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THE BINDING OF PYRIDOXAL PHOSPHATE ANALOGS TO GLUTAMIC OXALOACETIC TRANSAMINASE.

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THE BINDING OF PYRIDOXAL PHOSPHATE ANALOGS TO GLUTAMIC OXALOACETIC TRANSAMINASE

by

Francis Scott Furbish

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Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
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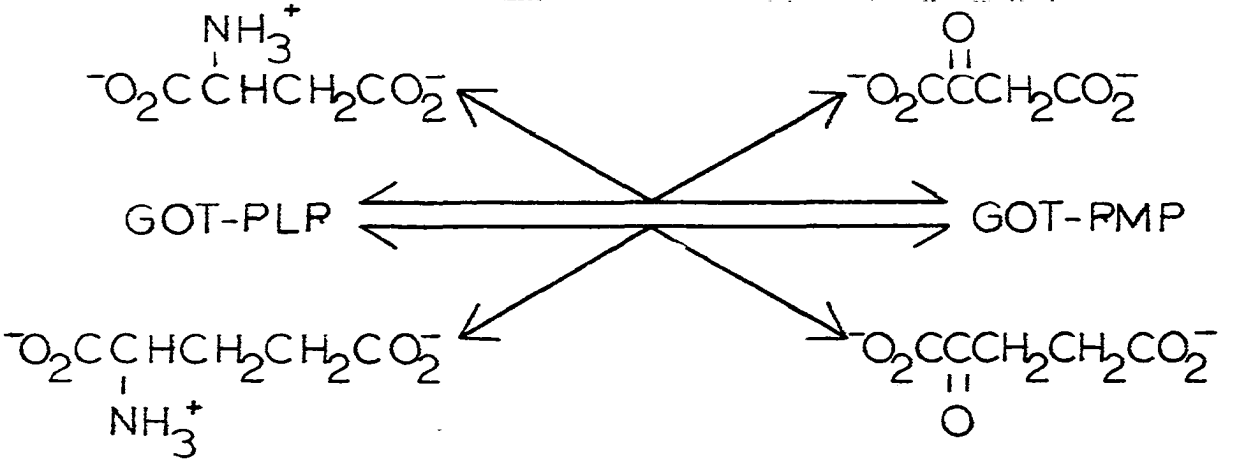
1969

TABLE OF CONTENTS

INTRODUCTION

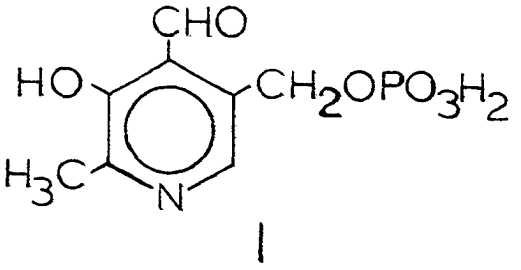
The enzyme, glutamic oxaloacetic transaminase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), has been intensively studied by a large number of investigators. Interest in this particular enzyme was stimulated by a number of factors: 1) the important position occupied by this transaminase in intermediary metabolism, 2) the experimental accessibility and comparatively easy preparation in contrast to other transaminases, 3) its role as a mechanistic prototype of group-transferring enzymes which operate through binary complexes, and 4) increased awareness of the role of the protein-bound cofactor, pyridoxal-5'-phosphate. In addition, the clinical importance of this enzyme has recently been stressed by workers in the medical areas.

Glutamic oxaloacetic transaminase (GOT) catalyzes the two half reactions shown below with the coenzyme serving as an amino group

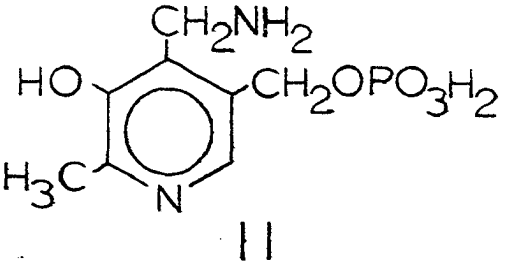


carrier. By reacting with amino $acid_1$, the aldehyde form of the

coenzyme, pyridoxal-5'-phosphate (PLP, I), is converted to the amine form, pyridoxamine-5'-phosphate (PMP, II), yielding keto acid$_1$ as product. The



I



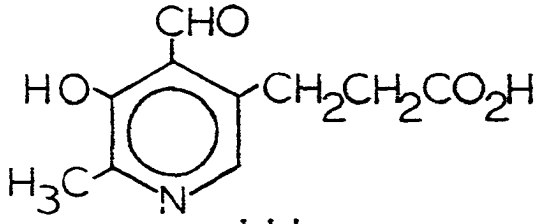
II

PMP-enzyme then reacts with keto acid$_2$ to form amino acid$_2$ thereby reproducing the PLP-enzyme. Since two forms of the enzyme are involved and the overall enzymic reaction is the sum of the two half reactions, GOT exhibits the kinetic characteristics of the Ping-Pong Bi Bi mechanism. The two amino acid substrates (L-glutamate and L-aspartate) are both focal points for intermediary nitrogen metabolism, and the two keto acids (α-ketoglutarate and oxaloacetate) are both components of the tricarboxylic acid cycle. All four metabolites are maintained in balance by this enzyme.

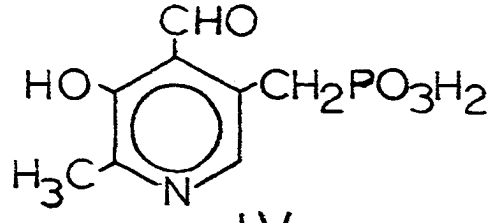
Although purified GOT preparations were not available until about 1960, the general features of the reaction mechanism were recognized some time before on the basis of the coenzyme's properties. Moreover, these features were confirmed in nonenzymic model systems simplifying mechanistic matters somewhat for the enzymologists. Nevertheless, model system studies could not portray the tremendous catalytic ability of the enzyme since the enzyme increases the rates of the slow steps in the reaction by factors of 10^8--10^9. Such studies did point out, however, the possible roles of functional groups presumed to be present at the active site of GOT. In particular, a group capable of general acid-base catalysis was predicted to be in the vicinity of the active center; it is now known that at least two such groups are available

It is apparent from its participation in enzymic transamination that PLP is bound to the protein in a manner as to give it a precise alignment with the enzyme-bound substrate and the catalytically functional groups of the protein.

