines at the top of the column.
3. Centrifuge the protein solution before adding it to the column to remove traces of precipitate which will clog the top.
4. The upper layer of the column may be stirred to unclog the column provided that the suspension does not become so dilute that the fines rise to the top.
Stability and reuse  Upon aging and repeated use some fine material was produced which could be readily removed by decantation. The hydroxyapatite was otherwise stable for at least six months.

After use the hydroxyapatite was washed with several portions of 1 M phosphate buffer, pH 6.8 to remove adsorbed material. Following thorough rinsing with water, the hydroxyapatite could be stored and reused.

AAT resolution

ApoAAT was prepared essentially according to the method of Scardi et al., (1963) as modified by Furbish et al., (1969).

ApoAAT so prepared could be reactivated with excess PLP to 85-100% of holoAAT specific activity. The residual specific activity was below 0.2%.

Assay

Routine assay was performed according to Furbish et al., (1969). Specific activity was expressed in μ moles/min/mg of enzyme. AAT prepared in this laboratory was found to have specific activity of 220 μ moles/min/mg.

Enzyme concentration

Protein concentrations were determined by measuring the absorbance at 35.8 kK. Dilution of enzyme samples were often avoided by employing cuvette spacers which were accurately manufactured to reduce the light path to either 0.2 or 0.1 cm. Molar absorptivity of $6.36 \times 10^4$ and
6.55 \times 10^4 \text{ (Furbish et al., 1969)} were used for the apoenzyme and holo-enzyme respectively. To express enzyme concentration in the unit of mg/ml, the value of 4.63 \times 10^4 \text{ (Ovchinnikov et al., 1973)} was used for the molecular weight. Throughout this work, a 'mole' of AAT was regarded as that amount of the enzyme containing 1 mole of bound pyridoxal phosphate.

**Analog binding**

Since most binding studies involved the monitoring of the reaction by UV-visible and circular-dichroism spectroscopy, it was most convenient to use apoAAT approximately $10^{-4}$ M in concentration.

To 0.8 to 1.0 ml of apoAAT in a semi-micro cuvette was added micro-liter quantities of the analog (concentration approximately $10^{-2}$ M). The exact amount of the analog added was calculated such that the molar ratio of the analog vs. apoenzyme was equal or less than the fraction of active apoenzyme, the fraction being typically 0.85-1.0. Addition of less than equivalent quantity of the analog to the apoenzyme minimized random binding which might occur due to the presence of inactive apoenzyme. Quantitative delivery of the analog was performed with a teflon tipped syringe (Unimetrics Universal Corporation) to avoid contact with the metal plunger.

Mixing of the analog and apoAAT was done by either inversion of the cuvette or gentle stirring with a small teflon stirrer.

**Displacement of bound analogs by PLP**

Several of the analogs possess reactive groups which could react
inreversibly with various side chains at locations other than the enzyme active site if these analogs were present in amounts in excess of the apoenzyme. To determine the displacibility by PLP of active site-bound analogs, it was therefore necessary to use these analogs in amounts calculated according to the method described in the preceding section (see Analog binding).

When the binding of the analog to the apoenzyme was complete, excess amounts (up to 200-fold) of PLP were introduced. Aliquots, typically 10 μl, were withdrawn at various time intervals and properly diluted, e.g. 40-fold, with 0.01 M tris buffer, pH 8.3. Ten μl aliquots of the diluted solution were assayed.

Under these conditions, part of the enzyme activity initially recovered would be due to the small amount of free apoenzyme present. Specific activity gained after the initial recovery was attributed to displacement of the analog by PLP.

**UV-visible spectrophotometric measurement**

UV-visible absorption spectra were recorded with a Cary 1501 Spectrophotometer equipped with a Datex SDS-1 Spectrophotometer Data Recording System which permitted automatic digitation of absorbance on IBM cards with an IBM-26 Printing Card Punch. Absorbance was reproducible within ± 0.001 unit. The wavenumber scale was calibrated against benzene vapor and against a solution of neodymium chloride. Peak positions were reproducible to ± 0.1 kK over a period of a year.
Absorbance, from 0 to 2.0 was recorded to the nearest 0.001 unit at regular intervals of wavenumber, usually 0.05 kK for spectra to be subjected to fine structure analysis and 0.2 kK for other spectra.

Circular dichroic measurement

Circular dichroism was recorded with a Jasco Model ORD/UV-5 spectropolarimeter equipped with a CD recorder and with Sproul Scientific SS-20-2 modification. Wavelength was measured to ± 1 nm.

Circular dichroism was determined to ± 0.1 millidegree ellipticity (3 x 10^-6 absorbance). Signal to noise ratio was optimized by scanning at a low speed, e.g. Gear No. 100 on shaft D, Gear No. 20 on shaft E, Scanning speed switch on position 1 to yield 1.8 nm/min., and with a high time constant of 16 or 64 seconds.

Ellipticity recorded on charts was transcribed at 1 nm intervals manually and subsequently punched on IBM cards for processing.

Preparation of active manganese dioxide

The method was basically that of Mancera et al., (1953). The nitric acid wash was adopted from Harfeist et al., (1954).

Twenty g of manganous sulfate monohydrate was placed in a 1-liter, 3-neck round bottom flask with a magnet. The flask was seated in a heating mantle on top of a magnetic stirrer. To the flask was added 300 ml of water. With stirring, the compound was dissolved and heated to 90° C. Four g of potassium permanganate dissolved in 100 ml of boiling water was transferred to a dropping funnel and allowed to drip into the flask over a period of approximately two minutes. Addition of hot
permanganate was repeated four more times, so that a total of 20 g of potassium permanganate in 500 ml of water was added.

Following the addition of the last of the permanganate, the content of flask was allowed to continue stirring at 90° for thirty minutes. Then, heating was discontinued while stirring was maintained until the mixture cooled to 60° (approximately 1 1/2 hours). The content was filtered through a 350-ml medium porosity sintered glass funnel. The precipitate was transferred to a 500-ml beaker containing 300 ml of 2.4 N nitric acid, stirred for five minutes and filtered with suction in the cleaned funnel. The acid treated manganese dioxide was removed to a beaker containing approximately 300 ml of 50° C water, stirred for five minutes and filtered with suction in the cleaned funnel. The warm water washing was repeated several times until the washing attained a pH of 6. The brownish black cake was removed from the funnel in as few pieces as possible and dried and stored in a 110-130° C oven. The chunks were pulverized just before use. Manganese dioxide thus prepared was suitable for the preparation of pyridoxal sulfate from pyridoxine sulfate (PN sulfate).

Washing of Dowex resin

One pound of Dowex 50 W x 8 was placed in a 1000-ml beaker and 300 ml of 6 N NaOH was added. With the acid of a magnet and a magnetic stirrer, the content was stirred for 30 minutes. The mixture was then poured into a 350-ml sinter-filter funnel, and the dark brown sodium hydroxide containing dissolved impurities was removed by filtration.
The resin was washed in the funnel with eight 250-ml portions of water. Each washing was accompanied by moderate stirring with a stirring rod before vacuum was applied. The resin was transferred back to the cleaned 1000-ml beaker and 330-ml of 6N HCl was introduced. The content was stirred in the same manner as described before. Again after 30 minutes, the resin was removed to the cleaned 350-ml funnel, rid of the HCl and washed with water. This cycle of NaOH-H₂O-HCl-H₂O was repeated twice more. At the end of the third cycle the resin was placed in a 2-liter beaker filled with H₂O and allowed to stir for an hour. The rinsing water was then decanted and the rinsing process was repeated once more. The Dowex 50 W x 8 in H⁺ form so obtained was suitable for purification of pyridoxal sulfate by column chromatography.

Preparation of pyridoxine 5'-sulfate (PN sulfate)

The procedure was adopted from Kuroda (1963).

One hundred percent sulfuric acid was prepared by mixing 100 g of 97% sulfuric acid and 68 g of 20% fuming sulfuric acid or proportions thereof. With stirring 2.1 g of isopropylidine pyridoxine was added, over a period of ten minutes, to 20 ml of 100% sulfuric acid in a 50-ml round-bottom flask in an ice bath. A drying tube packed with Drierite was fitted to the mouth of the flask and the content of the flask was allowed to stir in the ice bath for thirty minutes. Meanwhile 300 ml of anhydrous ether in a 500-ml round-bottom flask capped with a drying tube was chilled to below -30°C in a dry ice - acetone bath. With stirring, the sulfuric acid solution was gradually poured into the cold ether.
The mixture was allowed to stir until reaching room temperature, care being taken to exclude entrance of moisture. The ether was decanted and the coarse precipitate washed with two 100-ml portions of ether. The precipitate was dried in vacuo for 30 minutes to remove residual ether. The dried precipitate was dissolved in 50 ml of water. Excess sulfuric acid was titrated with a saturated solution of barium chloride. During the course of titration, several 1-ml portions of the turbid mixture were removed and millipored to yield a clear solution to which a drop of barium chloride solution was added to check for the completeness of the titration. The bulk of barium sulfate was removed in a clinical centrifuge. The supernatant was heated in a boiling water bath for thirty minutes during which time barium sulfate fines flocculated and precipitated out of solution. The precipitate was removed by filtration and the clear supernatant was concentrated in vacuo to dryness. The crude PN sulfate was dried in vacuo and purified by crystallization from water. Yield of crystalline substance: 1.85 g (74%).

Nmr spectrum of the compound showed chemical shifts (δ) in D₂O at 2.64, 5.02, 5.19 and 8.14 ppm which were readily assigned to 2-CH₃, 5-CH₂, 4-CH₂ and 6-H groups. The spectrum closely resembled that of PNP (Korytnyk and Ahrens, 1970) except no splitting of the 5-CH₂ was observed. Absorption spectra of the H₂P, HP and P forms of the compound exhibited maxima at 34.4, 30.7 and 32.3 kK. The peak positions as well as band shapes were found comparable to those of pyridoxine and pyridoxamine (Metzler and Snell, 1955). Thus, the spectral data were consistent with the structure of PN sulfate.
Oxidation of PN-sulfate to PL sulfate

Two hundred and fifty mg (1.0 mmole) of PN sulfate was dissolved in 25 ml of warm water in a 50-ml clinical centrifuge bottle with the aid of a tiny magnet (13 x 3 x 3 mm) and a magnetic stirrer. When the solution cooled to room temperature, 500 mg (5.7 mmole) of pulverized, active manganese dioxide was added and the mixture was allowed to stir in the dark. The progress of oxidation was monitored as follows. Ten µl aliquots of the mixture were removed into 3 ml of 0.1 N sodium hydroxide. After shaking, the basic mixture was pressed through a syringe equipped with a Millipore filter (HA 0.45 µ). The spectrum of the clear filtrate was recorded in a Cary 1501 spectrophotometer. The absorbance ratio $A_{25.8 \text{ kK}}/A_{32.5 \text{ kK}}$ was noted. This procedure was repeated until the ratio attained a value of 5.0. Approximately thirty minutes were required to attain this value (Figure 2). Further reaction would lead to the production of the corresponding acid which absorbed at 32.5 kK and reduce the ratio previously obtained. As soon as the ratio was maximized, the reaction vessel was placed in a clinical centrifuge and spun for two minutes to remove manganese dioxide. The precipitate was washed with 3 ml of water and again centrifuged. The combined supernatant was passed through another Millipore filter and the filtrate was concentrated in vacuo to 1 ml. The concentrate, intensely yellow and still with some suspended manganese dioxide, was then applied to a column (85 x 1.9 cm) of Dowex 50 W x 8 (100-200 mesh) in H⁺ form previously equilibrated with water. The column was eluted with water with a flow rate of 25 ml/hr.
Figure 2. Oxidation of PN sulfate to PL sulfate. Numbers designate time in minutes following the addition of manganese dioxide.
Five-ml fractions were collected. A 10 μl aliquot of each fraction was added to 1 ml of 0.1 N sodium hydroxide and the spectrum was recorded (Figure 3). The initial fractions with low $A_{25.8\,\text{kHz}}/A_{32.5\,\text{kHz}}$ ratio were discarded while the fractions with a constant ratio of 10 were pooled and concentrated in vacuo and lyophilized. Yield: 116 mg or 46%.

PL sulfate might be recrystallized from warm glacial acetic acid by addition of ethyl acetate and chilling. The powdery product did not seem to be of superior quality, judging from its slightly reduced $A_{25.8\,\text{kHz}}/A_{32.5\,\text{kHz}}$ ratio.

Nmr spectrum (Table 2) of the product showed chemical shifts comparable to those observed for PLP (Korytnyk and Ahrens, 1970) except no splitting of $5-\text{CH}_2$ was seen.

Table 2. Nmr spectral data of PL sulfate$^a$

<table>
<thead>
<tr>
<th>Chemical shift, δ</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42</td>
<td>2-CH$_3$</td>
</tr>
<tr>
<td>5.26</td>
<td>5-CH$_2$</td>
</tr>
<tr>
<td>7.60</td>
<td>6-H</td>
</tr>
<tr>
<td>10.44$^b$</td>
<td>4-CH</td>
</tr>
</tbody>
</table>

$^a$Spectra obtained in NaOD, pD = 7.50.

$^b$δ = 6.53 at pD 2, indicating formation of a hydrate.

Absorption spectra (Figure 40) of the compound are presented in RESULTS.
Figure 3. Purification of PL sulfate on Dowex column.
Elemental analysis yielded C 37.10, H 3.99, S 11.92 (calculated as 1/2 hydrate C 37.50, H 3.93, S 12.51).

High voltage electrophoresis showed that the compound migrated at the same rate as an authentic sample of PL sulfate.

Thus, all available data were consistent with the structure of PL sulfate.

Nephelometric determination of inorganic sulfate

To quantitate the release of inorganic sulfate from the reaction mixture of PL sulfate plus cysteine, barium ion was employed to trap minute amounts of free sulfate. The fine barium sulfate precipitate was kept in uniform suspension. Light scattering by this suspension was correlated with inorganic sulfate concentration in the reaction mixture by comparison with a calibrated standard curve.

The procedure was based on those of Meehan and Chiu (1964) and Vogel (1961) as adapted by Johnson (1973). Barium mixture was prepared by mixing, in the proportion of 1:2:7 (V/V/V), the following solutions: glycerol—absolute alcohol solution, 1:2 (V/V); NaCl—HCl solution, 60 g of NaCl and 5 ml of concentrated HCl in 250 ml of solution; barium chloride dihydrate solution, containing 0.43 g of the compound in 100 ml of solution. The mixture was passed through a Millipore filter prior to use. Care was exercised to minimize entrance of atmospheric dust. With stirring, 0.5 ml of the Millipore filtered reaction mixture of PL sulfate plus cysteine was added to 10 ml of the barium mixture in a capped vial. Stirring (with a tiny magnet) was continued for 90 seconds and then the content
was transferred to a light scattering photocell and left undisturbed for 20 minutes. Light scattering was then measured in a Sofia light scattering photometer Model 701.

**Data processing**

UV-visible and circular dichroism spectral data on IBM cards were processed employing several convenient FORTRAN IV programs (Thomson and Metzler, 1972). Computations were performed at the Iowa State Computation Center on an IBM 360-65 computer.

Initial processing of spectral data collected at regular intervals of wavenumber or wavelength yielded computer plots displaying UV-visible and circular dichroism spectra in terms of molar absorptivity and molar CD, respectively vs. wavenumber.

Acid dissociation constants of pyridoxal phosphate analogs were computed from UV-visible spectral data at various pH values. Spectra of these analogs at various stages of protonation were readily calculated and plotted.

**Curve fitting**

Where appropriate, absorption spectra were fitted with lognormal distribution curves by an iterative procedure (Siano and Metzler, 1969). Each lognormal curve (Figure 4) was described by four parameters: the peak position $\bar{\nu}_0$; the height, $e_0$; the width at half-height, $W$; and the skewness, $\rho$. For well resolved absorption bands, the values of $\bar{\nu}_0$ and $W$ were reproducible to $\pm 0.02$ kK and $\rho$ to $\pm 0.03$. The differences between each experimental point and the point at the same wavenumber on
Figure 4. Definition of lognormal parameters.
the fitted function were plotted as a percentage of the peak height.

**Fine structure peak height**

In fine structure or difference plots, it was often useful to calculate the height of the various peaks for the purpose of comparison. Peak height (Figure 5) was defined as follows:

\[
\text{Peak height} = (Y - Y_2) + (Y_2 - Y_1) \times \left( \frac{X_2 - X}{X_2 - X_1} \right).
\]

The unit of peak height was % error as in the fine structure plot.
Figure 5. Definition of fine structure peak height.
RESULTS

Experimental results are presented in two parts. Part I deals with the interaction of apoAAT with the various PLP analogs; Part II describes the analysis of electronic absorption spectra of AAT and aspartate transcarbamylase.

Part I. Interactions of ApoAAT with PLP Analogs

Pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP)

For the purpose of comparison, the absorption and CD spectra of the aldimine and the amine forms of AAT are presented.

Native AAT HoloAAT (Figure 6) displayed absorption maxima at 23.2 kK in the protonated form and 27.6 kK in the unprotonated form, the two forms being related by a pK of 6.2 (Jenkins et al., 1959). The CD spectra of holoAAT (Figure 7) showed peak positions corresponding to those of the absorption spectra. Although the band shapes of the absorption and CD spectra were in general similar, the former showed more prominent shoulders in the region of 28-30 kK. It appeared that some absorption bands comprising the shoulders did not contribute to optical activity. Contamination with β and γ subforms of the enzyme might be sufficient to cause this discrepancy.

In addition to the positive CD peak, a characteristic negative CD peak centered around 33.5-33.6 kK was also present in each holoAAT spectrum. The position of the peak corresponded well to those attributed to tryptophan residues in many proteins (Strickland et al., 1969;
Figure 6. Absorption spectra of holoAAT in 0.04 M acetate, pH 4.85 and in 0.02 tris HCl pH 8.46.
Figure 7. CD spectra of holoAAT in 0.04 M acetate pH 4.74 and in 0.02 tris HCl pH 8.70.
Reconstituted AAT  ApoAAT reconstituted with PLP closely approximated native AAT in terms of absorption spectra (Figure 8) as well as CD spectra (Figure 9), consistent with the fact that the apoenzyme could be nearly fully activated with excess PLP (see EXPERIMENTAL). However, in the reconstituted AAT, there was occasional increase in absorption in the 22-23 kK region, apparently due to 'non-specific binding' of PLP (Furbish et al., 1969). The amine form of the enzyme was obtained by reconstitution of apoAAT with PMP. This form of AAT possessed lower optical activity. The negative peak at 33.5-33.6 kK was small but unmistakable. Contrary to its claimed absence (Ivanov and Karpeisky, 1969), the apoenzyme (Figure 9) displayed a positive CD band at 33.6 kK. The appearance of the peak in this resolved form of AAT demonstrated that the origin of the CD band resided in the protein portion rather than the cofactor portion of the enzyme.