The nature of this coenzyme binding is the subject of the present investigation. Certain analogs of PLP were utilized in connection with the resolved enzyme (apoGOT) to ascertain the role of some of the functional groups of the cofactor. This approach has been taken before (see under Coenzyme Binding, Review of Pertinent Literature) and has yielded useful information. In particular, the binding of the 5'-phosphate was examined by use of analogs with varied substituents in the 5'-position. The analogs used for this study consisted of carboxymethyl-deoxypyridoxal (III), deoxypyridoxalyl phosphonic acid (IV),

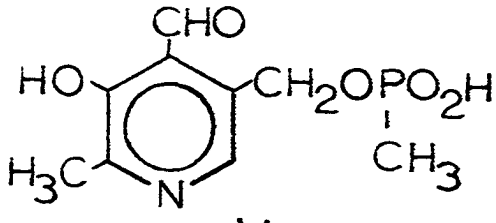


III

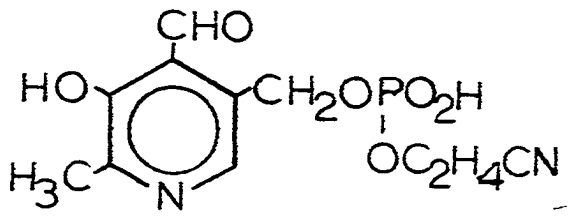


IV

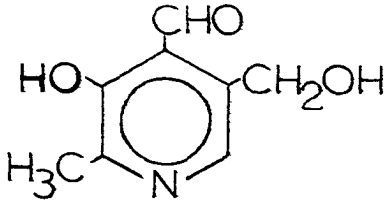
the methyl phosphonic acid of pyridoxal phosphate (V), the cyanoethyl ester of pyridoxal phosphate (VI), pyridoxal (VII), and deoxypyridoxal (VIII).



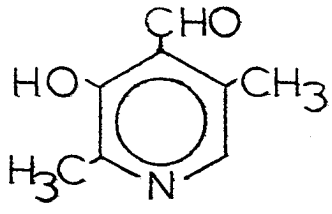
V



VI

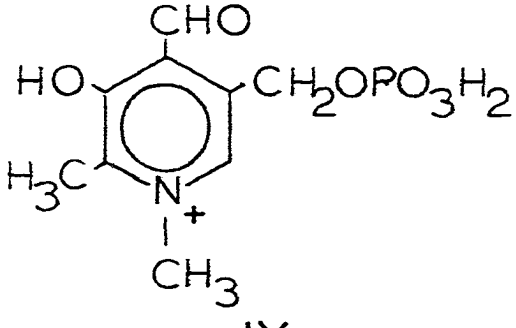


VII

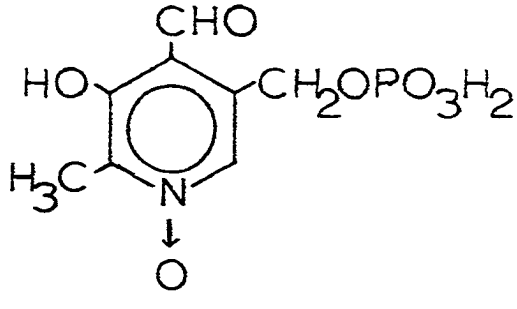


VIII

Also investigated was the effect of substituents on the pyridine nitrogen. The two analogs, N-methyl-pyridoxal phosphate (IX) and pyridoxal phosphate-N-oxide (X), were used for this study.



IX



X

REVIEW OF PERTINENT LITERATURE

The early history of enzymic transamination and the discovery of coenzyme functions of pyridoxal phosphate have recently been reviewed by Braunstein (1960) and by Guirard and Snell (1964). Since these reviews and that of Meister (1965) have discussed the role of GOT in amino acid metabolism, emphasis here will be placed on papers concerned with the isolation and structure of the GOT molecule, its mechanism, and the binding of coenzyme.

Isolation

Simple homogenates or crude fractions were utilized in early work with GOT as the enzyme proved difficult to purify; furthermore, the extensive ammonium sulfate precipitations usually led to resolution (O'Kane and Gunsalus, 1947). A highly purified preparation was finally obtained from pig heart by Jenkins et al. (1959b) who employed an impressively effective heat treatment in the presence of maleate followed by ammonium sulfate fractionation and chromatography on hydroxylapatite. Later preparations sometimes included precipitation by cold acetone (Jenkins, 1960). Banks and Vernon (1961) obtained a preparation about 95% pure by use of calcium phosphate gel and ammonium sulfate and ethanol fractionations; however, the enzyme was resolved during the purification and the yields were quite low. The Jenkins procedure was used until Turano and coworkers (1964) demonstrated that one thiol group (out of a total of six present in the molecule) was converted by maleate

into the corresponding S-(α,β-dicarboxyethyl) derivative; this particular thiol group was shown to be located at or near the active site by Ballio and coworkers (1966) since the S-(α,β-dicarboxyethyl)cysteine residue prevented the resolution of the enzyme under mild conditions. In order to avoid this structural modification, the enzyme is now prepared by using glutarate or succinate for protection during the heat treatment. While GOT has been prepared from several sources, most studies have concentrated on the pig heart enzyme.