Vinyl analog of PLP (VPLP)

Acid dissociation constants  Two acid dissociation constants of VPLP were determined spectrophotometrically. The pK of the 3-phenolic proton \( \text{H}_\text{gP} \rightarrow \text{HP} \) was determined to be 5.12; that of the pyridinium proton 9.86 \( \text{HP} \rightarrow \text{P} \). The spectra of the three ionic species, \( \text{H}_2\text{P} \), HP and P, according to their extent of protonation, are shown in Figure 10.

Binding by apoAAT  Mixing of the analog with apoAAT at pH 8.3 resulted in a bathochromic shift of the analog spectrum. The free analog
Figure 8. Absorption spectra of reconstituted AAT.
1. ApoAAT in 0.02 M tris HCl, pH 8.26
2. Reconstituted with PLP in 0.02 M tris HCl, pH 8.23
3. Reconstituted with PMP in 0.02 M tris HCl, pH 8.3
Figure 9. CD spectra of reconstituted AAT.
1. ApoAAT in 0.02 M tris HCl, pH 8.3
2. Reconstituted with PLP in 0.02 M tris HCl, pH 8.3
3. Reconstituted with PMP in 0.02 M tris HCl, pH 8.3
Figure 10. Spectra of VPLP.
at this pH had an absorption maximum at 29.7 kK. Upon binding by apoAAT the peak position was shifted to 28.0 kK (Figure 11). This shift was slow enough to be followed. Approximately two hours were required for the binding to be completed. The spectrum of the bound analog was found to be insensitive to pH variation, in the pH range of 5 to 8.5.

**Effects of low pH and boiling on enzyme bound VPLP**  
It has been reported (Fonda, 1971) that addition of NaOH to apoAAT-VPLP to a pH of 12 yielded a spectrum resembling that of the free analog.

When HCl was added to apoAAT-VPLP to a pH of 0.6 to denature the protein, the spectrum of the supernatant, adjusted to pH 7.2, displayed an absorption maximum at 29.7 kK (Figure 12). Not only was the peak position identical to that of the free analog at this pH but the recovery of the analog from E-VPLP was nearly quantitative as seen from a comparison of the values of molar absorptivity at 29.7 kK in Figures 10 and 12.

Similarly, boiling the enzyme at 100° released the bound analog. Again, the recovery was apparently quantitative.

Moreover, the supernatants of both acid- and boiling-denatured apoAAT-VPLP behaved in the same way towards pH variation as did the free analog, i.e., exhibiting absorption maxima at 33.8 and 30.8 kK at pH 1 and 12, respectively.

**Sodium borohydride treatment of apoAAT-VPLP**  
Addition of sodium borohydride to apoAAT-VPLP was carried out at pH 8.3 and 5.8. At pH 8.3 the spectrum of treated apoAAT-VPLP was found to be identical to the untreated sample. At pH 5.8, a small shift of the peak position to
Figure 11. Spectrum of AAT-bound VPLP in 0.02 M tris HCl, pH 8.24. Protein absorption has been subtracted.
Figure 12. Spectra of supernatant of acid-denatured apoAAT-VPLP, initially in 0.01 M tris HCl, pH 8.3.
1. 0.33 N in HCl, pH 0.63
2. 0.24 M in phosphate, pH 7.2
3. 0.36 N in NaOH, pH 11.9
28.6 kK was observed, however, the spectra before and after the treatment were similar otherwise. To check whether the binding between the apoenzyme and the analog was altered in any way, the treated solution was denatured by boiling. The resulting supernatant displayed an absorption maximum at 29.9 kK at pH 8, similar to that of the free analog. Thus it did not seem likely that a Schiff base had formed between apo-AAT and VPLP in the apoAAT-VPLP complex.

As a control, apoAAT reconstituted with PLP was similarly treated with sodium borohydride, immediate reduction of the Schiff base was evidenced by the resulting peak at 30.9 kK.

**Photolysis of free VPLP** Little change in the spectrum of the analog took place on exposure to light under acidic conditions. In neutral and basic solutions, the analog was found to be light sensitive. Figure 13 shows that exposure to light under these conditions greatly decreased the peaks at 29.7 and 30.7 kK, while increasing absorption at 34.8 and 35.3 kK respectively.

**Photolysis of apoAAT-VPLP** Figure 14 shows that in the laboratory daylight, the absorption maximum of the bound analog at 28.0 kK decreased significantly in 46 hours. Two additional hours of exposure to bright sunlight at a laboratory window further reduced the peak height to a level close to that of the apoenzyme. Accompanying the decrease of the 28.0 kK peak height, there was increased absorption above 33 kK. Thus close similarities existed in the photolysis of free and apoAAT-bound VPLP.
Figure 13. Photolysis of VPLP.
1. VPLP in phosphate, pH 6.8. After preparation
2. (1). After exposure to sunlight for one hour
3. VPLP in 0.01 N NaOH. After preparation
4. (3). After exposure to sunlight for 45 min.
Figure 14. Photolysis of apoAAT-VPLP in 0.01 M tris HCl, pH 8.3.
1. ApoAAT-VPLP before prolonged exposure to light
2. (1). After exposure to laboratory daylight for 46 hours
3. (2). After exposure to bright sunlight at a laboratory window for two hours
CD of apoAAT-VPLP  Except for the peak at approximately 33.6 kK, apoAAT-VPLP was found to be totally devoid of CD.

Aminotransferase activity of apoAAT-VPLP  AAT reconstituted with VPLP was completely unable to catalyze the transamination reaction.

Displacement of VPLP from apoAAT-VPLP by pyridoxal phosphate  AAT-bound VPLP was completely displaced by excess PLP (final molar ratio of AAT : VPLP : PLP = 1 : 0.70 : 170) on prolonged incubation as shown below;

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>% Recovery in specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 hour</td>
<td>56 ± 10</td>
</tr>
<tr>
<td>3.5 hour</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>10.5 hour</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>21.5 hour</td>
<td>98 ± 10</td>
</tr>
<tr>
<td>36.5 hour</td>
<td>99 ± 10</td>
</tr>
</tbody>
</table>

Since there was more enzyme than VPLP, the initial reactivation observed was largely due to reconstitution of the excess apoenzyme with PLP.

Total reactivation was not achieved when the molar ratio of AAT : VPLP : PLP was 1 : 3 : 10. In this case the mixture recovered only 25% of specific activity after 12 hours of incubation.

Reaction of VPLP with histidine, cysteine, alanine and hydroxylamine  Histidine (final concentration 5 x 10^{-2} M) was incubated with the analog (final concentration 8 x 10^{-5} M) at 50°C for 12 hours at pH 4, 6 and 8, respectively. The spectra of these mixtures resembled those of the free analog at these pH values both before and after incubation. Thus VPLP was unreactive toward histidine.
Cysteine also was tested with the analog (Figure 15). No reaction occurred following mixing at either pH 1.7 or 8.3. After 11 hours of incubation at 50°C, the peak at 30.0 kK (pH 8.3) was shifted to 31.4 kK. This hypsochromic shift bore no resemblance to the bathochromic shift observed when the analog was allowed to combine with the apoenzyme. At pH 1.7, the 33.6 kK peak was shifted to 34.1 kK with the appearance of two small shoulders at about 30 kK and 26 kK. Again the peak shift was hypsochromic although a shoulder appeared at lower wavenumbers than 28.0 kK.

At room temperature, VPLP was found to be unreactive towards alanine at pH 5 in the presence of alum.

The reactivity of VPLP toward hydroxylamine was found to depend on pH (Figure 16). At room temperature and at pH 6.8, a decrease of the 29.8 kK peak and a corresponding increase in absorption at 34.0 kK were observed over a period of two days. In addition, a shoulder at 25 kK appeared. However at pH 11, no change in the spectrum of VPLP was detected.

These results illustrate that VPLP either did not react with amino acids at all or reacted in ways quite different from that observed with apoAAT. The hypsochromic shifts observed with cysteine and hydroxylamine were consistent with the reaction mechanism in which a nucleophilic group added to the vinyl moiety of VPLP. A similar addition reaction occurs readily between sulfhydryl groups of proteins and 4-vinyl pyridine (Friedman et al., 1970). Therefore, it appeared unlikely that such an
Figure 15. Reaction of VPLP and cysteine (molar ratio = 1:2500).
1. After mixing, pH 8.3
2. After incubation at 50°C for 11 hours, pH 8.3
3. After mixing, pH 1.7
4. After incubation at 50°C for 11 hours, pH 1.7
Figure 16. Reaction of VPLP and hydroxylamine (molar ratio = 1:10,000), 0.01 M in alum.
1. After mixing
2. After two days
addition reaction would account for the interaction between apoAAT and VPLP.

**Spectra of free VPLP in water-methanol and water-dioxane mixtures**

The bathochromic shift due to binding as described above was mimicked by placing the analog in water-methanol and water-dioxane mixtures.

VPLP had an absorption maximum at 29.7 kK in 0% methanol. The peak position underwent a bathochromic shift as the proportion of methanol increased such that the peak position was 28.5 kK when the percentage of methanol was 99% (Figure 17).

In water-dioxane mixtures, the bathochromic shift from 29.7 to 28.0 kK resembled even more closely that of the apoAAT-VPLP binding reaction as the proportion of dioxane increased from 0 to 84%.

The solvent effect was completely reversible. A solution of the analog initially 45% in methanol was diluted with either water or methanol to final methanol concentration of 5 and 95% respectively. The resulting spectrum of the former solution fell between those of the compound in 0 and 25% methanol while that of the latter fell between those in 74 and 99% methanol.

The large solvent effect on the spectrum of VPLP was found to be independent of the 5'-phosphate moiety. The vinyl analog of pyridoxal (VPL) exhibited the same solvent effect and the spectra were indistinguishable from those of its phosphorylated analog.

In view of the ease with which unaltered VPLP was released from apoAAT-VPLP, the absence of an imine bond between the apoenzyme and the
Figure 17. VPLP in water-methanol mixtures, pH 7.9. Numbers designate volume percentage in methanol.

Figure 18. VPLP in water-dioxane mixtures, pH 7.9. Numbers designate volume percentage in dioxane.
analog, the similarity in the pattern of photolysis between the enzyme-bound and free analog, the disimilarity in the nature of reaction between VPLP with reactive nucleophiles and VPLP with apoAAT, and finally the mimicking of the interaction between apoAAT and VPLP by non-aqueous solvent effect on the analog, it seemed that the analog was bound by apoAAT through non-covalent forces, and that the environment of the enzyme-bound analog was hydrophobic like dioxane.

In addition to the shift in absorption maximum, the spectrum of VPLP in increasing amounts of methanol or dioxane underwent a change in ratio of the species absorbing maximally at 28-30 kK to that at 34-36 kK. This equilibrium appeared to be one between the zwitterionic (VII) and the uncharged species (VIII) of VPLP with regard to the charge on the phenolic and pyridinium moieties as illustrated below.

In aqueous solutions the zwitterion (VII) was the predominant species displaying strong absorption at 28-30 kK. As the solvent became more and more non-polar, the uncharged species (VIII) prevailed and the absorption was maximal at 34-36 kK. This type of equilibrium has been observed with 3-hydroxypyridines (Metzler and Snell, 1955; Nakamoto and Martelli, 1959a, 1959b).
The apoAAT-bound VPLP showed most of its absorption at 28-30 kK rather than 34-36 kK, indicating that the analog was bound by apoAAT in the zwitteronic form (VII).

The spectrum of AAT-bound VPLP (Figure 11) was obtained by subtracting the spectrum of apoAAT from that of apoAAT-VPLP. It seemed clear that the sharp peak at 33.8 kK and the deep valley at 34.5 kK represented perturbation of some tryptophan residues of the enzyme (see DISCUSSION).

**Cis-methylvinyl analog of PLP (Cis-Me-VPLP)**

**Acid dissociation constants** The three ionic forms (Figure 19) of cis-Me-VPLP, according to their extent of protonation, were computed from spectrophotometric data (Figures 20 and 21). The pK's relating these forms were 5.59 and 10.02. The absorption maxima of the three ionic forms of cis-Me-VPLP were very similar to those of B<sub>6</sub> analogs lacking conjugation outside the pyridine ring, such as pyridoxine and pyridoxamine (Metzler and Snell, 1955).

**Binding by apoAAT** Complete binding of cis-Me-VPLP to apoAAT was obtained immediately following mixing. At pH 8.5, the absorption maximum of the bound cis-Me-VPLP was 29.9 kK (Figure 22) representing a bathochromic shift from 30.8 kK of the free analog.

**Spectra of free cis-Me-VPLP** The bathochromic spectral shift arising from the binding reaction described above was mimicked by placing the analog in non-aqueous solvents such as dioxane. In 0% dioxane, the absorption maximum was 30.8 kK and in 84% dioxane, it was 29.9 kK
Figure 19. Spectra of cis-Me-VPLP.
Figure 20. Spectrophotometric titration curves of *cis*-Me-VLP. Numbers designate pH values.
Figure 21. Spectrophotometric titration curves of cis-Me-VPLP. Numbers designate pH values.
Figure 22. Absorption spectra of apoAAT-cis-Me-VPLP.
1. in 0.01 N tris HCl, pH 8.5
2. in 0.04 M acetate, pH 4.9
Protein absorption has been substracted.
(Figure 23). The solvent effect on this analog was reminiscent of that on VPLP, suggesting that the interaction of cis-Me-VPLP with apoAAT too was non-covalent in nature.

**Aminotransferase activity of apoAAT-cis-Me-VPLP**

ApoAAT-cis-VPLP yielded zero AAT activity.

**CD of apoAAT-cis-Me-VPLP**

Apo-AAT-cis-Me-VPLP displayed a sharp positive band (Figure 24) corresponding to its UV-visible absorption band.

**Displacement of cis-Me-VPLP from apoAAT-cis-Me-VPLP by PLP**

The analog in the apoAAT-cis-Me-VPLP complex was partially displaced by incubation with PLP (final molar ratio at AAT:cis-Me-VPLP: PLP = 1:4.6:7.8) as judged by a recovery of 39% enzyme activity following 18 hours of incubation. Total reactivation was not obtained under these conditions. A greater excess of PLP and longer incubation time would probably be required as in the case of apoAAT-VPLP.

**Effect of pH variation on AAT-cis-Me-VPLP**

pH variation affected the peak position of apo-AAT-cis-Me-VPLP spectrum. At pH 9.2 and 8.5, the peak position was 29.9 kK. At pH 4.9 the peak position was 28.5 kK (Figure 22).

**Trans-methylvinyl analog of PLP (trans-Me-VPLP)**

**Acid dissociation constants**

The three ionic forms (Figure 25) of trans-Me-VPLP, according to their extent of protonation were computed from spectrophotometric titration data. The forms were related by pK's of 5.44 and 10.14. The absorption maxima of trans-Me-VPLP are similar
Figure 23. *Cis*-Me-VPLP in water-dioxane mixtures, pH 7.7. Numbers designate volume percentage in dioxane.
Figure 24. CD spectrum of apoAAT-cis-Me-VPLP in 0.02 M tris HCl, pH 8.3.
Figure 25. Spectra of trans-Me-VPLP.
to those of VPLP, indicating conjugation between the vinyl double bond and the pyridine ring.

**Binding by Apo-AAT** Complete binding was obtained immediately following mixing of the analog with the enzyme. At pH 8.4 the absorption maximum of apoAAT-trans-Me-VPLP was 28.4 kK (Figure 26), representing a bathochromic shift from 29.7 kK of the free analog.

**Spectra of free trans-methylvinyl PLP in water-dioxane mixtures** The bathochromic spectral shift arising from the binding reaction described above was mimicked by placing the analog in non-aqueous solvents such as dioxane. In 0% dioxane, the absorption maximum was 29.7 kK and in 83% dioxane, it was 28.4 kK (Figure 27). As in the cases of apoAAT-VPLP and apoAAT-cis-Me-VPLP, it appeared that trans-Me-VPLP interacted with apoAAT through non-covalent forces.

**Aminotransferase activity of apoAAT-trans-Me-VPLP** AAT-trans-Me-VPLP yielded zero AAT activity.

**CD of apoAAT-trans-Me-VPLP** ApoAAT-trans-Me-VPLP displayed a positive CD band (Figure 28) corresponding to its UV-visible absorption band.

**Acetylenic analog of PLP (APLP)**

**Acid dissociation constants** Due to the limited supply of the phosphorylated analog, the computed spectra of the three ionic forms according to their extent of protonation and the acid dissociation constants relating these species were obtained from the non-phosphorylated analog, i.e. the acetylenic analog of pyridoxal (APL).
Figure 26. Absorption spectrum of apoAAT-trans-VPLP in 0.01 N tris HCl, pH 8.4. Protein absorption has been subtracted.
Figure 27. Trans-Me-VPLP in water-dioxane mixtures, pH 7.7. Numbers designate volume percentage in dioxane.
Figure 28. CD spectrum of apoAAT-trans-Me-VPLP in 0.01 M tris HCl, pH 8.4.
The computed spectra (Figure 29) of the three ionic forms and the pK's of 3.68 and 8.30 were assumed to closely approximate those of the phosphorylated analog VPLP (Morozov et al., 1965, 1968). It is known that spectra of PLP and 5-deoxypyridoxal closely resemble each other and their pK's are also very similar (Metzler and Snell, 1955; Williams and Nielands, 1954).

**Binding by apoAAT** At pH 5.07, mixing of the analog with apoAAT immediately lead to binding as judged by the bathochromic shift of absorption maximum from 28.4 kK to 27.6 kK (Figure 30). The spectrum of this first enzyme-analog complex (apoAAT-APLP-I) remained constant for at least ten hours.

At pH 6.10, the formation of apoAAT-APLP-I was also instantaneous. However, the intensity of the 27.6 kK peak soon began to decrease upon sitting at room temperature (Figure 31). This process continued for at least eight days. The decrease in absorption was not accompanied by significant increase in absorption in the visible range, however, there appeared to be elevated absorption in the region of protein absorption. This species, with low absorption in the visible region and increased absorption in the protein region was designated ApoAAT-APLP-II.

At pH 6.77 (Figure 32) the formation of apoAAT-APLP-I was also instantaneous. Furthermore, conversion of apoAAT-APLP-I to apoAAT-APLP-II was apparently rapid for decrease in absorption at 27.6 kK terminated approximately three and a half days following mixing and prominent shoulders at approximately 26.0 and 31.5 kK developed, representing a further conversion to a third enzyme-analog complex (apoAAT-APLP-III).
Figure 29. Spectra of APL.
Molar Absorptivity ($10^3$)

Wavenumber (KK)

Graph showing various absorption peaks labeled HP and H₂P.
Figure 30. Absorption spectrum of apoAAT-APLP-I in 0.02 M acetate, pH 5.07. Protein absorption has been subtracted.
Figure 31. Formation of ApoAAT-APLP-1 0.10 M in cacodylate and 0.016 M in acetate, pH 6.10. Numbers designate time in days following the mixing of apoAAT and APLP. Protein absorption has been subtracted.
Figure 32. Reaction of apoAAT and APLP, 0.1 M in cacodylate and 0.016 M in acetate, pH 6.77. Numbers designate time in hours following mixing. Protein absorption has been subtracted.
At pH 8.30 (Figure 33) the appearance of apoAAT-APLP-I was again instantaneous following mixing. The formation of apoAAT-APLP-II was not apparent. Conversion to apoAAT-APLP-III was readily observed. Approximately 16 hours were required for the complete formation of apoAAT-APLP-III which displayed a sharp absorption maximum at 26.0 kK and a smaller peak at 31.5 kK. The molar absorptivity of apoAAT-APLP-III at 26.0 kK was 12,000 M$^{-1}$ cm$^{-1}$, a considerable increase over that of apoAAT-APLP-I at 27.6 kK, which was 8,000 M$^{-1}$ cm$^{-1}$.

CD of apoAAT-APLP complexes. ApoAAT-APLP-I displayed only a trace amount of CD associated with its absorption maximum at 27.6 kK (Figure 34). However, the negative CD band centered around 33.7 kK was of opposite sign to and greater intensity than the corresponding band in the apoenzyme. The negative CD band was comparable in sign and intensity to that of holoAAT at pH 4.74 and 8.70 (Figure 7).

ApoAAT-APLP-II displayed a CD spectrum similar to that of apoAAT-APLP-I (Figure 34), the only discernible difference being the intensity of both the positive and the negative peaks. It was not known whether any change in optical activity was associated with the increase in ultraviolet absorption under the protein peak in the conversion of apoAAT-APLP-I to apoAAT-APLP-I.

ApoAAT-APLP-III possessed considerable optical activity (Figure 34) which did not correspond closely to its UV-visible absorption. The CD spectrum showed two overlapping positive bands centered around 26.0 and 29.5 kK and a striking negative band centered around 33.6 kK. The 26.0 kK
Figure 33. Absorption spectrum of apoAAT-APLP-III in 0.02 M tris HCl, pH 8.30. Protein absorption has been subtracted.
Figure 34. CD spectra of apoAAT–APLP complexes.
1. ApoAAT–APLP-I in 0.02 M acetate, pH 5.07, 30 minutes after the mixing of apoAAT and APLP
2. ApoAAT–APLP-II, 0.10 M in cacodylate and 0.016 M in acetate, pH 6.10, 8 days after mixing
3. ApoAAT–APLP-III in 0.02 M tris HCl, pH 8.30, 16 hours after mixing
4. (3) treated with NaBH$_4$
5. (4) treated with 2.2% SDS for 5 hours
peak appeared to be a composite band consisting of at least two components. The negative band showed a shoulder at approximately 31.6 kK. The intensity of the negative band is much greater than that found for the inactive holoAAT or AAT reconstituted with other PLP analogs.

**Aminotransferase activity of apoAAT-APLP-III**

ApoAAT-APLP-III possessed no AAT activity.

**Displacement of "APLP" from apoAAT-APLP by PLP**

The ability of PLP to displace the analog from apoAAT-APLP-III depended greatly on the lengths of time allowed for apoAAT and APLP to incubate before the addition of excess PLP. Short incubation time (10 minutes) made it possible to regain 25% of AAT specific activity by PLP. But, long incubation time (33 hours) permitted little (1-2%) reactivation.

**Sodium borohydride treatment of apoAAT-APLP-III**

Addition of sodium borohydride to apoAAT-APLP-III apparently resulted in the reduction of the bound analog as judged by the large hypsochromic shift from 26.0 to 30.4 kK (Figure 35). The sodium borohydride treated apoAAT-APLP-III possessed significant positive CD (Figure 34) corresponding well, in peak position to its UV-visible absorption. The comparable band in reduced holoAAT was much less intense (Torchinsky et al., 1968). The reducibility of apoAAT-APLP-III indicated that the linkage between the analog and apoAAT was likely an imine bond.

**Denaturation of apoAAT-APLP-III**

Boiling of apoAAT-APLP-III precipitated the protein but released the analog into supernatant. The spectrum of the released analog showed absorption maxima at 31.2, 31.5
Figure 35. Absorption spectrum of NaBH₄-treated apoAAT-APLP-III in 0.02 M tris HCl, pH 8.30.
and 34.8 kK at pH 8.3, 6.9 and 1.5 respectively (Figure 36). The possibility of this released analog being 4'-methyl PLP (IX) seemed quite improbable because the spectrum of 4'-methyl PLP displays absorption maxima at 30.0 and 33.9 kK at pH 7 and 1 respectively (Karpeisky et al., 1970). On the other hand, the 4-homolog of PLP (X) would be expected to have spectra with peak positions similar to those of PMP, i.e. 30.7 and 34.1 kK at pH 7 and 1 (Metzler and Snell, 1955). That the latter structure being more probable for the released analog suggested that Schiff base formation took place between some enzyme amino group and the β- rather than the α-carbon of APLP (see Discussion).

Denaturation of reduced apoAAT-APLP-III Although 0.1% sodium dodecylsulfate (SDS) did not significantly affect the CD of the reduced
Figure 36. Spectra of analog released from apoAAT-APLP-III.

1. 0.02 M in tris HCl, pH 8.30
2. 0.45 M in phosphate, 0.02 M in tris HCl, pH 6.93
3. 0.95 M in HCl, 0.45 M in phosphate, 0.02 M in tris HCl, pH 1.50
apoAAT-APLP-III, 2.2% SDS totally abolished the large CD band at 30.4 kK (Figure 34). 0.1 N NaOH had the same effect. Thus the optical activity in the reduced complex was induced by the enzyme as that of the co-factor in holoAAT (Fasella and Hammes, 1964) rather than inherent in some molecular asymmetry.

Elution of the denatured, reduced apoAAT-APLP-III. No APLP or its derivative was released from reduced apoAAT-APLP-III following treatment with SDS. The protein and the analog co-chromatographed on a Sephadex-G-25 column (Figure 37).

Pyridoxamine thiophosphate (PM thiophosphate)

Acid dissociation constants. The four ionic forms of PM thiophosphate according to their extent of protonation (Figure 38) were computed from spectrophotometric and pH data. The pK's of the phenolic, pyridinium and amino moieties were calculated to be 3.64, 8.50 and 10.84 respectively. These values are similar but not identical to the corresponding pK's of PMP which are 3.32, 7.68 and 10.02 determined in this laboratory.

Binding by apoAAT. At pH 8.3 the binding of PM thiophosphate to apoAAT occurred instantaneously following mixing with the enzyme. The resulting spectrum (Figure 39) showed an absorption maximum at 30.1 kK, representing a bathochromic shift from that of the free analog, which, at pH 8.3, would be somewhere between 30.5 and 32.0 kK, according to the spectra of the H$_2$P and HP forms and the pK of 8.50 of the analog (Figure 38).
Figure 37. Elution of denatured, reduced apoAAT-APLP-11, 2.2% in SDS, 0.02 M in tris HCl, pH 8.3. Column size: 47 x 0.9 cm; flow-rate: 14.5 sec/drop; fraction size: 40 drops (1.5 ml); eluent: 0.1% SDS.
Figure 38. Spectra of PM thiophosphate.
Figure 39. Absorption spectrum of apoAAT-PM thiophosphate in 0.01 M tris HCl, pH 8.3. Protein absorption has been subtracted.
Ami notransferase activity of apoAAT-PM thiophosphate

ApoAAT-PM thiophosphate was assayed to have 11% of the apoAAT-PMP activity, indicating that some spatial tolerance was available in the vicinity of the 5'-carbon, consistent with the observation that 5'-methyl PLP possessed 3% of AAT activity (Furbish et al., 1969).

Pyridoxal sulfate (PL sulfate)

Acid dissociation constants

The three ionic forms of pyridoxal sulfate (Figure 40) according to their extent of protonation were computed from spectrophotometric titration and pH data. The three species were related by the pK's of 3.54 and 7.39. The spectra bore close resemblance to those of the natural coenzyme PLP in band shape, absorption maxima and molar absorptivity. The pyridinium pK of PLP (8.33) was somewhat higher than that of pyridoxal sulfate.

Binding by apoAAT

At pH 8.20, the resultant spectrum (Figure 41) immediately following mixing of PL sulfate and apoAAT was broad in width and low in intensity. It consisted of a peak at 24.8 kK which showed prominent fine structure and two shoulders at approximately 23.2 kK and 29.2 kK. Subsequent spectra showed that the original absorption band underwent a time-dependent narrowing and sharpening such that the spectrum recorded two hours following mixing attained an absorption maximum at 24.9 kK with a width of 2.71 kK and a maximum height of $9.88 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Incubation beyond 15 hours apparently caused side reactions to take place, which gradually reduced the peak height at 24.8 kK accompanied by increase in absorption around 23 and 32.5 kK.
Figure 40. Spectra of PL sulfate.
Figure 41. Absorption spectra of apoAAT-PL sulfate, in 0.02 M tris HCl, pH 8.20. Numbers designate time in minutes following mixing of apoAAT and PL sulfate.
At pH 4.97, the spectrum (Figure 42) immediately following mixing of PL sulfate and apoAAT was also broad in width and low in intensity. In this case, the peak position appeared at 25.9 kK and shoulders at around 23.5 and 29.5 kK. Continuous monitoring of the absorption revealed similar narrowing and sharpening as the system at pH 8.20, although the extent of increase in peak height was significantly less at pH 4.97. The spectrum of apoAAT-PL sulfate was stabilized approximately 2.5 hours following mixing, characterized by peak position of 25.9 kK, peak height of $5.34 \times 10^3$ M$^{-1}$ cm$^{-1}$ and width of 3.502 kK. This final spectrum was stable for at least 11 hours. The final spectrum of apoAAT-PL sulfate at pH 4.97 differed from that at pH 8.20 in several respects. First of all, the peak position was different, being 25.9 kK at pH 4.97 and 24.9 kK at pH 8.20. Secondly the peak height was greater at pH 8.20 than at pH 4.97. Thirdly, the width showed that the pH 8.20 spectrum was much narrower than the pH 4.97 spectrum. Finally while the low pH spectrum displayed some fine structure, it was not nearly as prominent as the high pH spectrum.

**CD of apoAAT-PL sulfate**  
ApoAAT-PL sulfate possessed very weak optical activity. At pH 8.20, the CD spectrum (Figure 43) displayed a positive peak at 24.8 kK, corresponding to its UV-visible absorption maximum. In addition, there appeared to be a small negative band centered around 30.2 kK and a second negative band centered around 33.6 kK, the latter comparable in sign and intensity to the 33.6 kK band seen in the CD spectrum of the native holoenzyme (Figure 9). At pH 4.97, the CD
Figure 42. Absorption spectra of apoAAT-PL sulfate, in 0.04 M acetate, pH 4.97. Numbers designate time in minutes following mixing of apoAAT and PL sulfate.
Figure 43. CD spectra of apoAAT-PL sulfate.
1. 0.01 M in tris HCl, pH 8.20, 15 hours following mixing of apoAAT and PL sulfate
2. 0.04 M in acetate, 0.01 M in tris HCl, pH 4.97, 12 hours following mixing
spectrum (Figure 43) showed a negative band at 26.2 kK corresponding to its UV-visible absorption maximum. The remainder of the CD spectrum seemed to consist of two more negative bands, one centered around 32 kK and the other peaked clearly at 33.5 kK. Again, the 33.5 kK peak was very similar to the negative band in the holoenzyme.

Aminotransferase activity of apoAAT-PL sulfate AAT reconstituted with PL sulfate was assayed to have no aminotransferase activity.

Displacement of PL sulfate from apoAAT-PL sulfate by PLP Once bound, the analog could not at all be displaced by PLP.

Sodium borohydride treatment of apoAAT-PL sulfate Addition of sodium borohydride to apoAAT-PL sulfate at either pH 8.20 or 4.97 had no effect on the spectra.

Denaturation of apoAAT-PL sulfate Denaturation of apoAAT-PL sulfate in 1.3 N hydrochloric acid did not release the analog derivatized on the enzyme. The spectrum of the supernatant showed aside from a trace amount of absorption at 36 kK due to dissolved protein, no absorption band at all, indicating the absence of any chromophore. On the other band, the spectrum of the precipitate redissolved in guanidine hydrochloride displayed strong absorption with a peak at 26.7 kK, indicating that the analog remained on the enzyme during denaturation.

Reactions of PL sulfate with p-substituted amines Since PL sulfate seemed to label some residue following binding to apoAAT, it was thought useful to attempt mimicking the reaction in non-enzymic systems. Cysteine PL sulfate readily reacted with cysteine (Figure 44). At pH 9.0, the reaction could be followed in the spectrophotometer.
Figure 44. Reaction of PL sulfate and cysteine (molar ratio = 1:1,000), pH 9.0. Numbers designate time in minutes following the mixing of reactants.

Figure 45. Spectra of PL sulfate plus cysteine (molar ratio = 1:1,000).
1. pH 9.0, 18 hours following the mixing of reactants
2. (l), pH adjusted to 1.1 with concentrated HCl.
Initially, a sharp absorption band appeared at 31.0 kK, apparently representing the formation of a thiazolidine (XI) similar to that observed between pyridoxal phosphate and cysteine (Heyl et al., 1948; Buell and Hansen, 1960). The peak soon began to decrease in height accompanied by the appearance, and increase of a band at 24.8 kK, the latter band displaying unmistakable fine structure even as shortly as ten minutes following the mixing of the reactants, closely resembling that obtained as the analog was allowed to bind to apoAAT.

The peak position of the high pH spectrum shifted to 26.0 kK upon acidification (Figure 45). This transition was accompanied by a large decrease in molar absorptivity. Both the peak shift and the change in molar absorptivity in response to pH variation bore close similarity to the enzymic reaction (Figures 41 and 42).

β-mercaptoethylamine The carboxylate function of cysteine was not required for the reaction described above. In the case of mercaptoethylamine (cysteamine), the time course of reaction with DL sulfate, with respect to the 31.1 and 24.8 kK peaks (Figure 46), was
Figure 46. Reaction of PL sulfate and mercaptoethylamine (molar ratio = 1:2,000), pH 9.0. Numbers designate time in minutes following the mixing of reactants.

Figure 47. Spectra of PL sulfate plus mercaptoethylamine (molar ratio = 1:2,000).
1. pH 9.0, 16 hours following the mixing of reactants
2. (l), pH adjusted to 2.5 with concentrated HCl
indistinguishable from cysteine. The pH response was also very similar (Figure 47).

**Ethylenediamine** The β-substituent could also be an amino group as in ethylenediamine (Figure 48). Following mixing, there was transient absorption at around 23 kK, suggesting the initial formation of a Schiff base. Subsequent spectra showed that ethylenediamine reacted similarly to cysteine and mercaptoethylamine. However, the 29.8 kK peak, in this instance, represented an imidazolidine rather than a thiazolidine.

**Ethanolamine** Hydroxyl group was also an acceptable β-substituent. Ethanolamine reacted with PL sulfate in a manner similar to ethylenediamine (Figure 49). The characteristic peak position and fine structure were evident. Prolonged incubation of the reaction mixture, however, converted the shape spectrum to nondescript forms.

**Serine** At pH 7.0, the spectrum of the reaction mixture of PL sulfate plus serine showed a peak at 24.2 kK, a typical position for a Schiff base. There was no absorption peak in the range of 29 to 31 kK, a region where oxazolidines would be expected to absorb strongly. Prolonged incubation up to 18 hours at room temperature did not affect the spectrum.

At pH 9.0, Schiff base formation, as indicated by a fine structureless absorption maximum at 24.2 kK, was again instantaneous following the mixing of PL sulfate and serine. Two hours of incubation at room temperature shifted the absorption maximum to 24.6 kK accompanied by the
Figure 48. Reaction of PL sulfate and ethylenediamine (molar ratio = 1:15,000), pH 7.0. Numbers designate time in minutes following the mixing of reactants.
Figure 49. Reaction of PL sulfate and ethanolamine (molar ratio = 1:15,000) pH 10.0. Numbers designate time in minutes following the mixing of reactants.

Figure 50. Reaction of PL sulfate and serine (molar ratio = 1:1,000), pH 10. Numbers designate time in minutes following the mixing of reactants.
the appearance of the first sign of fine structure. In ten hours, the absorption band had further narrowed and sharpened and displayed its peak at 24.8 kK with the characteristic fine structure. As in the pH 7.0 case, the absorption at around 29-31 kK remained low and showed only small changes during the course of incubation. Thus the reaction at pH 9.0 between PL sulfate and serine mimicked the enzymic reaction.

At pH 10.0 (Figure 50) the conversion of the 24.2 kK, fine structure-less band to the 24.8 kK, fine structured band proceeded even more rapidly, reaching maximum height in an hour. Further reaction gradually decreased the 24.8 kK absorption. In 22 hours, the absorption spectrum became quite featureless showing only low absorption in the visible region and increasing absorption in the ultraviolet end of the spectrum.

Reaction of PL sulfate with arginine At pH 7.0, arginine reacted with PL sulfate instantaneously, giving rise to a spectrum with an absorption maximum at 24.0 kK along with a shoulder at 30 kK, indicating the formation of a Schiff base. Observed over a 3-day period, the absorption at 24.0 kK gradually decreased and was compensated by increases at 31 kK, suggesting the conversion to a species similar to thiazolidine or oxazolidine.

At pH 9.0, the formation of the PL sulfate-arginine Schiff base was unmistakable, however, its subsequent conversion to the species absorbing at 31.0 kK was not observable. Instead, the transition involved the shift of the absorption maximum from 24.0 to 24.8 kK, accompanied by the appearance of the characteristic fine structure and some
increase in molar absorptivity. A small decrease in absorption at 30.0
kK was associated with the transition. Incubation much beyond three
hours gradually reduced the spectrum to a nondescript form.

At pH 10 (Figure 51), the reaction resembled that at pH 9.0 except
for the accelerated rate which made possible maximal absorption at 24.8
kK in two hours. Conversion to some species with a nondescript spectrum
took place on prolonged incubation.

Other reactions of PL sulfate While reactions of the above β-
substituted amines and arginine with PL sulfate mimicked the binding of
the analog to apoAAT, amines without nucleophilic β-substituents did
not react with PL sulfate to yield spectra with the characteristic fine
structure.

Butylamine At pH 9.0, mixing of PL sulfate and butylamine
immediately lead to the formation of a Schiff base as evidenced by the
absorption maxima at 24.4, 36.5 and 43.3 kK and a low shoulder at approxi-
mately 29 kK (Figure 52). The Schiff base thus formed was stable for
up to an hour, beyond which time, the 24.4 kK peak was observed to de-
crease apparently as a result of slow transamination.

Valine PLP and valine readily formed a very stable Schiff
base (Metzler et al., 1954). The reaction of PL sulfate and valine was
identical in nature. At pH 9.0, the resulting spectrum displayed maxima
at 24.2, 36.0 and 43.3 kK and a shoulder at approximately 29 kK (Figure
53). The spectrum did not possess any fine structure. Furthermore, the
Schiff base was stable and showed no spectral change on prolonged
incubation.
Figure 51. Reaction of PL sulfate and arginine (molar ratio = 1:1,000), pH 10.0. Numbers designate time in hours following the mixing of reactants.
Figure 52. PL sulfate-butylamine Schiff base (molar ratio = 1:10,000), pH 9.0.