The discovery of the existence of several forms of GOT further complicated the effort to isolate a homogeneous preparation. A review of the early studies of GOT isozymes has been compiled by Wilkinson (1965, pp. 95-103). The GOT from pig heart has been the subject of intensive investigation by several workers (Nisselbaum and Bodansky, 1964, 1966; Wada and Morino, 1964) which has demonstrated the occurrence of two different enzymes, one associated with the cytoplasm and the other with mitochondria. The mitochondrial isozyme, which is cationic, is largely destroyed by the heat treatment, while the anionic cytoplasmic isozyme readily survives this step. Martinez-Carrion and others (1967) have shown that each of the isozymes contains at least three subforms and have employed a carboxymethyl--sephadex column procedure for the isolation of the main subforms of cytoplasmic GOT. These subforms were found to differ in their specific activity and spectral properties.

Structure

With the isolation of large amounts of highly purified GOT from pig heart, studies began on the chemical features of the enzyme. In the period 1960 to 1966 much work on the protein chemistry of GOT such as amino acid composition (Turano, et al., 1963), the role of sulfhydryl groups (Polyanovsky and Torchinsky, 1962; Turano, et al., 1963), optical rotatary dispersion (Fasella and Hammes, 1965), the sequence of amino acids at the PLP binding site (Hughes, et al., 1962; Polyanovsky and Keil, 1963) and quaternary structure (Polyanovsky and Makarova, 1966) was performed. However, these studies utilized preparations containing various mixtures of the GOT isozymes and their subforms. Martinez-Carrion, et al. (1967) repeated most of the basic structural work on purified preparations of the three major subforms (α, β, and γ) of cytoplasmic GOT. Their paper provides a convenient summary of present knowledge in this area and, unless otherwise noted, is the source for the following.

Primary structure

The content of individual amino acids is within the limits generally encountered in proteins except for a relatively low content of serine and a relatively high content of proline, arginine and tryptophan. Of interest is the fact that GOT contains twenty lysyl residues and four sulfhydryl groups (one of which is buried in the protein and is exposed in 8 M urea) per mole of PLP. Although no significant difference was found in the amino acid composition of the various subforms, it

cannot be excluded that the subforms may differ by a few residues because of the relatively large molecular weight (47,000 ± 3,000 g per mole of coenzyme) of the enzyme. Similarity of primary structure between the subforms was also demonstrated by fingerprints of tryptic digestions with the same 38 peptides appearing on the electrophoretogram of each subform.

Hughes, et al. (1962) provided the means to determine the amino acid sequence at the PLP binding site by reducing the lysyl residue-PLP Schiff base with sodium borohydride, thereby stably binding the coenzyme to the apoenzyme. Polyanovsky and Keil (1963) have reported this sequence to be -lys-pyridoxyllys-ser-asp (or aspNH_2)-phe-. The presence of two adjacent lysyl residues (one with coenzyme attached) has been demonstrated in another PLP enzyme, glutamic-pyruvic transaminase (EC 2.6.1.2) (Pfleiderer, et al., 1968).

Secondary and tertiary structure

Information relating to this area is particularly scanty and what exists is rather nebulous. Turano, et al. (1963) determined that GOT is composed of two polypeptide chains with no detectable differences between them. Optical rotatory dispersion (ORD) measurements by Fasella and Hammes (1965) in the ultraviolet region indicate that the enzyme has a considerable amount of ordered structure (i.e. helical content). No difference greater than experimental error was found in the UV ORD between the various subforms by Martinez-Carrion, et al. (1967). These authors also investigated the behavior of the transaminase subforms in concentrated urea. It had previously been determined that in the

presence of 8 M urea GOT rapidly and reversibly loses activity to about 25% of the original activity and then decays more slowly to the vanishing point. The rate of activity loss cannot be described by a simple equation (Fasella and Hammes, 1964) and changes in spectrum, optical rotation, and fluorescence suggest that an extensive conformation change occurs with the slow activity loss step. When repeated with the separate subforms, Martinez-Carrion and coworkers found that the rate of the slow conformational change was faster for the α subform than for the β. While these authors have intrepreted the results to show that the α subform has a less rigid or compact structure than the β, such a difference is not demonstrated by ORD. It is interesting to note that after extensive perturbation by exposure to concentrated urea, the subforms still retain their electrophoretic individuality. The authors suggest that the individuality of the subforms arises from a difference in the secondary and tertiary structure which occurs in that part of the protein molecule which retains some ordered structure even in concentrated urea.

Quaternary structure

On the basis of polarization of fluorescence techniques, Polyanovsky and Ivanov (1964) suggested that GOT dissociates into subunits upon dilution. Recent work by Banks et al. (1968c) has shown such dissociation to occur only at a very low level of concentration (10 to 1 mμM) and that both the dimer and the monomer units have the same specific activity. The dimeric molecule does dissociate into two monomers at extreme pH values (Polyanovsky and Makarova, 1966) and upon succinylation of the