Figure 53. PL sulfate-valine Schiff base (molar ratio = 1:10,000), pH 9.0.
Imidazole  Imidazole incubated with PL sulfate for up to ten hours did not alter the spectrum of the analog, indicating the lack of reactivity of the analog toward imidazole.

Histidine  Pyridoxal and histidine readily formed a tetrahydropyridine type of structure (Abbott and Martell, 1970) arising from cyclization of the initially formed Schiff base. PL sulfate and histidine reacted in a similar fashion. The spectrum of the mixture initially showed an absorption maximum at 24.0 kK, indicative of a Schiff base. On standing, absorption at 24.0 kK gradually decreased to zero while that at 32.5 kK increased, representing the formation of the tetrahydropyridine ring (XII). Reaction beyond this stage was not observed.

β-Mercaptoethanol  Addition of mercaptoethanol to PL sulfate immediately shifted the absorption maximum of the analog from 25.8 kK to 31.4 kK. Absorption remaining at 25.8 kK was close to zero, indicating nearly complete conversion of the free analog to the 31.4 kK species which appeared to be an adduct, i.e., a hemimercaptal (Bergeland Harrap, 1961). No further reaction occurred judging from the lack of spectral change.
β-Mercaptoethanol followed by butylamine. Since serine, ethanolamine and mercaptoethylamine all reacted in a way similar to that of the enzyme with PL sulfate, it was of interest to see if a mixture of mercaptoethanol and butylamine would do the same. To the reaction mixture of PL sulfate and mercaptoethanol described above was added excess butylamine. The spectrum underwent a gradual change. Absorption at 31.4 kK decreased and was compensated by a concurrent increase at 24.0 kK, suggesting the formation of a Schiff base by displacing mercaptoethanol from the adduct. No fine structure was observed. Therefore, it appeared that mimicking of the enzyme interaction with PL sulfate required the presence of both an amino and a nucleophilic substituents on the same molecule, suggesting the importance of proximity of these two groups for the reaction.

Sodium borohydride treatment of the product in the reaction mixture of PL sulfate plus cysteine. At pH 9.0, addition of sodium borohydride to the reaction product of PL sulfate plus cysteine reduced the absorption maximum at 24.8 kK to 60% of its original height in ten hours. Concomitant increase in absorption occurred in the region of 32 to 33 kK. Thus treatment with sodium borohydride appeared to reduce the species absorbing at 24.8 kK. The reduction was only partial, indicating that the reaction proceeded with difficulty. The remaining absorption at 24.8 kK represented the unreacted species rather than a new substance for it still possessed the characteristic fine structure and shifted to 26.0 kK on acidification.
At pH 1.1, sodium borohydride also reduced the reaction product of PL sulfate plus cysteine as evidenced by the diminution of the 26.0 kK peak and increase in absorption at around 34 kK. The lowered pH adversely affected the extent of the reaction. In three hours, more than 80% of the absorption at 26.0 kK remained unchanged.

**Sodium borohydride treatment of the reaction product of PL sulfate plus mercaptoethylamine**

Sodium borohydride treatment of the reaction product of PL sulfate plus mercaptoethylamine at pH 7.5 also resulted in reduction as judged from the gradual decrease of absorption at 24.8 kK. Compared to the system of PL sulfate plus cysteine, the reduction in this case progressed somewhat more rapidly. A 75% decrease of the 24.8 kK peak occurred in two hours.

Although sodium borohydride treatment was not done on other systems which mimicked the enzyme binding reaction of PL sulfate, it was assumed that partial reduction would also take place.

**Release of inorganic sulfate in the reaction of PL sulfate and cysteine**

Alkyl acid sulfates are known to undergo elimination of sulfuric acid to give alkenes (Roberts and Caserio, 1965). To elucidate the nature of the reaction of PL sulfate with β-substituted amines in forming the species which displayed an absorption maximum at 24.8 kK with fine structure, it was of interest to learn whether the sulfate moiety at the 5'-position was released.

Nephelometric determination of free sulfate trapped as barium sulfate suspension was conducted along with monitoring of absorption at 24.8 kK. The time course of the production of inorganic sulfate paralleled
the increase in absorption at 24.8 kK (Figure 54). Obviously, in the formation of the 24.8 kK species, the sulfate moiety of the analog was simultaneously released. Moreover, by comparison with the standard curve (Figure 55) the total amount of sulfate released was equal to that originally present in PL sulfate, indicating complete release of sulfate from the analog.

Reaction of PL sulfate and cysteine as followed by nmr  The nmr spectrum of the thiazolidine obtained from pyridoxal phosphate and cysteine has been reported (Abbott and Martel, 1970). For the corresponding thiazolidine formed between PL sulfate and cysteine to proceed to the product with the characteristic UV-visible absorption spectrum via loss of the 5'-sulfate moiety, it seemed likely that proton loss and rearrangement would also be involved and thus the course of the reaction could be subjected to monitoring with nmr spectroscopy.

PL sulfate incubated with 2-fold excess of cysteine at pH 7.5 gave rise to the following time course of nmr spectra (Table 3).

The chemical shifts and their assignments for the nmr spectrum at time = 6 min. were in good agreement with those attributed to the thiazolidine formed between PLP and cysteine. Thus the nmr data were consistent with UV-visible absorption spectra, indicating that the initial complex formed possessed the thiazolidine structure (XI).

Following the formation of the thiazolidine (XI), the reaction was accelerated by an addition of sodium deuteroxide and incubation at 60°. The nmr spectrum recorded 15 minutes after the initiation of the reaction
Figure 54. Release of inorganic sulfate and production of the 24.8 kK species in the reaction of PL sulfate (final concentration 1.13 mM) plus L-cysteine (final concentration = 0.1 M), pH 9.0.

1. Light scattering curve
2. Absorbance at 24.8 kK curve
Figure 55. Standard curve for inorganic sulfate determination.
Table 3. Nmr spectral data on PL sulfate\textsuperscript{a} plus cysteine\textsuperscript{b}  

<table>
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<th>Time</th>
<th>Chemical shift, $\delta$</th>
<th>Assignment</th>
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<td>2-CH$_3$ of free cysteine</td>
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<td>$\beta$-H$_2$ of free cysteine</td>
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<tr>
<td></td>
<td>3.59</td>
<td>$\beta$-H$_2$ of thiazolidine</td>
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<td></td>
<td>4.05</td>
<td>$\alpha$-H of free cysteine</td>
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<tr>
<td></td>
<td>4.36</td>
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<tr>
<td></td>
<td>5.13</td>
<td>5-CH$_3$</td>
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<td></td>
<td>6.10</td>
<td>4-CH$_2$ of free cysteine</td>
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<td>15 minutes</td>
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\textsuperscript{a}PL sulfate hydrate lyophilyzed from D$_2$O. Final concentration 0.05 M, pD $\sim$ 7.5 with NaOD.

\textsuperscript{b}L-cysteine·HCl·H$_2$O. Final concentration 0.1 M, pD $\sim$ 7.5 with NaOD.
showed that the resonance of 5-CH$_2$ at 5.15 ppm and of 4-CH at 6.10 ppm decreased considerably, whereas a new resonance appeared at 5.44 ppm, a position that might be satisfactorily ascribed to a = CH$_2$ group (Chamberlain, 1959). These changes were consistent with the conversion of the thiazolidine (XI) to an orthoquinoid intermediate (XIII).

One and a quarter hours later, the reaction proceeded past the orthoquinoid intermediate. The nmr spectrum at this time showed only residual resonance attributable to the CH$_2$ group. By this time, the resonance due to 5-CH$_2$ of the thiazolidine (XI) was entirely absent, but another previously unseen chemical shift now appeared at 2.21 ppm, indicating the emergence of a methyl group, the peak size of which suggested that the methyl protons had partially exchanged with the media. At this stage, α-H of either free or complexed cysteine could not be readily distinguished from machine noise. These spectral changes pointed to the formation of a cyclic Schiff base (XIV).

Further incubation (5 1/2 hours) merely lead to a more complete formation of the cyclic Schiff base (XIV) as indicated by the total
disappearance of the resonance at 5.40 ppm due to $\text{CH}_2$ and the increase in peak size at 2.24 ppm due to $5\text{-CH}_2\text{D}$.

Compounds XIII and XIV possessed the necessary conjugation to show UV-visible absorption bands at 24-25 kK (Heinert and Martell, 1963a, 1963b). Thus the nmr results appeared to be congruous with those of the nephelometric and the spectrophotometric experiments.

Part II. Band Shape Analysis of Protein Spectra

Shapes of many electronic absorption spectra can be adequately described as the sum of several lognormal distribution curves, one for each absorption band (Siano and Metzler, 1969). Here the method is applied to the analysis of UV-visible absorption spectra of proteins.

Properties of the lognormal curve

Near-uniqueness of the fit The protein peak of the absorption spectra of enzymes was readily fitted with a lognormal distribution curve whose parameters, peak position ($\bar{\nu}$), peak height ($c_0$), width (W) and skewness ($\rho$) were nearly unique for any given spectrum as illustrated in the following example.

A typical spectrum of active apoAAT (Figure 56) was first truncated at 38.00 kK, a wave number above which the spectrum contained contribution from the higher-energy band. In order not to place undue emphasis on the "foot" of the spectrum, which might arise from spectrophotometric error and slight misfit of the lognormal, the spectral data below the wave number $\bar{\nu}$-1.00 kK, i.e. 33.00 kK were automatically overlooked in computing the best fit. Initial estimates of the parameters were
Figure 56. Spectrum of active apoAAT in 0.02 M tris HCl, pH 8.26 showing experimental points (XXX), the two lognormal curves (---) and the sum of the curves.
supplied and the standard deviation (S.D.) was minimized by the computer in fifteen iterations (Table 4).

The eight sets of initial estimates of the parameters represented some of the possible ways one would be expected to make such estimates. In trials 4-8, initial value of $\tilde{\nu}_o$ was first selected at or near the experimental absorption maximum of 35.80 kK, the remaining parameters were then picked from the digitized data for the protein peak with no attempt to estimate what the final values for $e_o$, W and $p$ might be. Thus, these trials included nearly all initial values one would likely choose, that is to say, the trials represented the worst possible estimates of W and $p$ starting with some selected value of $\tilde{\nu}_o$ whose range was also amply allowed between 35.60 and 36.00 kK. Trials 2 and 3 adequately covered the range of W and $p$ - approximately 10% on either side of the best values. Trial 1 allowed the possibility of making good initial estimates. Judging from the standard deviation (Table 4), it was obvious that the same goodness of fit was obtained no matter how close to or far from, within reasonable bounds, the best final values the initial values were. Furthermore, the final values of the four parameters obtained in all trials were quite similar. Peak position ($\tilde{\nu}_o$) could be measured without any ambiguity; peak height ($e_o$) to $\pm 0.1 \times 10^3$ M$^{-1}$ cm$^{-1}$ width (W) to $\pm 0.01$ kK and skewness ($p$) to $\pm 0.01$. On percentage basis, these standard deviations were $\pm 0$, $\pm 0.1$, $\pm 0.2$ and $\pm 0.4\%$ respectively. To the extent that these parameters were easily reproducible, it was clear that the lognormal fitting of the spectrum was nearly unique.
<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Feature of initial values</th>
<th>Initial values</th>
<th>Final values</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Good estimates</td>
<td>35.77 63.2 4.74 1.72</td>
<td>35.77 63.1 4.67 1.68</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>Large W &amp; ρ</td>
<td>35.77 63.2 5.20 1.90</td>
<td>35.77 63.1 4.67 1.67</td>
<td>0.62</td>
</tr>
<tr>
<td>3</td>
<td>Small W &amp; ρ</td>
<td>35.77 63.2 4.20 1.50</td>
<td>35.77 63.2 4.66 1.68</td>
<td>0.58</td>
</tr>
<tr>
<td>4</td>
<td>Peak at 35.60 kK</td>
<td>35.60 63.1 4.78 2.06</td>
<td>35.77 63.1 4.69 1.69</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>Peak at 35.70 kK</td>
<td>35.70 63.1 4.78 1.88</td>
<td>35.77 63.1 4.68 1.68</td>
<td>0.60</td>
</tr>
<tr>
<td>6</td>
<td>Peak at 35.80 kK</td>
<td>35.80 63.2 4.78 1.72</td>
<td>35.77 63.2 4.66 1.68</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>Peak at 35.90 kK</td>
<td>35.90 63.1 4.78 1.37</td>
<td>35.77 63.1 4.68 1.68</td>
<td>0.60</td>
</tr>
<tr>
<td>8</td>
<td>Peak at 36.00 kK</td>
<td>36.00 62.7 4.80 1.44</td>
<td>35.77 63.0 4.68 1.69</td>
<td>0.61</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>35.77 63.1 4.67 1.68</td>
<td>± 0.00 ± 0.1 ±0.01 ±0.01</td>
<td></td>
</tr>
</tbody>
</table>

a Same sample as apoAAT-LM, pH 8.26 (see Table 12).

b Unit is kK in this and subsequent tables.

c Unit is $10^3$ M$^{-1}$ cm$^{-1}$ in this and subsequent tables.

d Unitless in this and subsequent tables.
The near-uniqueness of the lognormal fitting was also manifested in features of the fine structure plots, such as fine structure peak position and height (Figure 57). For the same eight trials, all the fine structure peak positions were located without any ambiguity (Table 5).

Table 5. Fine structure peak positions of active apoAAT

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Feature of initial values</th>
<th>Fine structure peak position&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Good estimates</td>
<td>34.40 35.30 35.90 (36.70)&lt;sup&gt;b&lt;/sup&gt; 37.25 37.80</td>
</tr>
<tr>
<td>2</td>
<td>Large W &amp; p</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>3</td>
<td>Small W &amp; p</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>4</td>
<td>Peak at 35.60 kK</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>5</td>
<td>Peak at 35.70 kK</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>6</td>
<td>Peak at 35.80 kK</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>7</td>
<td>Peak at 35.90 kK</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
<tr>
<td>8</td>
<td>Peak at 36.00 kK</td>
<td>34.40 35.30 35.90 (36.70) 37.25 37.80</td>
</tr>
</tbody>
</table>

| Average   | 34.40 35.30 35.90 35.70 37.25 37.80 | + 0.00 + 0.00 + 0.00 + 0.00 + 0.00 + 0.00 |

<sup>a</sup> Measured to the nearest 0.05 kK in this and subsequent tables.

<sup>b</sup> Parentheses denote a shoulder or a very weak peak in this and subsequent tables.

The height of the two prominent peaks arising from tryptophan and tyrosine at 34.40 and 35.30 kK were 3.02 ± 0.03% and 2.32 ± 0.01% respectively (Table 6). On percentage basis, the standard deviation amounted to only ± 0.9 and ± 0.6% of the mean.

Such near-uniqueness in the lognormal fitting of absorption spectra made it possible for the parameters and the fine structure features of
Figure 57. Fine structure plot of active apoAAT in 0.02 M tris HCl, pH 8.26. Arrow indicates position of V_o in this and subsequent figures.
various spectra to be compared.

Table 6. Fine structure peak heights of active apoAAT

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Feature of initial values</th>
<th>Fine structure peak height&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>34.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>Good estimates</td>
<td>3.03</td>
</tr>
<tr>
<td>2</td>
<td>Large W and ρ</td>
<td>3.08</td>
</tr>
<tr>
<td>3</td>
<td>Small W and ρ</td>
<td>3.00</td>
</tr>
<tr>
<td>4</td>
<td>Peak at 35.60 kK</td>
<td>2.98</td>
</tr>
<tr>
<td>5</td>
<td>Peak at 35.70 kK</td>
<td>3.02</td>
</tr>
<tr>
<td>6</td>
<td>Peak at 35.80 kK</td>
<td>3.04</td>
</tr>
<tr>
<td>7</td>
<td>Peak at 35.90 kK</td>
<td>3.01</td>
</tr>
<tr>
<td>8</td>
<td>Peak at 36.00 kK</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>3.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> Unit is percent (%) of peak height (ε<sub>0</sub>) in this and subsequent tables.

<sup>b</sup> Such numbers designate fine structure peak positions in kK in this and subsequent tables.

Tolerance of the parameters In obtaining the preceding results, the computer was allowed to find the best fit without any constraint according to the method of Fletcher and Powell (1963). It was, however, possible to fix one parameter, e.g. the skewness (ρ) at certain pre-selected value and still obtain a lognormal curve with the same goodness of fit (standard deviation ~ 0.60). Thus, the parameters appeared to have a certain degree of tolerance. For the example used above, the tolerance in skewness (ρ) was approximately ± 2% (Table 7) of the optimal
Table 7. Tolerance of skewness (\( \rho \))

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Constraint</th>
<th>( \nu_0 )</th>
<th>( e_0 )</th>
<th>Final values</th>
<th>( \rho )</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \rho = 5% &gt; 1.68 )</td>
<td>35.75</td>
<td>62.9</td>
<td>4.79</td>
<td>1.764*(^a)</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>( \rho = 3% &gt; 1.68 )</td>
<td>35.76</td>
<td>62.9</td>
<td>4.74</td>
<td>1.730(^*)</td>
<td>0.63</td>
</tr>
<tr>
<td>3</td>
<td>( \rho = 2% &gt; 1.68 )</td>
<td>35.76</td>
<td>63.0</td>
<td>4.72</td>
<td>1.714(^*)</td>
<td>0.61</td>
</tr>
<tr>
<td>4</td>
<td>( \rho = 1% &gt; 1.68 )</td>
<td>35.76</td>
<td>63.2</td>
<td>4.69</td>
<td>1.697(^*)</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>( \rho = 1% &lt; 1.68 )</td>
<td>35.77</td>
<td>63.2</td>
<td>4.65</td>
<td>1.667(^*)</td>
<td>0.60</td>
</tr>
<tr>
<td>6</td>
<td>( \rho = 2% &lt; 1.68 )</td>
<td>35.78</td>
<td>63.3</td>
<td>4.62</td>
<td>1.650(^*)</td>
<td>0.61</td>
</tr>
<tr>
<td>7</td>
<td>( \rho = 3% &lt; 1.68 )</td>
<td>35.78</td>
<td>63.4</td>
<td>4.60</td>
<td>1.633(^*)</td>
<td>0.63</td>
</tr>
<tr>
<td>8</td>
<td>( \rho = 5% &lt; 1.68 )</td>
<td>35.79</td>
<td>63.4</td>
<td>4.55</td>
<td>1.596(^*)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

\(^a\) Asterisk (\(^*\)) denotes fixed skewness (\( \rho \)) in this and subsequent tables.

Value found (1.68) when no initial estimate was fixed.

Associated with the \( \pm 2\% \) tolerance in skewness (\( \rho \)), the peak position (\( \nu_0 \)), the peak height (\( e_0 \)) and the width (\( W \)) had tolerances of \( \pm 0.03 \), \( \pm 0.3 \) and \( \pm 1\% \) respectively.

Fixing of the skewness (\( \rho \)) in this way had no effect at all on the position of the fine structure peaks which remained at 34.40, 35.30, 35.90, (36.70), 37.25, and 37.80 kK. However, it was found to change the peak height by approximately \( \pm 5 \) and \( \pm 1\% \) at 34.40 and 35.30 kK respectively (Table 8).

Even though such tolerance existed in the various features of the lognormal, they appeared, to a great extent, quite reproducible.
Table 8. Effect of skewness ($p$) fixing on fine structure peak heights

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Constraint</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p = 2% &gt; 1.68$</td>
<td>$2.88$ $2.29$</td>
</tr>
<tr>
<td>3</td>
<td>$p = 1% &gt; 1.68$</td>
<td>$2.95$ $2.32$</td>
</tr>
<tr>
<td>4</td>
<td>$p = 1% &lt; 1.68$</td>
<td>$3.09$ $2.31$</td>
</tr>
<tr>
<td>5</td>
<td>$p = 2% &lt; 1.68$</td>
<td>$3.17$ $2.29$</td>
</tr>
</tbody>
</table>

*Referring to the same numbers as those of Table 7.*

**Spectra of reference compounds**

**Lognormal parameters** For the purpose of comparison, the experimental and the fitted spectra of N-acetyl ethyl esters of tryptophan (trp), tyrosine (tyr) and phenylalanine (phe) (Metzler et al., 1972) are presented (Figure 58). Each experimental spectrum was fitted with two lognormal curves, the second of which was far from unique due to the fact that it described only the leading edge of another higher-energy absorption band. The band parameters for the first absorption bands of these amino acid derivatives in water and in methanol are tabulated below (Table 9).

**Fine structure peak positions** The difference plots (Figures 59 and 60) showed distinct vibronic fine structure. The principal fine structure peak position of the three spectra are tabulated below (Table 10).
Figure 58. Spectra of the reference compounds in methanol.
1. N-Ac-Trp-OEt
2. N-Ac-Tyr-OEt, × 2
3. N-Ac-phe-OEt, × 20
Figure 59. Fine structure plots of the reference compounds in methanol.
1. N-Ac-trp-OEt
2. N-Ac-tyr-OEt
3. N-Ac-phe-OEt
Figure 60. Fine structure plots of the reference compounds in water.
1. N-Ac-trp-OEt in 0.05 M phosphate, pH 6.8
2. N-Ac-tyr-OEt in 0.05 M phosphate, pH 6.8
3. N-Ac-tyr-OEt in 0.01 M NaOH
4. N-Ac-phe-OEt in 0.05 M phosphate, pH 6.8
Table 9. Parameters of the reference compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>ν₀</th>
<th>ε₀</th>
<th>W</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ac-Trp-OEt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>35.72</td>
<td>6.11</td>
<td>4.77</td>
<td>1.87</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>35.87</td>
<td>5.57</td>
<td>5.16</td>
<td>1.95</td>
</tr>
<tr>
<td>N-Ac-Tyr-OEt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>35.85</td>
<td>1.57</td>
<td>3.00</td>
<td>1.65</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>36.22</td>
<td>1.32</td>
<td>3.03</td>
<td>1.63</td>
</tr>
<tr>
<td>in 0.01 N NaOH</td>
<td>34.08</td>
<td>2.18</td>
<td>3.72</td>
<td>1.74</td>
</tr>
<tr>
<td>N-Ac-Phe-OEt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>38.67</td>
<td>0.160</td>
<td>3.92</td>
<td>1.96</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>38.78</td>
<td>0.168</td>
<td>3.85</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Table 10. Fine structure peak positions of the reference compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fine structure peak position</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ac-Trp-OEt</td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>34.40 35.35</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>34.55 35.55</td>
</tr>
<tr>
<td>N-Ac-Tyr-OEt</td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>34.90 36.15</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>35.35 36.45</td>
</tr>
<tr>
<td>N-Ac-Phe-OEt</td>
<td></td>
</tr>
<tr>
<td>in MeOH</td>
<td>37.30 37.80 38.70 39.65 40.45 41.35</td>
</tr>
<tr>
<td>in H₂O pH 6.8</td>
<td>37.40 37.90 38.85 39.75 40.60 41.50</td>
</tr>
</tbody>
</table>
Each compound displayed fine structure peaks at its characteristic positions although some overlapping of N-Ac-trp-OEt and N-Ac-tyr-OEt occurred in the region of 35-36 kK.

The solvent effect of methanol was to shift the fine structure peak position bathochromically. The magnitude of the shift differed in the three compounds. The shift for N-Ac-tyr-OEt was the greatest, approximately 0.30-0.45 kK. Next followed N-Ac-trp-OEt, with 0.15-0.20 kK. The fine structure peak position of N-Ac-phe-OEt was least affected by solvents, the shift amounting to only 0.10-0.15 kK.

Several important features of these parameters seemed clear. First of all, N-Ac-trp-OEt had the greatest peak height ($\varepsilon_o$), followed by N-Ac-tyr-OEt and N-Ac-phe-OEt. Thus, for proteins containing comparable amounts of the three aromatic residues, the protein band would be dominated by tryptophan absorption and its lognormal parameters would bear strong resemblance to those of N-Ac-trp-OEt. Secondly, compared to those in water, the parameters were significantly altered by methanol. The peak position ($\bar{\nu}_o$) was shifted bathochromically. For N-Ac-tyr-OEt and N-Ac-tyr-OEt, the peak height ($\varepsilon_o$) increased considerably. The width ($W$) of N-Ac-trp-OEt greatly narrowed.

Dissociation of the phenolic proton in N-Ac-tyr-OEt had a profound effect on the fine structure. The fine structure plot (Figure 60) consisted of three broad and rather featureless peaks. In comparison with the sharp and narrow peaks seen in the protonated form of N-Ac-tyr-OEt in either methanol (Figure 59) or water (Figure 60), it might be
concluded the deprotonation of the phenolic proton "washed out" the fine structure of tyrosine (Metzler et al., 1972).

**Fine structure peak heights**

The fine structure peak heights (Table 11) clearly manifested the effect of solvent change. Methanol had the general effect of intensifying the vibronic fine structure in the spectra of all three compounds. This effect was most pronounced in N-Ac-trp-OEt where the increase of fine structure peak height was 75-85%. The increase was much less dramatic in N-Ac-tyr-OEt, amounting to 0-15%. Methanol also greatly intensified the fine structure peaks (25-65%) of N-Ac-phe-OEt. However, proteins which are not unusually rich in phenylalanine received small over-all contribution to their absorption spectra due to the very low molar absorptivity of phenylalanine (Table 9). Fine structure peaks attributable to phenylalanine residues were generally low and thus inadequate as a probe of protein perturbation.

**Spectra of aspartate aminotransferase**

**Spectra of active apoAAT**

The apoenzyme of AAT prepared according to the method of Furbish et al., (1969) could be fully reactivated with PLP and was termed active apoAAT.

Active apoAAT prepared from different batches of holoAAT was subjected to spectral analysis. Each experimental spectrum (Figure 56) was fitted with two lognormal curves, the sum of which closely approximated the experimental spectrum. Vibronic fine structure was vividly displayed in the difference plot (Figure 57). The peak at 34.4 kK clearly
Table 11. Fine structure peak heights of the reference compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ac-Trp-OEt</td>
<td>34.40-50 35.35-55</td>
</tr>
<tr>
<td>in MeOH</td>
<td>25.84 9.28</td>
</tr>
<tr>
<td>in H₂O, pH 6.8</td>
<td>14.06 5.34</td>
</tr>
<tr>
<td>N-Ac-Tyr-OEt</td>
<td>34.90-35.35 36.15-45</td>
</tr>
<tr>
<td>in MeOH</td>
<td>12.34 4.53</td>
</tr>
<tr>
<td>in H₂O, pH 6.8</td>
<td>10.74 4.87</td>
</tr>
<tr>
<td>N-Ac-Phe-OEt</td>
<td>37.30-40 37.80-90 38.70-85 39.65-75 40.45-60 41.35-50</td>
</tr>
<tr>
<td>in MeOH</td>
<td>17.18 29.14 37.91 20.08 8.66 4.78</td>
</tr>
<tr>
<td>in H₂O, pH 6.8</td>
<td>10.38 17.83 27.18 14.54 6.14 3.80</td>
</tr>
</tbody>
</table>
corresponded to the major fine structure peak of N-Ac-trp-OEt in methanol (Table 11). The 35.3 kK peak appeared between a tryptophan and a tyrosine fine structure peaks as observed in the respective reference compounds in methanol and appeared to contain contributions from both residues. The 35.9 kK peak closely corresponded to the minor fine structure peak of N-Ac-tyr-OEt in methanol. The remaining peaks between 27 and 41 kK could be readily assigned to phenylalanine by comparison with those of N-Ac-phe-OEt in methanol. The large peak at 33 kK was found to be present in all the enzyme spectra examined and thus might represent a systematic misfit of the lognormal fitting method. However, in many proteins (Ananthanarayanan and Bigelow, 1969; Strickland et al., 1969, 1971; Metzler et al., 1972) tryptophan residues have been postulated to absorb in this region.

Lognormal parameters of the apoenzyme obtained from various batches of holoAAT at separate times and under different conditions are summarized below (Table 12).

ApoAAT-III, IV, and V (Numbers 1-3) were resolved from earlier preparations of holoAAT, whereas the series and apoaAT at various pH values (Numbers 4-8) and those used in the Lawrence Livermore Laboratories (Numbers 9 and 10) were prepared more recently from holoAAT of superior quality. It appeared that the lognormal parameter were dependent on the quality of the enzyme. ApoAAT-III, IV and V showed greater width (W) and skewness (p), suggesting the presence of subforms other than α. Spectra recorded in Livermore possessed higher peak
Table 12. Parameters of active apoAAT

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>$\bar{v}_o$</th>
<th>$e_o$</th>
<th>W</th>
<th>p</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ApoAAT-III, pH 8.30</td>
<td>35.75</td>
<td>63.8</td>
<td>4.74</td>
<td>1.72</td>
<td>0.68</td>
</tr>
<tr>
<td>2</td>
<td>ApoAAT-IV, pH 7.80</td>
<td>35.74</td>
<td>63.8</td>
<td>4.71</td>
<td>1.71</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>ApoAAT-V, pH 8.18</td>
<td>35.74</td>
<td>63.6</td>
<td>4.77</td>
<td>1.73</td>
<td>0.65</td>
</tr>
<tr>
<td>4</td>
<td>ApoAAT, pH 5.01$^a$</td>
<td>35.75</td>
<td>63.2</td>
<td>4.68</td>
<td>1.69</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>ApoAAT, pH 6.11$^a$</td>
<td>35.72</td>
<td>63.6</td>
<td>4.65</td>
<td>1.67</td>
<td>0.62</td>
</tr>
<tr>
<td>6</td>
<td>ApoAAT, pH 7.09$^a$</td>
<td>35.71</td>
<td>63.4</td>
<td>4.61</td>
<td>1.67</td>
<td>0.56</td>
</tr>
<tr>
<td>7</td>
<td>ApoAAT, pH 7.97$^a$</td>
<td>35.72</td>
<td>63.0</td>
<td>4.62</td>
<td>1.69</td>
<td>0.58</td>
</tr>
<tr>
<td>8</td>
<td>ApoAAT, pH 9.11$^a$</td>
<td>35.78</td>
<td>63.2</td>
<td>4.77</td>
<td>1.72</td>
<td>0.71</td>
</tr>
<tr>
<td>9</td>
<td>ApoAAT-LM, pH 4.91$^b$</td>
<td>35.77</td>
<td>63.2</td>
<td>4.67</td>
<td>1.68</td>
<td>0.62</td>
</tr>
<tr>
<td>10</td>
<td>ApoAAT-LM, pH 8.26$^b$</td>
<td>35.77</td>
<td>63.2</td>
<td>4.67</td>
<td>1.68</td>
<td>0.59</td>
</tr>
</tbody>
</table>

$^a$Obtained from the same batch of apoAAT.

$^b$Spectra recorded in the Livermore Laboratories (LM).

position ($\bar{v}_o$), most likely arising from small errors in instrument calibration. Considerable variation could be observed in molar absorptivity ($e_o$) obviously reflecting errors in dilution and turbidity correction, judging from its randomness.

The effect of pH on the spectral parameters was rather small. The spectra recorded at pH 5.01, 6.11, 7.09 and 7.97 showed that the peak position ($\bar{v}_o$) seemed to be the lowest near neutrality. The values of width (W) and skewness (p) were quite similar in the pH range although there appeared to be real differences in the width. Closer scrutiny of these differences are presented in a later section.
At pH 9.11, the spectrum of apoAAT showed more clearly the effect of relatively high basicity. The width (W) and the skewness (p) had increased significantly. Although it was possible to obtain a nearly equally good fit with reduced width (W) by assigning to the spectrum a smaller skewness (p), the width (W) so obtained was still greater than those obtained for spectra at lower pH values (Table 13). This broadening of the spectrum could be readily attributed to the partial dissociation of the tyrosine phenolic protons whose pK of 10.05 (Izatt and Christensen, 1968) was not far from the pH of the solution. Dissociation of the phenolic proton caused a bathochromic shift of approximately 2 kK (Table 9) in the model compound.

In order to determine more definitively whether the lognormal parameters of the apoenzyme spectra changed as a function of pH, it was deemed advantageous to fix the skewness (p) of each spectrum. This operation was justified by the following reasons. First of all, the differences were small in the skewness (p) of the apoenzyme spectra at pH 5.01, 6.11, 7.09, 7.97 (Numbers 4-7), 4.91 and 8.26 (Numbers 9 and 10) suggesting that the slight lack of uniqueness of the lognormal fitting method rather than real differences accounted for the skewness (p) variation. Secondly, after fixing the skewness (p) at an average value of 1.695, the goodness of the fit for each spectrum remained as sound as before as judged by the standard deviation (Tables 12 and 13). Having fixed the skewness, another set of lognormal parameters was obtained (Table 13).
Table 13. Parameters of apoAAT with fixed skewness (p)

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>$\bar{v}_O$</th>
<th>$\epsilon_O$</th>
<th>W</th>
<th>$p^*$</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ApoAAT-III, pH 8.30</td>
<td>35.76</td>
<td>63.9</td>
<td>4.70</td>
<td>1.695*</td>
<td>0.68</td>
</tr>
<tr>
<td>2</td>
<td>ApoAAT-IV, pH 7.80</td>
<td>35.74</td>
<td>63.8</td>
<td>4.70</td>
<td>1.695*</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>ApoAAT-V, pH 8.18</td>
<td>35.75</td>
<td>63.7</td>
<td>4.73</td>
<td>1.695*</td>
<td>0.66</td>
</tr>
<tr>
<td>4</td>
<td>ApoAAT, pH 5.01</td>
<td>35.74</td>
<td>63.2</td>
<td>4.68</td>
<td>1.695*</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>ApoAAT, pH 6.11</td>
<td>35.71</td>
<td>63.5</td>
<td>4.68</td>
<td>1.695*</td>
<td>0.63</td>
</tr>
<tr>
<td>6</td>
<td>ApoAAT, pH 7.09</td>
<td>35.70</td>
<td>63.5</td>
<td>4.64</td>
<td>1.695*</td>
<td>0.57</td>
</tr>
<tr>
<td>7</td>
<td>ApoAAT, pH 7.97</td>
<td>35.72</td>
<td>63.0</td>
<td>4.63</td>
<td>1.695</td>
<td>0.58</td>
</tr>
<tr>
<td>8</td>
<td>ApoAAT, pH 9.11</td>
<td>35.79</td>
<td>63.2</td>
<td>4.74</td>
<td>1.695*</td>
<td>0.71</td>
</tr>
<tr>
<td>9</td>
<td>ApoAAT-LM, pH 4.91</td>
<td>35.77</td>
<td>63.2</td>
<td>4.69</td>
<td>1.695*</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>ApoAAT-LM, pH 8.26</td>
<td>35.77</td>
<td>63.1</td>
<td>4.68</td>
<td>1.695*</td>
<td>0.57</td>
</tr>
</tbody>
</table>

It seemed clear that the width (W) of the apoAAT spectrum was indeed affected by the pH of the solution. The spectrum was narrowest near neutrality. Thus, perturbation of AAT structure by pH variation could be detected by spectral analysis.

In addition to the lognormal parameters, the fine structure of difference plots (Figure 61) of apoAAT provided another avenue for examining the possible effect of pH on the apoenzyme. The fine structure peak positions were found to be quite independent of pH. The tabulated values along with their assignments (Table 14) encompassed the ten samples described above.

An observable difference in the fine structure plots at various pH values was the fine structure peak at 36.55-36.60 kK. This peak was
Figure 61. Fine structure plots of active apoAAT.
1. ApoAAT-III, pH 8.30 in 0.01 M tris HCl
2. ApoAAT-IV, pH 7.80 in 0.01 M tris HCl
3. ApoAAT-V, pH 8.18 in 0.01 M tris HCl
4. ApoAAT, pH 5.01 in 0.02 M acetate
5. ApoAAT, pH 6.11 in 0.02 N cacodylate
6. ApoAAT, pH 7.09 in 0.02 N cacodylate
7. ApoAAT, pH 7.97 in 0.02 M tris HCl
8. ApoAAT, pH 9.11 in 0.02 M bicarbonate
9. ApoAAT- LM, pH 4.91 in 0.1 M acetate-0.02 M tris HCl
10. ApoAAT- LM, pH 8.26 in 0.02 M tris HCl
Table 14. Fine structure peak positions and assignments of active apoAAT

<table>
<thead>
<tr>
<th>Peak position</th>
<th>34.35-45</th>
<th>35.25-30</th>
<th>35.80-90</th>
<th>36.55-60&lt;sup&gt;a&lt;/sup&gt;</th>
<th>37.20-25</th>
<th>37.75-80</th>
<th>38.60-70</th>
<th>39.55-65</th>
<th>40.25-35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assignment</td>
<td>trp</td>
<td>trp+tyr</td>
<td>tyr</td>
<td>tyr</td>
<td>phe</td>
<td>phe</td>
<td>phe</td>
<td>phe</td>
<td>phe</td>
</tr>
</tbody>
</table>

<sup>a</sup>Seen in only some of the samples.
small in intensity making it somewhat difficult to distinguish from machine noise. However, the peak was obviously more pronounced at neutral pH (7.09) than under either more acidic or more basic conditions. The peak position bore good correspondence to that of the minor fine structure peak of N-Ac-tyr-OEt in water (Table 11) suggesting that some tyrosine residue(s) of active apoAAT under neutral conditions might be in an aqueous environment.

Another feature of the difference plot that could be regarded as an indicator of perturbation was the height of the fine structure peaks (Table 15).