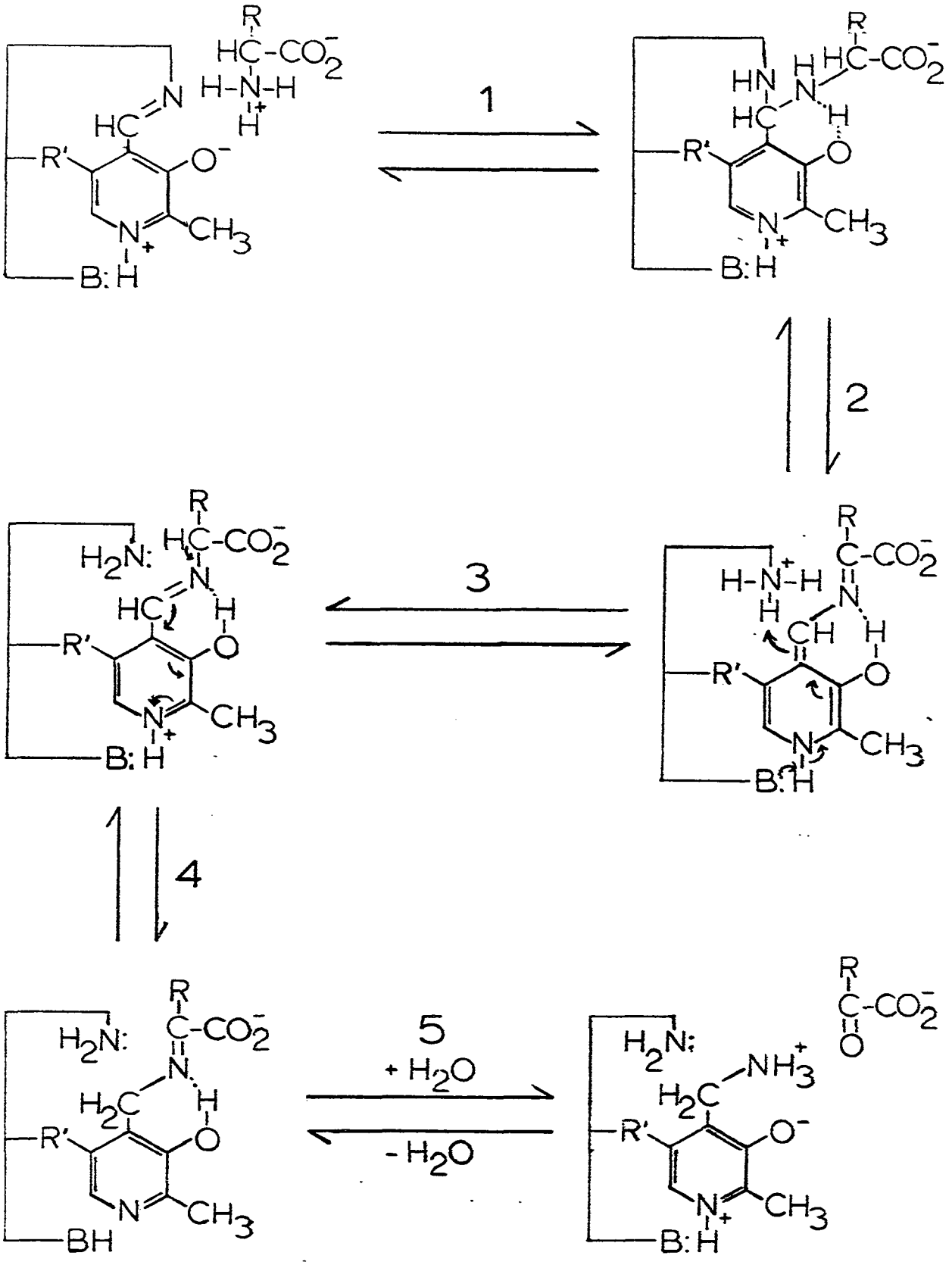
protein (Polyanovsky, 1965). Dissociation into subunits does not occur under the conditions normally employed in GOT studies.

Mechanism

The establishment of the generally accepted mechanism of pyridoxal phosphate-catalyzed transamination (sometimes called the PLP-PMP shuttle mechanism) as proposed by Braunstein and Shemyakin (1953) and independently by Metzler et al. (1954) has been extensively reviewed (Meister, 1962; Braunstein, 1960, 1964; Guirard and Snell, 1964). Hence, the general mechanism for GOT is summarized in Figure 1 and is only briefly described here. An imine is formed between the coenzyme and the enzyme-bound substrate amino acid by a transaldimination reaction (reactions 1 and 2 in Figure 1) which ruptures the aldimine linkage between a lysyl ε-amino group of the protein and the 4' carbon of the coenzyme. The transaldimination is thought to proceed via an intermediate with the 4'-carbon of the coenzyme in a tetrahedral configuration (referred to as a tetrahedral addition intermediate). Tautomerization of the enzyme-amino acid Schiff base--the rate limiting step--produces an intermediary enzyme-keto acid Schiff base, a ketimine (reactions 3 and 4 in Figure 1). It is reasonable to suppose that this conversion proceeds through a resonance-stabilized carbanion after loss of a proton from the enzyme-amino acid Schiff base. Hydrolysis of the ketimine yields a keto acid-enzyme complex with the coenzyme in the aminic form (reaction 5 in Figure 1). Release of the keto acid completes the half reaction.

Figure 1. Binary mechanism of pyridoxal phosphate function in GOT (Guirard and Snell, 1964)

Reactions: 1,2. Transaldimination
3,4. Tautomerization
5. Hydrolysis


1
2
3
4
5
+ H2O
- H2O

The detailed mechanism of the reaction catalyzed by GOT with normal substrates has been investigated essentially by three approaches: steady state kinetics, fast reaction kinetics, and equilibrium studies of the reaction between high concentrations of the enzyme and the substrates.

Steady state kinetics

Steady state kinetics of GOT has been studied by several investigators (Henson and Cleland, 1964; Wada and Morino, 1964; Nisselbaum and Bodansky, 1964, 1966; Velick and Vavra, 1962). While some numerical values of kinetic constants varied considerably, all the results were consistent with the binary mechanism. Velick and Vavra (1962) also included product and substrate inhibition (as did Henson and Cleland, 1964) and pH dependence studies which showed that only the non-protonated aldimine GOT reacts with amino acids (the pK is 6.2 for the protonation). These authors also demonstrated competitive inhibition of the amino acid substrates by keto acids. Banks et al. (1968a) have reported on the isotope effects induced by replacing the hydrogen atom bound to the substrate's α-carbon with deuterium; they conclude that the rate limiting step involves loss of the α-hydrogen from the amino acid. These authors take the view that a carbanion intermediate does not exist.