Table 15. Fine structure peak heights of active apoAAT

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>34.35-45</td>
</tr>
<tr>
<td>1</td>
<td>ApoAAT-III, pH 8.30</td>
<td>3.82 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>ApoAAT-IV, pH 7.80</td>
<td>3.96 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>ApoAAT-V, pH 8.18</td>
<td>3.78 ± 0.13</td>
</tr>
<tr>
<td>4</td>
<td>ApoAAT, pH 5.01</td>
<td>3.82 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>ApoAAT, pH 6.11</td>
<td>3.66 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>ApoAAT, pH 7.09</td>
<td>2.96 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>ApoAAT, pH 7.97</td>
<td>3.54 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>ApoAAT, pH 9.11</td>
<td>4.12 ± 0.12</td>
</tr>
<tr>
<td>9</td>
<td>ApoAAT-LM, pH 4.91</td>
<td>3.42 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>ApoAAT-LM, pH 8.26</td>
<td>2.94 ± 0.07</td>
</tr>
</tbody>
</table>

Clearly, the 34.35-45 kK (trp) and 35.25-30 kK (trp + tyr) fine structure peaks were least intense near neutrality and became sharpened
at extremes of pH. At pH 9.11, the fine structure peaks possessed much increased heights. This observation, coupled with the finding that the spectrum of apoAAT was clearly broader at pH 9.11 than those in the pH range of 5.01 to 8.26 (Table 13) indicated perturbation of protein structure by base.

The general reduction of fine structure peak heights of the two samples from Livermore (Numbers 9 and 10) appeared to arise from the differing tuning of the instrument (see DISCUSSION).

Whether the considerable differences in the two peak heights between the two Livermore samples were due to pH variation could not be ascertained although ApoAAT at pH 5.01 (Number 4) and pH 7.97 (Number 7) appeared to show a consistent trend.

Spectra of denatured apoAAT Complete denaturation of proteins converts folded native conformations to open random coils. Such gross changes could be shown in terms of the enzyme spectral characteristics.

ApoAAT was denatured in urea, guanidine hydrochloride (GueHCl), sodium dodecylsulfate (SDS) and sodium hydroxide respectively. Except for sodium hydroxide (Figure 62), the denaturants did not grossly alter the over-all shape of the apoenzyme spectrum. However, some of the important changes wrought by denaturation could be detected by examining the lognormal parameters (Table 16).

The numbers given were obtained by fixing the skewness at a reasonable value of 1.671. Similar results were obtained when all parameters were free. The standard deviations were nearly identical in both cases.
Table 16. Parameters of denatured apoAAT

<table>
<thead>
<tr>
<th>Description</th>
<th>$\nu_0$</th>
<th>$\epsilon_0$</th>
<th>W</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoAAT in 10 M urea</td>
<td>35.97</td>
<td>63.5</td>
<td>4.88</td>
<td>1.671*</td>
</tr>
<tr>
<td>ApoAAT in 6 M Gu-HCl</td>
<td>35.97</td>
<td>64.0</td>
<td>4.89</td>
<td>1.671*</td>
</tr>
<tr>
<td>ApoAAT in 0.1 M SDS</td>
<td>35.86</td>
<td>65.7</td>
<td>4.83</td>
<td>1.671*</td>
</tr>
<tr>
<td>ApoAAT in 0.1 N NaOH</td>
<td>35.18</td>
<td>62.5</td>
<td>5.24</td>
<td>1.671*</td>
</tr>
<tr>
<td></td>
<td>(35.08)</td>
<td>(63.1)</td>
<td>(4.11)</td>
<td>(1.27)</td>
</tr>
</tbody>
</table>

Fixing of skewness permitted more rigorous comparison of the results tabulated. In the case of apoAAT in 0.1 N NaOH, values obtained when all the parameters were free were also given. Because of the extensive overlapping (Figure 62) of the two bands in the spectrum, the two sets of widely different width (W) and skewness (p) both made good fit of the experimental spectrum possible but did not necessarily represent the true values.

Compared with active apoAAT (Table 13), the peak position of denatured apoAAT was shifted hypsochromically by approximately 0.2 kK, i.e. from 35.70-77 kK to 35.86-97 kK, a shift resembling that seen in the model compounds (Table 9) going from methanol to water. The broadening of the width (W) due to denaturation, i.e. from 4.63-74 kK to 4.83-89 kK was similarly seen in N-Ac-trp-0Et and N-Ac-tyr-0Et due to solvent change.

Fine structure plots (Figure 63) of denatured apoAAT also offered close scrutiny on the effect of denaturation. In the fine structure plots of active apoAAT, the peak at 36.55-60 kK was often ambiguous
Figure 62. Spectrum of apoAAT denatured in 0.1 N NaOH, pH 12.4 for 48 hours.
Figure 63. Fine structure plots of denatured apoAAT.
1. In 10 M urea, pH 5.6 for 55 hours
2. In 6 M Gu·HCl, pH 6.2 for 48 hours
3. In 0.1 M SDS, pH 8.12 for 71 hours
4. In 0.1 N NaOH, pH 12.4 for 50 hours
(Figure 61), however, the fine structure plots of denatured apoAAT, with the exception of apoAAT in sodium hydroxide, showed conspicuously a peak at 36.50 kK. Examination of the fine structure peak positions (Table 10) of the model compounds revealed that no similar peak could be found in the N-acetyl ethyl ester of either tryptophan or phenylalanine in either methanol or water. However, an unmistakable peak was found in N-Ac-tyr-OEt at 36.45 kK in water and 36.15 kK in methanol. Thus, the appearance of this peak in the fine structure plots of denatured apoAAT suggested that denaturation caused exposure of some tyrosine residue(s) to a more aqueous environment.

Another conspicuous difference in the fine structure plots of active and denatured apoAAT is the presence of a 35.80-90 kK peak in the former and the absence of it in the latter. This peak appeared to correspond to the 36.15 kK peak in the fine structure plot of N-Ac-tyr-OEt in methanol (Figure 59). Thus, it appeared likely that denaturation of apoAAT shifted some tyrosine absorption from 34.80-90 kK to 36.50 kK corresponding to the shift from 36.15 kK to 36.45 kK in the tyrosine derivative on changing solvent.

Fine structure peak positions of apoAAT denatured under different conditions were found to be nearly identical (Table 17) and were readily assigned to the aromatic residues.

In general, these fine structure peak positions did not differ greatly from those of the active apoAAT (Table 14). The peaks assigned to phenylalanine appeared to have undergone a small (0.05 kK)
Table 17. Fine structure peak positions of denatured apoAAT

<table>
<thead>
<tr>
<th>Peak position</th>
<th>34.35-40</th>
<th>35.30-45</th>
<th>36.45-50</th>
<th>37.25</th>
<th>37.70-80</th>
<th>38.65-70</th>
<th>39.50-70</th>
<th>40.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assignment</td>
<td>Trp</td>
<td>Trp+Tyr</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
<td>Phe</td>
<td>Phe</td>
<td>Phe</td>
</tr>
</tbody>
</table>
hypsochromic shift in denaturation. However, the magnitude of the shift was less than that observed in N-Ac-phe-OEt going from methanol to water (Table 10). The peak (35.30-45 kK) of mixed origin represented a clear hypsochromic shift from its corresponding position (35.25-30 kK) in active apoAAT. Contrary to the apparent trend of shifting in the direction of higher energies in denaturation, the tryptophan peak (34.35-40 kK) largely remained stationary. In fact, compared to most apoAAT samples, there seemed to be a slight (0.05 kK) bathochromic shift in denaturation, the significance of which remains to be seen.

A very striking difference in the fine structure plots of active and denatured apoAAT was the much enhanced peak heights at 34.35-40 and 35.30-45 kK (Table 18) due to denaturation.

**Table 18. Fine structure peak heights of denatured apoAAT**

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.35-40</td>
</tr>
<tr>
<td>ApoAAT in 10 M urea</td>
<td>7.02 ± 0.11</td>
</tr>
<tr>
<td>ApoAAT in 6 M Gu·HCl</td>
<td>8.59 ± 0.08</td>
</tr>
<tr>
<td>ApoAAT in 0.1 M SDS</td>
<td>11.04 ± 0.07</td>
</tr>
<tr>
<td>ApoAAT in 0.1 N NaOH</td>
<td>10.60 ± 0.11</td>
</tr>
</tbody>
</table>

In the pH range of 5 to 8, the peak height at 34.35-40 kK intensified from 3.0-4.0 to 7.0-11.0 and that at 35.30-45 kK from 2.5-2.8 to 5.0-7.1, representing an average increase of over two-fold at each wavenumber. This sharp rise in peak height indicated the homogenization
of tryptophan and tyrosine microenvironments in the process of denaturation (see DISCUSSION).

While comparison of various parameters between active and denatured apoAAT offered insights into the general nature of denaturation, examination of certain criteria revealed differences in the process of denaturation arising from the different denaturants employed.

In terms of fine structure peak height, denaturation in 0.1 M SDS offered the highest peaks at 34.35-40 and 35.30-45 kK, followed by 6 M guanidine hydrochloride and then 10 M urea. Since fine structure peak height seemed to be an indicator of the homogeneity of aromatic residue environments as suggested previously, it appeared that 0.1 M SDS was a better denaturant than guanidine hydrochloride which in turn was better than 10 M urea.

The fine structure plot of apoAAT in 0.1 N NaOH (Figure 63) possessed some special significance. It showed two prominent peaks at 34.35-40 and 35.45 kK. Although qualitatively these fine structure peaks were no different from those seen with other denaturants, quantitatively, the peak height at 35.45 kK was much smaller (Table 18). Examination of the tyrosine reference compound showed that almost all fine structure was lost upon dissociation of the phenolic proton in 0.1 N NaOH (Figure 60). Thus, the difference plot of apoAAT in 0.1 N NaOH showed only tryptophan fine structure at 34.35-40 and 35.45 kK, accounting for the reduced intensity at the latter position. It was also clear that the 36.45-50 kK peak present in other fine structure plots was absent from that in 0.1 N
NaOH, thus, supporting the previous suggestion that the peak originated from some tyrosine residue(s).

**Spectra of AAT-bound cofactor**  Active apoAAT could be readily reconstituted with PLP. Experimentally less PLP (mole/mole) was used such that the resulting mixture contained an excess of apoAAT (see EXPERIMENTAL, Methods). By subtracting the spectrum of the original apoAAT from that of the reconstituted AAT, a spectrum representing largely the enzyme-bound cofactor was obtained (Figure 64). Although considerable machine noise was evident, the reproducibility of the spectrum insured the real existence of some of the major features such as the peaks at 33.9 and 34.9 kK and the valley at 34.4 kK.

Since binding of PLP by apoAAT seemed to involve perturbation of certain aromatic residues (Ivanov et al., 1967), the spectrum obtained by subtraction was not simply that of the bound cofactor. In the ultraviolet region, i.e. from 32 to 42 kK, the spectrum included also fine structure arising from perturbation of the protein absorption.

Spectrum of AAT-bound factor was fitted with four lognormal bands (Figure 65). All the parameters (Table 19), except those of the first band, could not be known with certainty because of the heavy overlapping of the last three bands. Rather arbitrary values were given to facilitate computer iterations.

The width \( w \) of the first band appeared to be fairly constant \( (4.01 \pm 0.07) \) for the three samples. The large discrepancy in the peak height \( e_o \) of the band apparently was a result of gross measurement error.
Figure 64. Spectra of AAT-bound cofactor.
1. AAT-bound cofactor - M in 0.01 M tris HCl, pH 8.30
2. AAT-bound cofactor - V in 0.01 M tris HCl, pH 8.18
3. AAT-bound cofactor - LM in 0.02 M tris HCl, pH 8.23
Figure 65. Lognormal fitting of AAT-bound cofactor - LM in 0.02 M tris HCl, pH 8.23.
Table 19. Parameters of AAT-bound cofactor

<table>
<thead>
<tr>
<th>Description</th>
<th>Band No.</th>
<th>$v_0$</th>
<th>$e_0$</th>
<th>$W$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT-bound cofactor-III $^a$</td>
<td>1</td>
<td>27.49</td>
<td>7.06</td>
<td>4.09</td>
<td>1.484$^*$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.63</td>
<td>1.52</td>
<td>4.29</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>39.18</td>
<td>2.41</td>
<td>4.000$^*$</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>42.07</td>
<td>10.67</td>
<td>4.000$^*$</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td>AAT-bound cofactor-V $^b$</td>
<td>1</td>
<td>27.46</td>
<td>7.97</td>
<td>3.98</td>
<td>1.484$^*$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.53</td>
<td>1.67</td>
<td>3.85</td>
<td>1.400$^*$</td>
</tr>
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<tr>
<td></td>
<td>4</td>
<td>42.09</td>
<td>10.75</td>
<td>4.000$^*$</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td>AAT-bound cofactor-LM $^c$</td>
<td>1</td>
<td>27.52</td>
<td>8.55</td>
<td>3.97</td>
<td>1.484$^*$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.15</td>
<td>1.75</td>
<td>5.80</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>39.20</td>
<td>2.43</td>
<td>4.000$^*$</td>
<td>1.400$^*$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>42.11</td>
<td>12.17</td>
<td>4.000$^*$</td>
<td>1.400$^*$</td>
</tr>
</tbody>
</table>

$^a$Apoenzyme used was apoAAT-III, pH 8.30 (see Table 12).
$^b$Apoenzyme used was apoAAT-V, pH 8.18 (see Table 12).
$^c$Apoenzyme used was apoAAT-LM, pH 8.26 (see Table 12).

The difference plot (Figure 66) showed the fine structure arising from the bound cofactor and/or the protein component. Most prominent were the peaks at 33.85 and 34.90 kK which embraced a deep valley at 34.45 kK. Another peak which might originate from perturbation due to cofactor binding rather than noise was that at 40.35 kK.

Although it was different to differentiate contribution to the fine structure from the cofactor and the protein, at least one feature appeared
Figure 66. Fine structure plot of AAT-bound cofactor in 0.02 M tris HCl, pH 8.23.
to have come from perturbation of the protein absorption - the steep valley located at 34.45 kK.

The position of the valley corresponded to that of the first fine structure peak of tryptophan residues (Table 14). Perturbation of some tryptophan residue(s) in the binding of the cofactor, leading to a reduced fine structure peak at around 34.4 kK would account for the valley observed. This postulation was consistent with the observation that the fine structure plots of reconstituted AAT did in fact display a dwarfed 34.40-45 kK peak relative to that seen in active apoAAT, as discussed in the next section. Although it could just as easily come from cofactor fine structure, the unusual height of the peak at 33.85 kK might also be attributed to perturbation of protein spectrum. It would compensate for the decreased height at 34.45 kK. In terms of the binding process, it would be seen as a shift of some tryptophan residue(s) from absorbing maximally at 34.40-45 to 33.80-85 kK. This view was congruous with the decrease in peak height at 34.40 kK and the appearance of a shoulder just under 34 kK in the spectra of reconstituted AAT discussed below.

**Spectrum of reconstituted AAT**  
The analysis of the spectrum of AAT reconstituted with PLP was performed in two ways. In the first, six lognormal bands were employed to fit the entire spectrum, four belonging to the bound-cofactor and two the protein component (Table 20).

Actually, no fitting, as by computer iteration, was involved since all twenty-four parameters for each spectrum were prefixed. Parameters for bands 1, 2, 4 and 5 were those which fitted the spectrum of AAT-bound
Table 20. Parameters of reconstituted AAT, 6-band fit

<table>
<thead>
<tr>
<th>Description</th>
<th>Band No.</th>
<th>$w_0$</th>
<th>$e_0$</th>
<th>$W$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recon AAT-III&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>27.49*</td>
<td>7.059*</td>
<td>4.090*</td>
<td>1.484*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.63*</td>
<td>1.522*</td>
<td>4.292*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>35.76*</td>
<td>63.880*</td>
<td>4.702*</td>
<td>1.695*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.18*</td>
<td>2.411*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>42.07*</td>
<td>10.674*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>42.69*</td>
<td>80.833*</td>
<td>2.288*</td>
<td>0.819*</td>
</tr>
<tr>
<td>Recon AAT-V&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>27.46*</td>
<td>7.974*</td>
<td>3.985*</td>
<td>1.484*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>34.53*</td>
<td>1.673*</td>
<td>3.852*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>35.75*</td>
<td>63.661*</td>
<td>4.728*</td>
<td>1.695*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>38.93*</td>
<td>1.923*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>42.09*</td>
<td>10.746*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>42.78*</td>
<td>80.626*</td>
<td>2.459*</td>
<td>0.827*</td>
</tr>
<tr>
<td>Recon AAT-LM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>27.52*</td>
<td>8.552*</td>
<td>3.968*</td>
<td>1.484*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.15*</td>
<td>1.748*</td>
<td>5.801*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>35.77*</td>
<td>63.141*</td>
<td>4.682*</td>
<td>1.695*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.20*</td>
<td>2.433*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>42.11*</td>
<td>12.168*</td>
<td>4.000*</td>
<td>1.400*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>42.39*</td>
<td>80.064*</td>
<td>1.491*</td>
<td>0.614*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Apoenzyme used was apoAAT-III, pH 8.30 (see Table 12).

<sup>b</sup>Apoenzyme used was apoAAT-V, pH 8.18 (see Table 12).

<sup>c</sup>Apoenzyme used was apoAAT-LM, pH 8.23 (see Table 12).
cofactor (Table 19). Parameters for bands 3 and 6 were obtained previously (Table 13) in fitting the spectrum of active apoAAT. Thus the operation was merely performed to obtain the fine structure plot whose protein region, i.e. 32 to 41 kK, differed from that of the apoAAT by precisely what had been obtained in the fine structure plot of the bound cofactor. Therefore, the fine structure plot of the reconstituted AAT (Figure 67) represented an indicator of perturbation due to cofactor binding. Such perturbation was already evident from the examination of the spectrum of the bound cofactor discussed in the previous section. However, display of the perturbation in the present form permitted direct comparison with the fine structure plots of apoAAT.

In the second method, only the protein portion of each reconstituted AAT spectrum was considered. Furthermore, no attempt was made to sort out contributions from the bound cofactor and the apoenzyme, rather, the absorption spectrum between 32 and 41 kK was fitted with two log-normal bands (Figure 68). Clearly, the parameters (Table 21) for such a spectrum would be different from those of the corresponding apoenzyme (Table 13). The important point was that the features of the fine structure.

Table 21. Parameters for reconstituted AAT, 2-band fit

<table>
<thead>
<tr>
<th>Item</th>
<th>$\tilde{\nu}_0$</th>
<th>$e_0$</th>
<th>$W$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recon AAT-III</td>
<td>35.76</td>
<td>65.1</td>
<td>4.85</td>
<td>1.707*</td>
</tr>
<tr>
<td>Recon AAT-IV</td>
<td>35.74</td>
<td>65.1</td>
<td>4.86</td>
<td>1.707*</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>35.78</td>
<td>64.8</td>
<td>4.86</td>
<td>1.707*</td>
</tr>
</tbody>
</table>

*Significant
Figure 67. Fine structure plots of reconstituted AAT.
1. Recon AAT-III, 6-band fit
2. Recon AAT-V, 6-band fit
3. Recon AAT-LM, 6-band fit
4. Recon AAT-III, 2-band fit
5. Recon AAT-V, 2-band fit
6. Recon AAT-LM, 2-band fit
Figure 68. Reconstituted AAT-V fitted with two lognormal bands.
structure plot were nearly identical in both the 6-band and the 2-band fitting methods (Figure 67). Fine structure peak positions were found to be constant (Table 22).

Fine structure peak heights varied only very slightly in the two methods of fitting (Table 23).

Thus, it appeared that fine structure peak positions and peak heights remained relatively constant when greatly different lognormal methods were applied to fit the spectrum.

Slight quantitative differences appeared to exist in the fine structure peak heights of Recon AAT-III and V. It probably reflected different enzyme quality in the two preparations. Recon AAT-LM obviously displayed significantly less intense fine structure peak heights at both positions. This discrepancy was attributed to the different tuning of the Livermore instrument (see DISCUSSION).

Perturbation of the enzyme because of cofactor binding was examined by comparing the fine structure plots of active apoAAT and reconstituted AAT. The two sets of fine structure plots (Figures 61 and 67) bore general similarity. The fine structure peak positions were found identical. However, significant difference in the heights of the 34.4 kK peak could be readily detected (Table 24).

Relative to those of the corresponding apoAAT (Table 15), the fine structure peak heights at 34.4 kK in reconstituted enzymes-III, V and LM had decreased by approximately 20%. Although part of this difference could arise from fitting, (see DISCUSSION), the fact that greatly
Table 22. Fine structure peak positions of reconstituted AAT

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.40 35.20 35.90 36.70 37.20 37.70 38.60 39.60 40.20</td>
</tr>
<tr>
<td>Recon AAT-III</td>
<td>34.45 35.20 35.90 37.20 37.75 38.10 39.55 40.25</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>34.40 35.30 35.90 (36.55) 37.25 37.80 38.65 39.70 40.35</td>
</tr>
</tbody>
</table>

2-Band fit

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recon AAT-III</td>
<td>34.40 35.20 35.90 36.70 37.20 37.70 38.60 39.60 40.20</td>
</tr>
<tr>
<td>Recon AAT-V</td>
<td>34.45 35.20 35.90 37.20 37.75 38.65 39.55 40.50</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>34.40 35.30 35.80 37.25 37.80 38.65 39.65 40.35</td>
</tr>
</tbody>
</table>
Table 23. Fine structure peak heights of reconstituted AAT

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.40-45</td>
<td>35.20-30</td>
</tr>
<tr>
<td><strong>6-Band fit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recon AAT-111</td>
<td>3.47 ± 0.05</td>
<td>2.72 ± 0.11</td>
</tr>
<tr>
<td>Recon AAT-V</td>
<td>3.12 ± 0.08</td>
<td>2.68 ± 0.08</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>2.36 ± 0.11</td>
<td>2.29 ± 0.03</td>
</tr>
<tr>
<td><strong>2-Band fit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recon AAT-111</td>
<td>3.26 ± 0.09</td>
<td>2.80 ± 0.08</td>
</tr>
<tr>
<td>Recon AAT-V</td>
<td>2.90 ± 0.15</td>
<td>2.66 ± 0.11</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>2.30 ± 0.08</td>
<td>2.37 ± 0.04</td>
</tr>
</tbody>
</table>

Table 24. Average fine structure peak height of reconstituted AAT

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.40-45</td>
<td>35.20-30</td>
</tr>
<tr>
<td>Recon AAT-111</td>
<td>3.36 ± 0.14</td>
<td>2.76 ± 0.09</td>
</tr>
<tr>
<td>Recon AAT-V</td>
<td>3.01 ± 0.16</td>
<td>2.67 ± 0.08</td>
</tr>
<tr>
<td>Recon AAT-LM</td>
<td>2.32 ± 0.08</td>
<td>2.33 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average of 6-band and 2-band values.
different methods of fitting of reconstituted AAT resulted in closely similar peak heights for a given spectrum as demonstrated earlier, indicated that the 20% decrease in the peak height at 34.4 kK indeed showed perturbation due to the binding of PLP.

Accompanying the decrease in fine structure peak height at 34.4 kK, there seemed to be a slight broadening of the peak and a shoulder was occasionally visible just below 34 kK. Thus, the decrease in peak height at 34.4 kK and a slight increase at around 33.8 kK were both consistent with the sharp peak and valley at 33.8 and 34.4 kK seen in the plots of AAT-bound cofactor (Figure 64).

Spectra of holoAAT The portion of holoAAT spectrum of interest was the protein region, thus only absorption above 32 kK was considered. Two lognormal bands were employed to fit each spectrum. Since the absorption included contribution from the bound cofactor, and such contribution was dependent on pH, the parameters obtained (Tables 25) appeared somewhat random. However, at comparable pH values, e.g. 4.9 and 4.85 rather similar width (W) and skewness (ρ) were obtained for different preparations of the enzyme. The apparent difference in the peak position (ν_o) most certainly arose from erroneous instrument calibration in Livermore. The situation was similar for the preparations at pH 8.2 and 8.45. Moreover when the skewness (ρ) was fixed at 1.707, the width (W) for all holoAAT preparations at around pH 8 and all reconstituted AAT (all at pH ~ 8) (Table 21) was nearly unique (4.86 ± 0.005, illustrating the soundness of AAT reconstitution.

In the fine structure plots (Figure 69), several features were
Figure 69. Fine structure plots of holoAAT.
1. HoloAAT in 0.04 M acetate, pH 4.9
2. HoloAAT in 0.05 M tris HCl-0.01 M acetate, pH 8.2
3. HoloAAT in 0.04 M acetate, pH 4.85
4. HoloAAT in 0.05 M cacodylate-0.01 M acetate, pH 6.33
5. HoloAAT in 0.05 M tris HCl-0.01 M acetate, pH 8.46
Table 25. Parameters of holoAAT

<table>
<thead>
<tr>
<th>Description</th>
<th>$\bar{v}_0$</th>
<th>$\varepsilon_o$</th>
<th>$W$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaloAAT, pH 4.9</td>
<td>35.70</td>
<td>66.5</td>
<td>4.75</td>
<td>1.66</td>
</tr>
<tr>
<td>HaloAAT, pH 8.2</td>
<td>35.74 (35.73)</td>
<td>64.9 (65.0)</td>
<td>4.88 (4.86)</td>
<td>1.71 (1.707*)</td>
</tr>
<tr>
<td>HaloAAT-LM, pH 4.85</td>
<td>35.75</td>
<td>66.0</td>
<td>4.76</td>
<td>1.64</td>
</tr>
<tr>
<td>HaloAAT-LM, pH 6.33</td>
<td>35.76</td>
<td>66.3</td>
<td>4.81</td>
<td>1.67</td>
</tr>
<tr>
<td>HaloAAT-LM, pH 8.46</td>
<td>35.79 (35.78)</td>
<td>63.6 (63.6)</td>
<td>4.86 (4.86)</td>
<td>1.70 (1.707*)</td>
</tr>
</tbody>
</table>
immediately clear. First of all, the fine structure peak positions (Table 26) were quite independent of pH and were nearly identical to those found for active apoAAT (Table 14) and reconstituted AAT (Table 22). Secondly, the 34.4 kK fine structure peak heights (Table 27) at pH 8.2 and 8.46 were found comparable to those of reconstituted AAT (3.05 vs. 3.01-36 and 2.44 vs. 2.32, respectively) and less than the corresponding heights of the apoenzyme (3.05 vs. 3.78-96 and 2.44 vs. 2.94, respectively), consistent with the contention that perturbation of some tryptophan residue(s) occurred on binding of PLP.

Spectra of aspartate transcarbamylase catalytic subunit

The catalytic subunit of aspartate transcarbamylase (ATCase) contains two tryptophan and eight tyrosine residues per peptide (M.W. 33,500) (Changeaux and Gerhart, 1968; Weber, 1968). Perturbation of both types of residues by carbamyl phosphate (CP) and the substrate analog succinate (succ) was demonstrated by difference spectroscopy (Collins and Stark, 1969). Addition of succinate alone to the enzyme gave rise to a difference spectrum with peaks at 35.00 and 36.01 kK and was interpreted to reflect perturbation of a tyrosine residue, whereas, the combination of carbamyl phosphate and succinate caused the appearance of peaks at 34.59 + 0.01 and 35.50 kK which were postulated to arise from perturbation of a tryptophan residue.

Perturbation observed by the lognormal method

The availability of the enzyme made it possible to compare the results obtained through lognormal fitting procedure with those from conventional difference spectroscopy.
Table 26. Fine structure peak positions of holoAAT

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak position</th>
</tr>
</thead>
<tbody>
<tr>
<td>All holoAAT samples</td>
<td>34.40-45 35.20-30 35.80-95 (36.50-70) 37.20-25 37.80 38.60-70 39.50-60 40.30</td>
</tr>
</tbody>
</table>

Table 27. Fine structure peak heights of holoAAT

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height 34.40-45 35.20-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoloAAT, pH 4.9</td>
<td>2.83 ± 0.03 2.87 ± 0.03</td>
</tr>
<tr>
<td>HoloAAT, pH 8.2</td>
<td>3.05 ± 0.03 2.74 ± 0.02</td>
</tr>
<tr>
<td>HoloAAT-LM, pH 4.85</td>
<td>2.28 ± 0.02 2.57 ± 0.02</td>
</tr>
<tr>
<td>HoloAAT-LM, pH 6.33</td>
<td>2.28 ± 0.06 2.52 ± 0.007</td>
</tr>
<tr>
<td>HoloAAT-LM, pH 8.46</td>
<td>2.44 ± 0.02 2.43 ± 0.02</td>
</tr>
</tbody>
</table>
Addition of the perturbant did not affect the spectrum (Figure 70) visibly. The lognormal parameters (Table 28) remained nearly unchanged.

### Table 28. Parameters of ATCase catalytic subunit

<table>
<thead>
<tr>
<th>Description</th>
<th>$\tilde{\nu}_0$</th>
<th>$\tilde{e}_0$</th>
<th>$W$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCase</td>
<td>35.91</td>
<td>23.2</td>
<td>4.32</td>
<td>1.557*</td>
</tr>
<tr>
<td>ATCase + succ</td>
<td>35.92</td>
<td>23.2</td>
<td>4.32</td>
<td>1.557*</td>
</tr>
<tr>
<td>ATCase + succ + CP</td>
<td>35.89</td>
<td>23.1</td>
<td>4.30</td>
<td>1.557*</td>
</tr>
</tbody>
</table>

Some information could be gained from fine structure peak positions (Figure 71; Table 29). Firstly, the 35.45 kK peak of ATCase + succ + CP represents a hypsochromic shift from the corresponding peaks in ATCase or ATCase + succ. Secondly, the shoulder at around 35.75 kK was just discernible in ATCase. It became readily recognizable on addition of succinate and appeared to be present albeit masked when carbamyl phosphate was added.

### Table 29. Fine structure peak positions of ATCase catalytic subunit

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak position</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCase</td>
<td>34.60 35.35 (35.75) 37.25 37.75 38.65 39.50</td>
</tr>
<tr>
<td>ATCase + succ</td>
<td>34.60 35.30 (35.75) 37.30 37.80 38.65 39.50</td>
</tr>
<tr>
<td>ATCase + succ + CP</td>
<td>34.60 35.45 37.25 37.80 38.65 39.50</td>
</tr>
</tbody>
</table>
Figure 70. Spectrum of ATCase catalytic subunit in 0.02 M glycylglycine, containing 2 mM β-mercaptoethanol and 0.02 mM EDTA, pH 7.0. Spectra of the enzyme with succinate (90 mM) or succinate plus carbamyl phosphate (4 mM) added are approximately the same.
Figure 71. Fine structure plots of ATCase catalytic subunit.
1. ATCase
2. ATCase + succ
3. ATCase + succ + CP
By far, the perturbation by succinate and/or carbamyl phosphate was most readily seen in the fine structure peak heights (Table 30).

Table 30. Fine structure peak heights of ATCase catalytic subunit

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.60</td>
</tr>
<tr>
<td>ATCase</td>
<td>2.25 ± 0.01</td>
</tr>
<tr>
<td>ATCase + succ</td>
<td>2.12 ± 0.04</td>
</tr>
<tr>
<td>ATCase + succ + CP</td>
<td>4.57 ± 0.07</td>
</tr>
</tbody>
</table>

Comparison of the first two samples showed that the effect of adding succinate was to decrease the fine structure peak height at 35.30-45 kK by 10% while keeping the height at 34.60 kK constant, consistent with the interpretation that tyrosine and only tyrosine residues) was perturbed. Subsequent addition of carbamyl phosphate induced large changes as manifested by the two-fold increase in height at 34.60 kK and the 15% increase at 34.30-45, again substantiating the postulation that perturbation of tryptophan residue(s) occurred.

Generation of difference spectra Spectral data digitally collected could be readily used to generate conventional difference spectra. In this application, only one sample solution was required and the task of matching the sample against the reference was largely eliminated.

The difference spectrum showing the effect of succinate (Figure 72) was quite close to that obtained by difference spectroscopy (Collins and Stark, 1969), displaying peaks at 35.00 and 36.00 kK. Similarly the
difference spectrum (Figure 72) showing the perturbation by succinate and carbamyl phosphate bore the same features as those referred to earlier, such as the prominent peaks at 34.60 and 35.55 kK.

From the results thus far presented on aspartate transcarbamylase, it could be seen that the fine structure peaks positions were not identical to those obtained from conventional difference spectroscopy. Peak positions in the fine structure plot acquired by the lognormal fitting method appeared to have a closer correspondence to the actual location of the vibronic fine structure (see DISCUSSION).
Figure 72. Difference spectra of ATCase.
1. ATCase + succ minus ATCase
2. ATCase + succ + CP minus ATCase
DISCUSSION

Topics discussed will be divided into two parts as in RESULTS for the sake of consistency and clarity.

Part 1. Interaction of apoAAT with PLP Analogs

5'-Analogs

Pyridoxamine 5-thiophosphate (PM thiophosphate) and pyridoxal 5'-sulfate (PL sulfate) were the only analogs with modification at the 5' position. Structurally both analogs bear close resemblance to PLP.

PM thiophosphate In this analog, sulfur has replaced the oxygen between the 5'-carbon and phosphorus. Oxygen and sulfur, belonging to the same family, are rather similar in electronic configuration. The significant difference between these two atoms is the size as reflected in their covalent radii which are 0.70 and 1.07 Å (Pauling, 1960) respectively for oxygen and sulfur.

The finding that PM thiophosphate possessed 11% of PMP activity as the cofactor of AAT illustrated that there appears to be a significant amount of spatial tolerance in the vicinity of the 5'-carbon. This view is consistent with the reports that 5'-Me-PLP (Furbish et al., 1969) and 5-homo-PLP (Fonda, 1971) have 3 and 1% cofactor (PLP) activity respectively.

PL sulfate This analog is sterically very similar to PLP. Sulfate and phosphate groups are both essentially tetrahedral in configuration and the bond lengths differ by only 0.03 Å (Pauling, 1960). Electronically, PL sulfate and PLP differ in that the former has only a single
strongly acidic ionizable proton whereas the latter has in addition a second reversibly dissociable proton with a pK of 6.2.

The ability of PL sulfate to serve as the cofactor in PLP-dependent enzymes varies greatly from enzyme to enzyme. While it was found to have 10 and 23% cofactor activity in tryptophanase and arginine decarboxylase (Groman et al., 1972), it failed to activate the apoenzymes of brain glutamic acid decarboxylase (Matsuda and Makino, 1961) phosphorylase (Shaltiel et al., 1969), aspartate-β-decarboxylase (Tate and Meister, 1969) and D-serine dehydratase (Groman et al., 1972).

Whereas it is not known how PL sulfate fails to activate the apoenzymes described above, the mode of inactivation of apoAAT by PL sulfate seems clearer, in view of the model reactions of PL sulfate. It seems likely that, by way of sulfate elimination, the analog attaches covalently to the protein, presumably via the ε-amino group of the same lysine residue that participates in the normal Schiff base formation. Formation of a stable covalent linkage of this kind makes PL sulfate a promising active site label for AAT and possibly other PLP-dependent enzymes.

Histidine is known to react with PLP to form a tetrahydropyridine derivative (XII) on ring closure involving the imidazole moiety (Abbott and Martell, 1970). Similar reaction occurs with PL sulfate judging from its absorption spectrum. The question arises as to why the reaction does not proceed further to form the product whose spectrum possesses the characteristic fine structure. It seems likely that the explanation resides in the steric hindrance between the imidazole ring of the histidine moiety and the 5-substituent of PL sulfate, preventing the formation
of a coplanar structure involving the pyridoxyl and the imidazole-tetrahydropyridine rings. The lack of coplanarity precludes the required step, loss of the $4'$-proton, for the elimination of the sulfate moiety (Dunathan, 1966).

Under neutral conditions, arginine reacts very slowly with PL sulfate in a manner similar to histidine, apparently giving rise to a piperidine derivative (XV). However under basic conditions, a further reaction readily takes place and leads to the product with the characteristic spectrum, presumably via the loss of the $4'$-proton and the $5'$-sulfate moiety. This last reaction is possible, most likely because the steric hindrance as described for histidine is not present in arginine.

Although no proof is available to show that the formation of the piperidine ring involves the making of a bond between the $4'$-carbon and the $δ$-carbon, such a linkage seems plausible because the guanidino moiety normally carries a positive charge and thus making the protons on the $δ$-carbon acidic.
4'-Analogs

The vinyl analog of PLP (VPLP), cis-Me-VPLP, trans-Me-VPLP and the acetylenic analog of PLP (APLP) were all synthesized with the intention to chemically modify residues directly surrounding the active sites of AAT and other PLP-dependent enzymes. However, APLP appears to be the only covalent label of the coenzyme site, at least, as far as AAT is concerned.

Acetylenic analog of PLP (APLP) This analog reacts with apoAAT to form three complexes, apoAAT-I, II and III. These complexes can be "trapped" for observation in the spectrophotometer by selecting appropriate pH values for the reaction mixture. That these three complexes are not the same chemical species with different extents of protonation is evidenced by the observation that apoAAT-I is very slowly converted into apoAAT-II at pH 6.10.

The identity of these complexes and their formation is postulated to be as follows.

Initially, mixing of APLP with apoAAT immediately produced an absorption complex as evidenced by the bathochromic shift from greater than
28.4 kK to 27.6 kK. This type of shift arising from analog adsorption has also been observed with VPLP. The peak position at 27.6 kK is independent of pH as is the 28.0 kK peak of apoAAT-VPLP.