Fast reaction kinetics

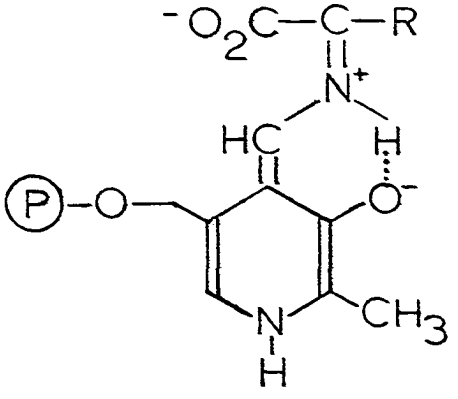
Temperature jump experiments on GOT in the presence of each amino acid-keto acid pair were performed by Fasella and Hammes (1967). Three relaxation processes were observed for each half reaction:

lower bounds of 10^7 - 10^8 M^{-1} sec^{-1} and 10^5 - 10^6 sec^{-1} were estimated for the rate constants associated with the bimolecular and dissociation processes involving amino acids while the corresponding lower bounds for keto acids were 10^8 M^{-1} sec^{-1} and 10^4 sec^{-1}. The wavelength dependence of the amplitudes of the relaxation effects was also investigated; intermediates absorbing at 360, 430, and 490 mμ were suggested to occur on one side of the slow step and an intermediate absorbing at 330 mμ to be on the other side. This conclusion is supported by use of the pseudosubstrates α-methylaspartate and β-hydroxyaspartic acid in equilibrium studies; the former which can combine with GOT as a Schiff base, but cannot transaminate, has absorption peaks at 430 and 365 mμ (Fasella, et al., 1966) while the latter compound exhibited an intermediate absorbing at 490 mμ (Jenkins and Taylor, 1965; Jenkins, 1961, 1964).

Equilibrium studies

Jenkins and coworkers (1965, 1966a) demonstrated that the amino acid substrates do not complex with the aminic enzyme, while the keto acid substrates react with the aldimine enzyme (predominately with the protonated enzyme but also somewhat with the nonprotonated GOT) to form abortive complexes. Furthermore, the enzyme-substrate intermediates were shown to have absorption maxima at 490, 430, 365, and 330 mμ by combining spectroscopic analyses with kinetic methods. These authors suggest that the 430 and 360 mμ absorbancies are due to aldimine derivatives, while the absorbancy at 330 mμ is due to ketimine derivatives or to tetrahedral addition products between PLP and amino

acids. The 490 mμ absorbing species has been suggested to have a quinoid structure (Jenkins, 1964) as shown below.



Equilibrium studies have also been performed on pseudosubstrates. Two of these (α-methylaspartate and β-hydroxyaspartate) were mentioned above under fast reaction kinetics. The erythro and threo isomers of β-hydroxyglutamate were investigated by Khomutov and others and reported by Fasella (1968). Both isomers reacted with GOT rapidly to form a 490 mμ absorbing species followed by slow transamination. The threo isomer produced a larger amount of quinoid intermediate than did the erythro. These results suggest, in the case of the normal substrates, that a proton removed from the α-carbon to form the quinoid intermediate would then be transferred to the 4'-carbon atom of the coenzyme to give the ketimine.

The binding of glutaric acid to GOT has been studied by Jenkins and D'Ari (1966b). One carboxylate group of glutaric acid is bound

to the protein by displacement of a buffer anion; the other is bound at low pH values, but not at high pH. The binding site of the second carboxylate group is thought to be the quaternary nitrogen atom of the protonated lysyl ε-amino group that is involved in the aldimine linkage between the protein and the aldehyde group of pyridoxal phosphate. Jenkins and D'Ari (1966b) further state that there is no protein conformational change upon binding of substrate, but that a configurational change in the substrate is required and that the ability to undergo this change determines the substrate specificity.

An intriguing study of the stereochemistry of the transamination reaction has been recently undertaken by Dunathan and coworkers (1966, 1968a, 1968b). The 1,3-prototropic shift of the tautomerism of the amino acid aldimine to the ketimine intermediate must occur within the confines of five stereochemical variables: a) the configuration of the α-carbon of the amino acid (C_p), b) the configuration of the proton added to the 4'-carbon of the coenzyme ($C_{4'}$), c) the conformation about the C-N single bond, d) the conformation of the $C_{4'} = N$ double bond, and e) the stereochemistry of the proton transfer (called "cis" if the CH bond making and breaking both occur on the same side of the π system plane or "trans" if on opposite sides). The first variable (a) is known for L-amino acids and the third (c) was assumed to be trans by Dunathan. Progress in determining (b) and (e) was reported for GOT (Dunathan, 1968a, 1968b) and for pyridoxamine-pyuvate transaminase (Ayling et al., 1968); investigation of the latter enzyme indicated a "cis" removal and addition of a proton presumably by a single group of

the enzyme which functions as a general acid-base catalyst.

Thus, in spite of the complex nature of an enzyme-catalyzed reaction, a detailed picture of GOT's mode of action in terms of substrate binding, formation and tautomerization of the Schiff bases, and release of product is emerging.

Coenzyme Binding

From its extensive role in GOT's catalytic action, it is obvious the PLP is bound to the protein in a highly specific manner. The nature of the coenzyme's binding has been the subject of much investigation and controversy. The discovery that PLP was bound to the protein as a Schiff base with the ε-amino group of a lysine resideu (Hughes et al., 1962) raised objections to the proposed formation of enzyme-substrate complexes as Schiff bases (Evangelopoulos and Sizer, 1963). However, imine formation was found to enhance the reactivity of PLP toward carbonyl reagents (Cordes and Jencks, 1962); furthermore, amino acids and many carbonyl reagents reacted more readily with the bound PLP of GOT than with free PLP (Jenkins et al., 1959a).

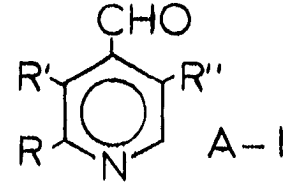
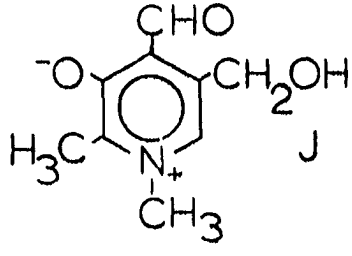
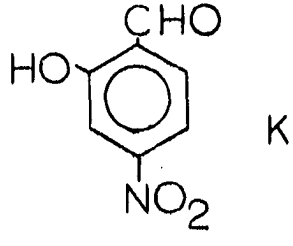
Spectral and chemical evidence indicated that PLP could be bound to the ε-amino group of a protein in at least three ways: 1) as a protonated imine with an absorption maximum in the 410-430 mμ region, 2) as a nonprotonated imine with an absorption maximum at about 360 mμ, and 3) as a substituted aldimine with an absorption maximum at about 330 mμ. The latter mode of binding does not permit reduction by sodium borohydride or react with carbonyl reagents (Fischer et al., 1963) and

is not present in catalytically active GOT (although it most likely may be responsible for the inactive γ-subform). GOT exhibits pH dependent absorption spectra that are characteristic of the unsubstituted imines from which Jenkins and coworkers (1959b) obtained a pK value of 6.3 for the protonation of the PLP-protein imine of GOT.