Formation of apoAAT-APLP-II involves the addition of an ε-amino group of some lysine residue, presumably the same one that participates in the normal Schiff base formation, to the β-carbon of the acetylenic moiety. The reasoning for the β-rather than the α-addition will be given in the ensuing discussion. The existence of this complex is largely based on the spectrum of the mixture of apoAAT and APLP at pH 6.10. This spectrum consists of a low absorption band at 27.4 kK and an apparently much higher absorption band centered between 34 and 36 kK (Figure 31). In addition to absorption due to apoAAT-APLP-II, the 27.4 kK peak probably contained contributions from the remnant of apoAAT-APLP-I as well as a small quantity of apoAAT-APLP-III judging from its shoulder at around 26 kK. The high-energy band most likely represents the second electronic transition of apoAAT-APLP-II by comparison with the spectra of similar compounds such as VPLP (Figure 10). It is not clear why the intensity of the high-energy peak is apparently considerably greater than that of the 27.4 kK peak.

Prototropic shift of apoAAT-APLP-II results in apoAAT-APLP-III. The semiquinoid structure is substantiated as follows. First of all, the absorption maximum at 26.0 kK of apoAAT-APLP-III is at lower energies than that of any of the enzyme-analog Schiff bases thus far observed at pH 8.3. It is not as low as that of the complex formed between AAT and erythro-β-hydroxyaspartate, i.e. 20.3 kK, possibly because
in the present system, conjugation terminates at the ε-amino nitrogen, whereas in the dicarboxylate semiquinoid, conjugation extends further to the α-carboxyl group of hydroxyaspartate. The effect of reversing the order of C and N in the imine bond is not known, but it may possibly affect the peak position. Secondly, the width of the 26.0 kK band of apoAAT-APLP-III is approximately 3.0 kK, significantly narrower than that of the AAT aldimine band at 27.6 kK, i.e. about 3.9 kK, although still wider than that of the AAT-hydroxyaspartate semiquinoid band, about 1.6 kK. Thirdly, although molar absorptivity of 12,000 cm$^{-1}$ M$^{-1}$ of apoAAT-APLP-III is not as large as that of the dicarboxylate semiquinoid (21,000 cm$^{-1}$ M$^{-1}$), it is much greater than those commonly observed for Schiff bases, i.e. 7,800 cm$^{-1}$ M$^{-1}$ (Metzler, 1957). Finally, in view of the trapping of this type of semiquinoid in organic solvent (Matsumoto and Matsushima, 1972) and the recent isolation of such a product between PL and diethyl aminomalonate (Abbott and Bobrik, 1973), it does not seem unreasonable for such a structure to be maintained by the enzyme although in model systems in aqueous solution, semiquinoid intermediates are apparently too unstable to be seen.

The above discourse equating apoAAT-APLP-III to a semiquinoid structure constitutes arguments for the addition of the amino group to the β-carbon of the acetylenic moiety in the formation of apoAAT-APLP-III. Although α-addition seems as likely, such an addition will not lead to the semiquinoid structure.

Another line of evidence in this regard is the absorption maxima of the analog released from apoAAT-APLP-III. While the peak positions
do not establish the identity of the released analog, they rule out the compound being $4'$-methyl PLP whose absorption maxima as a function of protonation are known (see RESULTS). If the addition reaction occurred at the $\alpha$-carbon, $4'$-methyl PLP would be the expected compound released from apoAAT-APLP-III.

Thirdly, the observation that the CD of reduced apoAAT-APLP-III is abolished by SDS denaturation indicates that inherent asymmetry does not exist in the reduced complex, consistent with the structure postulated for apoAAT-APLP-III. $\alpha$-addition would yield a Schiff base which, upon reduction, would possess an asymmetric center at the $4'$-carbon providing that the reduction is done asymmetrically - a likely possibility in view of the stereospecificity of proton transfer reactions in AAT.

Circular dichroism of apoAAT-$4'$-analogs Some correlation appears to exist between the structure of the $4'$-analogs and the intensity of the CD of the enzyme "reconstituted" with these analogs.

The similarity of the absorption spectra of VPLP (Figure 10), trans-Me-VPLP (Figure 25) and APLP (Figure 29) indicates that the pi-bond between the $\alpha$- and the $\beta$-carbon in each of these compounds is in conjugation with the pi-bonds of the pyridine ring, i.e. coplanarity exists between the pyridine ring and the double or triple bond outside the ring. The spectra of the $H_2P$, HP and P forms of cis-Me-VPLP (Figure 19) possess absorption maxima significantly blue-shifted as compared to those of the other $4'$-analogs. Thus, in this analog, there appears to be a lack of coplanarity present in the other analogs, obviously due to the steric hindrance between the pyridine ring and the $\beta$-methyl group.
ApoAAT-VPLP possessed no optical activity in the range of 20 to 33 kK. ApoAAT-trans-Me-VPLP and ApoAAT-APLP-I both possess little optical activity (Figures 28 and 34). ApoAAT-APLP-I rather than the other complexes of this analog is chosen for comparison because ApoAAT-APLP-I apparently represents an adsorption complex, similar in nature to those formed between apoAAT and the three vinyl analogs.

In contrast, apoAAT-cis-Me-VPLP displayed intense CD (Figure 24).

Thus, it seems that the intensity of CD associated with these analogs is directly proportional to the non-planarity of the analog conformation.

Whether this correlation holds for PLP and other analogs that form aldimine linkages with the enzyme is not known. Such a correlation would be contrary to the planar structure generally assumed for internal Schiff bases of PLP-dependent enzymes (Dunathan, 1971).

What becomes abundantly clear is the fact that the origin of CD of holoAAT does not reside in the imine bond per se. Adsorption complexes as described above are sufficient to give rise to optical activity, providing that certain conditions, such as non-planarity of the adsorbed analog, are met, consistent with the observation that the amine form of holoAAT is also optically active (Torchinsky et al., 1968).

Part II. Band Shape Analysis of Protein Spectra

Discrepancy in fine structure peak heights

Fine structure peak heights at 34.4 and 35.3 kK of various samples of active apoAAT (Table 15), reconstituted AAT (Table 23) and holoAAT
Table 27 clearly show a consistent discrepancy between spectra recorded in Ames on a Cary - 15 spectrophotometer and those recorded in Livermore Laboratories using a Cary - 16. The Livermore spectra, without exception, show dampened fine structure peak heights. It is highly unlikely that this difference reflects variation in AAT quality. Variation of this type is usually small. Furthermore, such variation is also present in the samples whose spectra were recorded in Ames, yet large differences in the fine structure peak heights among comparable spectra are not observed. The cause of the discrepancy most probably resides in the differing tuning of the instruments involved. The Cary - 15 spectrophotometer is operated with a variable slit-width which automatically adjusts itself in response to the intensity of the incident light and the absorption by the sample. On the other hand, the Cary - 16 is a fixed-slit instrument. For a given measurement, the slit-width is set manually and the entire spectrum is scanned at this pre-fixed slit-width. Depending on the setting, the intensity of the vibronic fine structure may be sharpened or dampened.

In spite of the discrepancy between the Ames data and the Livermore Laboratory data, each set is internally consistent and thus, comparison of active apoAAT, reconstituted AAT and holoAAT can be validly made. In general, very similar conclusions can be drawn from these two sets of data with regard to any given parameter such as the reduction of the 34.4 kK peak of active apoAAT on coenzyme binding.

Data from the Livermore Laboratories definitely contain less noise as reflected in the various fine structure plots and serve to ascertain
certain spectral features previously suspected to be real rather than noise, such as the 36.4-7 kK peak of AAT (Figures 61 and 63) and the peak-valley-peak at 33.8-34.4-34.9 kK of the bound cofactor (Figure 64).

**Dependence of fine structure peak height on the fitting method**

The lognormal computer program employed in fitting the spectra in this work performs iteration, i.e. minimization of standard deviation, over the wavenumber range of $\bar{\nu}_r - 1.0$ to $\bar{\nu}_{\text{truncation}}$ kK (Figure 56). Thus, data points below $\bar{\nu}_r - 1.0$ kK are ignored as are those above $\bar{\nu}_{\text{truncation}}$. The region just below $\bar{\nu}_r$ is where the leading edge of the spectrum commences to rise sharply and how this portion of the spectrum is fitted by the computer program has significant bearing on the lognormal parameters and the fine structure peak heights. Thus, for a given protein spectra, different lognormal parameters (Table 31) as well as different fine structure peak heights (Table 32) are obtained when the lower wavenumber limit is set at $\bar{\nu}_r - 1$ or $\bar{\nu}_r - 0.5$ kK, although fine structure peak positions remained unchanged.

The variation in fine structure peak heights due to these two different fitting methods is particularly of concern because the magnitude of such variation approximates that used in this work to imply perturbation of protein molecules. However, the dependence of fine structure peak height on the fitting method seems unusually large in this instance where the difference in fitting is the lower wavenumber limit. In this work, the limit is set at $\bar{\nu}_r - 1.0$ kK for all spectra, and error arising from fitting appears to be negligible as seen in the fine structure peak
Table 31. Parameters of apoAAT-LM, pH 8.26

<table>
<thead>
<tr>
<th>Description</th>
<th>$v_o$</th>
<th>$e_o$</th>
<th>$W$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoAAT-LM, pH 8.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower limit = $v_0 - 1.0$ kK</td>
<td>$35.77 \pm 0.00$</td>
<td>$63.12 \pm 0.07$</td>
<td>$4.674 \pm 0.009$</td>
<td>$1.684 \pm 0.006$</td>
</tr>
<tr>
<td>Lower limit = $v_0 - 0.5$ kK</td>
<td>$35.75 \pm 0.00$</td>
<td>$63.16 \pm 0.05$</td>
<td>$4.716 \pm 0.008$</td>
<td>$1.758 \pm 0.004$</td>
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</table>

Table 32. Fine structure peak heights of apoAAT-LM, pH 8.26

<table>
<thead>
<tr>
<th>Description</th>
<th>Fine structure peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.40</td>
</tr>
<tr>
<td>ApoAAT-LM, pH 8.26</td>
<td></td>
</tr>
<tr>
<td>Lower limit = $v_0 - 1.0$ kK</td>
<td>$3.022 \pm 0.028$</td>
</tr>
<tr>
<td>Lower limit = $v_0 - 0.5$ kK</td>
<td>$2.658 \pm 0.008$</td>
</tr>
</tbody>
</table>
heights of reconstituted AAT (Table 23) where two drastically different fitting procedures, the 6-band and the 2-band fits, produce rather similar peak heights at 34.4 and 35.3 kK. All of the fine structure peak heights reported in RESULTS section each represents the average of several determinations obtained by procedures differing in upper wavenumber limit (\( \nu_{\text{truncation}} \)) and number of parameters held constant during iteration. The smallness of the standard deviations seen throughout this work further illustrates the limited degree of error originating from the fitting procedure.

**Effect of denaturation**

AAT is rich in aromatic residues containing 10 tryptophans, 12 tyrosines and 23 phenylalanines per subunit. Undoubtedly, for each kind, all residues do not reside in the same kind of environment. Such heterogeneity is probably a major cause for the subdued fine structure peak heights of active apoAAT at 34.4 and 35.3 kK (Table 15). Denaturation places the aromatic residues in a more uniform environment, in which corresponding vibronic transitions, e.g. the 0-0 transition of tryptophan residues, are likely to be located at the same wavenumber, resulting in great intensification of fine structure, as observed in denatured apoAAT (Table 18).

The effect of denaturation on the intensity of vibronic fine structure becomes even more dramatic when the solvent change is taken into consideration. Upon exposure to denaturants, the aromatic residues, as a whole, enter a more aqueous environment compared to where they reside prior to denaturation. In N-Ac-try-OEt, a similar solvent change, i.e.
from methanol to water, results in a 45% reduction in the height of the 34.4 kK peak (Table II). However, in AAT, denaturation in guanidine hydrochloride or SDS is accompanied by a two-fold increase of the fine structure peak height at the same wavenumber.

Thus, it appears that the effect of the loss of heterogeneity of the tryptophan micro-environments on the fine structure peak height more than compensates for the effect of the solvent change.

Some slight uncertainty exist. The effect of the denaturants on the model aromatic compounds has not been dealt with in this work. The effect of more non-polar solvents than methanol on model compounds also remains to be studied.

**Comparison of conventional difference spectroscopy and lognormal fitting**

The fine structure plot obtained by the lognormal fitting method, to be sure, is a different plot. However, it is the difference between the spectrum of a sample and a smooth, mathematically constructed baseline. The conventional difference spectroscopy subtracts the reference spectrum from that of the sample, the former usually possessing fine structure as well.

Perturbation of a sample, e.g. protein, often results in changes in both vibronic fine structure peak positions and peak heights of the absorption spectrum. Such combined changes make interpretation of the difference spectrum difficult (Wetlaufer, 1962). The peaks and valleys in the difference spectrum often do not correspond to those in the parent spectra.
The fine structure plot obtained from the lognormal fitting method generally bears a more readily recognizable resemblance to the vibronic fine structure of the parent absorption spectrum. Perturbation is observed by comparing the two fine structure plots, obtained from the spectra before and after the introduction of the perturbant. Thus, the peaks in the fine structure plot of N-Ac-phe-OEt in both methanol and water (Table 9) agree closely with those reported by Strickland et al. (1971) for N-Ac-phe amide at 77°K; and the fine structure plots of apo-AAT (Table 14) and holoAAT (Table 26) show corresponding fine structure peaks at the same positions although perturbation due to cofactor binding seems evident.

For detecting very small perturbations, the conventional difference spectroscopy is thought to be more sensitive (Metzler et al., 1972).

**Fine structure of bound analogs**

The spectra of bound PLP (Figure 64) and VPLP (Figure 11) have been presented. An additional one is shown here (Figure 73). Insufficient incubation of APLP with apoAAT renders the spectrum somewhat shapeless. However, contributions to the spectrum can be readily traced to apoAAT-APLP-I, II and III.

These three spectra share a common feature, the peak-valley-peak at 33.8-34.4-34.9 kK. Since the binding of these compounds to apoAAT can cause perturbation in both the analog and the apoenzyme, the apparent fine structure at 33.8-34.4-34.9 kK may theoretically arise from perturbation of either moiety.
Figure 73. Spectrum of apoAAT-APLP in 0.02 M tris HCl, pH 8.24. Incubation time approximately six hours.
However, the following observations support the perturbation of the protein moiety. First of all, the valley is located at 34.4 kK, a position identical to that observed for the major fine structure peak of tryptophan residues in nearly all fine structure plots of AAT. Secondly, the two-band fitting of holoAAT (Table 27) and reconstituted AAT (Table 23) spectra yielded fine structure peak heights at 34.4 kK consistently lower than those of corresponding apoAAT preparations (Table 15). Thirdly, the CD spectra of reconstituted AAT (Figure 9), and apoAAT-APLP (Figure 34) all display a negative transition at 33.3-33.9 kK. The transition is induced by the apoenzyme in some aromatic residue(s).

Finally, the nature of binding of the three compounds in their respective apoAAT-analog complexes is non-identical. In reconstituted AAT, the linkage is an imine bond. In apoAAT-APLP, the binding is mostly covalent, although some non-covalent interaction may be present due to the presence of apoAAT-APLP-1. In apoAAT-VPLP, the interaction is non-covalent. The three compounds, in such different combinations with apoAAT, are not likely to display perturbation of their high-energy bands at identical positions. Certainly their lower energy bands are not located at the same wavenumber.

The possibility of perturbation in the spectra of the analog moiety can not be completely overlooked. In this case, the perturbation may be obscured by the prominent protein fine structure at 33.8-34.4-34.9 kK and thus not readily apparent.
The origin of the low-energy CD band

In all CD spectra of apoAAT-analog complexes, including the aldimine and amine forms of holoAAT, a common feature is the low-energy band centered around 33.6 kHz. Since this optical activity is induced by the enzyme (Ivanov and Karpeisky, 1969), tryptophan or tyrosine residues may be the origin. Low-temperature studies of CD and absorption spectra of tyrosine derivatives place the lowest-energy 0-0 transition at 34.7 kHz (Horwitz et al., 1970). For RNase-A and RNase-S, this vibronic transition occurs at 34.6 kHz (Horwitz and Strickland, 1971). Hydrogen bonding produces a red shift of 0.1 to 0.5 kHz in N-stearyl-L-tyrosine n-hexyl ester (Strickland, 1972). Nevertheless no tyrosine transitions occur as low as 33.6 kHz. On the other hand, the 0-0 \( ^1L_a \) transition of tryptophan derivatives have been observed at 33.7 kHz (Strickland et al., 1970).

In horseradish peroxidase isoenzymes, bovine and horse heart cytochrome C, the 0-0 \( ^1L_a \) band is clearly seen between 33.1 and 34.2 kHz (Strickland et al., 1971). Thus, for AAT, the peak position of the CD band is consistent with that of a tryptophan residue. The fine structure at 33.8-34.4-34.9 kHz in the absorption spectra of AAT-bound PLP, VPLP and APLP and the reduced 34.4 kHz peak in reconstituted AAT and holoAAT compared to that of apoAAT are also in accordance with the perturbation of a tryptophan residue upon analog binding.

Ivanov and Karpeisky (1969) assign the low-energy CD band to a tyrosine residue which is of critical importance in their postulated model for enzymatic transamination. The tyrosine residue is proposed to have an ionized phenolic side chain in order to assume a maximum at
33.3-9 kK. The width of the CD band is claimed to be too narrow for tryptophan. In this work, the peak position of tyrosine anion spectrum has been measured to be 34.08 kK (Table 9) and width of the CD band approximately 1.3 kK (Figures 7 and 43). Width of the anion spectrum is 3.7 kK and that for tryptophan in methanol is 4.8 kK (Table 9). Thus, close correspondence of the lower-energy CD band to the spectrum of tyrosine anion appears tenuous. Earlier results of chemical modification with TNM showed the importance of a tyrosine residue. However, the effect on the low-energy band is not known. Moreover, the importance ascribed to the tyrosine residue is currently in question (Birchmeier et al., 1973).
CONCLUSIONS

The study on the interaction of apoAAT with the PLP analogs indicated that VPLP, cis- and trans-Me-VPLP and APLP at pH 5 form adsorption complexes with the apoenzyme. Enzyme bound cis-Me-VPLP alone displays a prominent CD band suggesting that non-coplanarity between the pyridine ring and the double-bond outside the ring is related to the induction of optical activity.

In a multi-step reaction, APLP under neutral and basic conditions forms a Schiff base with some lysine residue of the protein, thus making it a covalent label for the coenzyme site of AAT.

PM thiophosphate is the only analog, among those investigated in this work, that shows coenzyme activity, illustrating the spatial tolerance in the vicinity of the 5'-carbon.

Model reactions of PL sulfate with β-substituted amines and other nucleophiles suggest that the analog labels a residue at the enzyme active site in addition to the lysine residue involved in the aldimine formation. The reaction appears to proceed by the loss of the 5'-sulfate moiety. PL sulfate seems to be a promising reagent for covalently labeling the active site of AAT and other PLP-dependent enzymes.

Band shape analysis of AAT spectra demonstrates that vibronic fine structure of the aromatic residues can be vividly displayed by the log-normal curve-fitting method. Perturbation of a trytophan residue in AAT upon PLP or analog binding is suggested.
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