Other optical properties of the bound coenzyme have been studied. PMP has a characteristic luminescence spectrum which is quenched when bound at the active site, but not when bound in an unspecific manner to other proteins (Churchich, 1964). A positive Cotton effect is associated with bound PLP and is centered about the coenzyme's absorption band as shown by optical rotatory dispersion (Fasella and Hammes, 1965) and circular dichroism (Breusov et al., 1964). The Cotton effect is diminished by substrate, carbonyl reagents, borohydride reduction, and concentrated urea. These studies have emphasized the highly specific nature of the enzyme-coenzyme interaction.

Analogs of PLP have been utilized for a definition of the structural requirements of the coenzyme. Morino and Snell (1967) reported that ω-methyl-pyrodoxal phosphate and norpyridoxal phosphate reactivated apoGOT (see Table 1 for structures of these derivatives); hence, the methyl group is not required for catalysis. O-methyl-pyridoxal phosphate (see Table 1) did not reactivate GOT, but whether the reason lies in its failure to combine with or to activate apoGOT has not been determined. ApoGOT was found to catalyze the two half reactions (1) and (2) (Wada and Snell, 1962)

Table 1. Activity of pyridoxal analogs as substrates for pyridoxamine pyruvate transaminase (Ayling and Snell, 1968)

Structure	Name	Substituents R	R'	R''	Active as Substrate
 A–I	A. Pyridoxal	CH_3	OH	CH_2OH	Yes
	B. ω-Methylpyridoxal	CH_3CH_2	OH	CH_2OH	Yes
	C. Norpyridoxal	H	OH	CH_2OH	Yes
	D. 5-Deoxypyridoxal	CH_3	OH	CH_3	Yes
 J	E. 3-Hydroxy-4-pyridine aldehyde	H	OH	H	Yes
	F. Pyrodixal phosphate	CH_3	OH	$CH_2OPO_3^{=}$	(Yes)
	G. 3-Deoxypyridoxal	CH_3	H	CH_2OH	No
	H. Pyridine-4-aldehyde	H	H	H	No
 K	I. O-Methylpyridoxal	CH_3	OCH_3	CH_2OH	No
	J. N-Methylpyridoxal		(see column I)		No
	K. 4-Nitrosalicylaldehyde		(see column I)		No

(1) Pyridoxal and glutamate $\rightleftarrows$ pyridoxamine and α-ketoglutarate

(2) Pyridoxamine and oxaloacetate $\rightleftarrows$ aspartate and pyridoxal

by using substrate-level concentrations of pyridoxal and pyridoxamine. The K_M value for pyridoxal (about 2.5 mM) was given as about 1.7×10^3 times higher than that for PLP (K_{PLP} = 0.15 μM); this showed the tremendous effect of the 5'-phosphate of PLP upon the affinity of the coenzyme for apoGOT. These authors also concluded that the phosphate group played only a binding role and was not required for enzymic transamination. Because of the low affinity of apoGOT for non-phosphorylated pyridoxal analogs, Ayling and Snell (1968) utilized pyridoxamine pyruvate transaminase which catalyzes reaction (3) and which

(3) Pyridoxamine and pyruvate $\rightleftarrows$ pyridoxal and L-alanine

has high affinity for pyridoxal and pyridoxamine to analyze the effects of several pyridoxal analogs as shown in Table 1. Compounds A through E were excellent substrates, F was poor, while G through K were inactive as substrates. These investigators also obtained dissociation constants for each analog. Their conclusions are summarized as follows.

1) Neither the 2-methyl group or the 5-hydroxymethyl of pyridoxal plays a role in transamination.

2) The substituent at position 2 of the analogs strongly affects binding to the enzyme.

3) Removal of the 5 substituent affects binding to a lesser extent.

4) A phenolic group in the 3 position is required for efficient binding and seems to be essential for transamination.

5) The minimum structural requirements are met by 3-hydroxy-pyridine-4-aldehyde.

In brief, the essential structural features of pyridoxal required for enzymic transamination are the same as those required for nonenzymic transamination in aqueous solutions: a formyl group adjacent to a phenolic group and conjugated to the electron-withdrawing heterocyclic nitrocyclic nitrogen of the pyridine ring (Metzler et al., 1954).

EXPERIMENTAL

Materials

Pyridoxal phosphate analogs

Carboxymethyl-deoxypyridoxal and the corresponding amine were synthesized by Dr. Chuzo Iwata of this laboratory. The syntheses have been described (Iwata and Metzler, 1967).

The phosphonic acid derivative of deoxypyridoxal was also supplied by Dr. Iwata. The synthesis has not been described in the literature.

Deoxypyridoxal was synthesized by Mr. Robert J. Johnson of this laboratory using the method of Iwata (1968).

Pyridoxal hydrochloride and pyridoxal phosphate were obtained from Sigma Chemical Company.

The methyl phosphonic acid of PLP and the cyanoethyl ester of PLP were prepared by Dr. Walter Korytnyk, Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York.

N-methyl-PLP was synthesized by Dr. Anna Pocker of the Department of Biochemistry, University of Washington, Seattle, Washington and was the generous gift of Dr. Esmond E. Snell of the Department of Biochemistry, University of California, Berkeley, California.

PLP-N-oxide was synthesized and supplied by Dr. Saburo Fukui of the Department of Industrial Chemistry, Kyoto University, Kyoto, Japan. The synthesis has been described (Ohishi, et al., 1968).

Buffers and other chemicals

Buffers, substrates, inhibitors, and other chemicals were obtained

from commercial sources. Triethanolamine hydrochloride was prepared by dissolving reagent grade triethanolamine in a minimal amount of water in the presence of an excess of HCl, precipitating the hydrochloride salt with addition of ethanol (75% v/v, final concentration), and recrystallizing three times from water-ethanol. Glutaric acid was recrystallized three times from benzene after treatment with activated charcoal. All other chemicals were reagent grade.

Enzyme

Fifty pound lots of fresh pig heart were obtained from Des Moines packing plants and trimmed free of auricles and fat and minced in a meat grinder. Two kilogram portions of minced muscle were combined with two liters of 0.05 M glutarate, 0.005 M EDTA buffer, pH 6.0, and homogenized for sixty seconds in a chilled one gallon Waring blender. The thick homogenate was transferred to stainless steel beakers in an 85° water bath and stirred continuously. When the temperature of the homogenate reached 60°, solid α-ketoglutarate (0.5 g. per 8 l.) was stirred in to maintain the enzyme in the aldehyde form. When the temperature of the homogenate reached 75° (about 20 minutes) the temperature of the bath was reduced to about 77° and the homogenate was allowed to remain at 75° for twenty minutes with intermittent stirring. The denatured protein and other solids were removed by straining through a cheesecloth bag and the supernatant was cooled to 5°. Solid ammonium sulfate (310 g. per l., 50% saturation) was added to the chilled supernatant and the precipitate was collected by centrifugation.

The precipitate was discarded and additional ammonium sulfate (150 g. per l., 72.5% saturation) was added to the supernatant. The yellow precipitate was collected by centrifugation and was taken up in 0.02 M glutarate buffer, pH 6.0, and dialyzed overnight against water in the cold. After dialysis fifty moles of α-ketoglutarate were added to the preparation and the solution was made 0.08 M in phosphate, pH 6.8. This preparation was applied to a hydroxylapatite column (4.5 x 15 cm) which had been previously equilibrated with water. The hydroxylapatite was prepared by the method of Jenkins, (1960). After the column had drained, the yellow band of GOT was eluted with 0.08 M phosphate, pH 6.8, and the elutent was collected with a fraction collector. The bright red-yellow fractions containing the enzyme were pooled, concentrated by ammonium sulfate precipitation (500 g. per l.), and dialyzed against 0.04 M acetate, pH 5.4. The major subforms were separated on a carboxymethyl Sephadex (CM-50) column (1.5 x 30 cm) which had been equilibrated with 0.04 M acetate, pH 5.4. After allowing the enzyme to absorb slowly into the column, the column was washed with 100 ml. of 0.04 M acetate, pH 5.4. The γ-fraction was completely removed and separation of the β-GOT from the α-GOT was performed by elution with 2 liters of 0.06 M acetate, pH 5.4. The separated α and β forms were eluted with a linear gradient from 0.06 M to 0.11 M acetate (one liter each solution). Fractions containing the α-subform were collected, concentrated by ammonium sulfate precipitation, and stored in 0.01 M acetate, pH 5.4.

The α-GOT used for the major amount of experimental results

presented in this dissertation was prepared as above by Dr. W. Terry Jenkins of the Department of Chemistry, Indiana University, Bloomington, Indiana and very generously given for this study. This preparation had an absorption ratio (430 mμ/340 mμ) of 4.3 in 0.8 M acetate, pH 4.8, and a specific activity of 40.4 mM/min./mg.

Methods

Resolution

The native enzyme was resolved by the method of Scardi et al. (1963). For a typical preparation twenty-four ml. of 0.2 M L-glutamate, pH 8.3, were added to one ml. of α-GOT stock solution (35 mg. per ml.); this solution was allowed to remain at room temperature for ten minutes to produce the amine form of the enzyme. Twenty-five ml. of 1.0 M phosphate, pH 4.8, was then added and the preparation was incubated at 30° for thirty minutes. After incubation the protein was precipitated by addition of 150 ml. of saturated ammonium sulfate. The precipitate was collected by centrifugation, taken up in 0.01 M triethanolamine HCl, pH 8.3, and the incubation with phosphate with subsequent precipitation repeated. This precipitate was also dissolved in 0.01 M triethanolamine HCl and passed through a short (1.5 x 10 cm) G-25 Sephadex column to remove the remaining ammonium sulfate. If a significant amount of absorption at 330 mμ (greater than one-fortieth of the absorption at 280 mμ) was detected in the preparation, the incubation procedure was repeated once more.

The resolved GOT preparations utilized for cofactor studies had an absorption at 330 mμ of less than one-fortieth than that at 280 mμ and no enzymic activity. These preparations could be reactivated with PLP to 90-99% of the activity of the native enzyme.

Reduction

The aldimine linkage between some cofactors and the protein was reduced with sodium borohydride. Solid $NaBH_4$ (one mg. per ml.) was added to enzyme solutions in 0.1 M triethanolamine HCl, pH 8.3, at 0° and the solution was dialyzed against 0.01 M triethanolamine HCl, pH 8.3, overnight.

Spectrophotometric measurements

Absorption spectra were obtained on a Cary 15 recording spectrophotometer equipped with a digital output device which recorded spectral data directly on punched cards. Those spectra which were recorded on cards were available for normalization with respect to protein concentration by computer. Computer treatment of data was performed by Mr. Robert J. Johnson of this laboratory.

Circular dichroism was measured in some preparations with a modified Jouan dichrograph described by Johnson and Graves (1966) with a sensitivity of approximately 2×10^{-5} absorbance units under the conditions employed. A Jasco Model ORD/UV-5 with CD attachment was utilized for the remainder of the CD spectra. Fluctuations in the recorder tracings were 1.0×10^{-4} or less. While the instruments were calibrated to $\pm$ 1 mμ, peak positions could not be determined to this accuracy (especially the low, broad peaks). The peak positions reported are approximately

± 5 mμ. Solutions of five to ten mg. per ml. in protein were used.

Assay

Routine assays were performed by the method of Jenkins et al. (1959b). Enzyme (0.1 ml.) was added with an adder-mixer to a cuvette containing 200 μmoles of tris(hydroxymethyl)aminomethane, pH 8.3, 20 μmoles of α-ketoglutarate, and 20 μmoles of L-asparate in a total volume of 3 ml. Oxaloacetate produced was followed at 280 mμ utilizing a Beckman DU equipped with a Gilford Model 220 absorbance indicator and automatic recorder with multispeed chartdrive. The absorption coefficient of oxaloacetate as given by Velick and Vavra (1962) (0.57 mM^{-1} cm^{-1}) was used to express activity as mM/min. per mg. of enzyme.

Assays for amino acid substrates and some kinetic studies utilized oxaloacetate and L-glutamate as substrates. In these assays the decrease of oxaloacetate was followed and the activity was expressed as above.

Protein concentration

Protein concentrations were determined by measuring the absorbancy at 280 mμ. A solution of 0.74 mg./ml. of apoenzyme has an absorbancy of 1.0; for the holoenzyme, the value is 0.71 mg./ml. (Banks, et al., 1968c).

The molecular weight was assumed to be 4×10^4 g./mole per mole of coenzyme (Banks, et al., 1968c).

RESULTS

Spectral data are presented here by illustrating each entire absorption or CD spectrum within the wave-length range of 300 to 500 mμ. A summary of spectral and enzymic data is presented at the end of this section. Where appropriate, the spectra have been corrected for dilution.

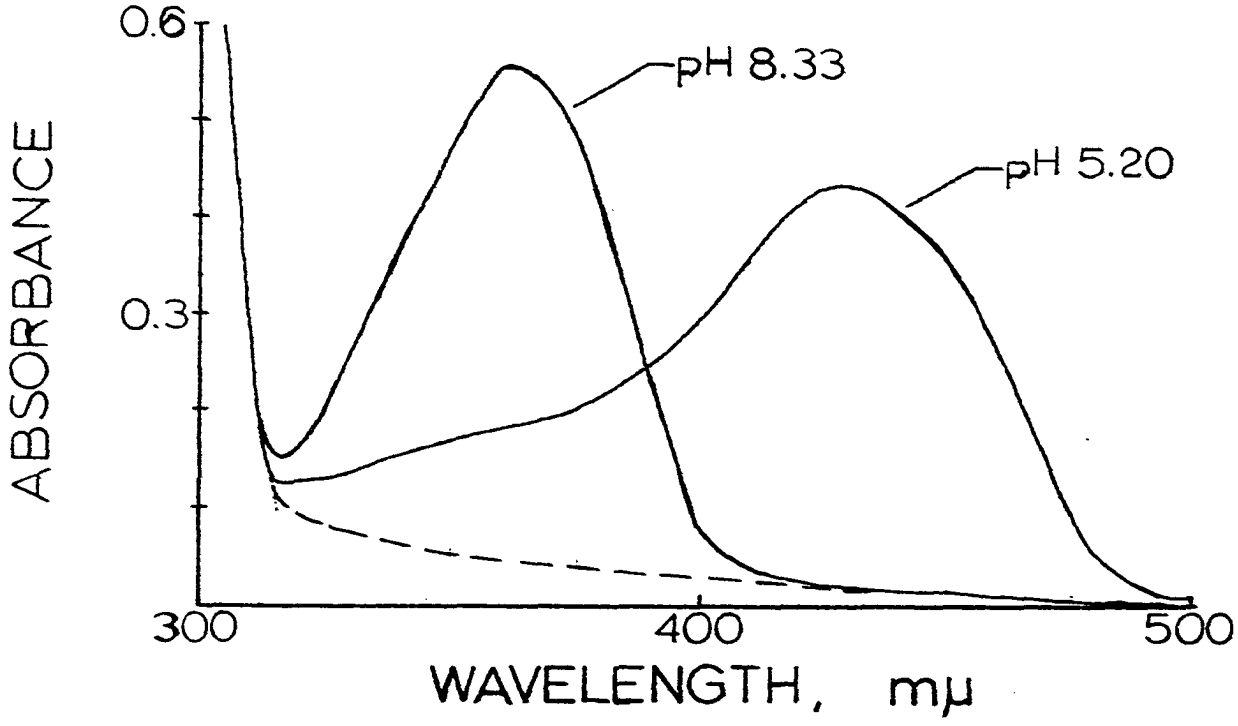
I and II Pyridoxal Phosphate and Pyridoxamine Phosphate

Native GOT

For purposes of comparison, data on the PLP-containing native enzyme are included. The pH dependence of the enzyme's (α subform) absorption spectrum is shown in Figure 2. The corresponding CD spectra (Figure 3) were obtained on a GOT preparation in which the subforms had not been separated (the $A_{430\ m\mu}/A_{340\ m\mu}$ ratio was 1.2 at pH 4.8). The spectra of the abortive complex formed between α-GOT (aldehyde species) and α-ketoglutarate are illustrated in Figures 4 and 5. The absorption and CD spectra of the PMP form of the enzyme, shown in Figure 6, were obtained on a preparation of GOT in which the subforms had not been separated (the $A_{430\ m\mu}/A_{340\ m\mu}$ ratio was 1.2 at pH 4.8). The reactivity of α-GOT toward L-glutamate at a saturating level of oxaloacetate (halving the oxaloacetate concentration had no effect on the initial velocity) was investigated. From the abscissa intercept and the slope of a double reciprocal plot of $1/V_i$ vs 1/S (Lineweaver-Burke plot, Figure 7), a value of 2.15 (± 0.5) mM was obtained for K_{Glu}. This value was in close agreement with that (4 mM) of Velick and

Figure 2. Absorption spectra of α-GOT in 0.1 M acetate, pH 5.20, and in 0.01 M triethanolamine HCl, pH 8.33, (solid lines); and apoGOT in 0.01 M triethanolamine HCl, pH 8.3, (broken line). Protein concentrations are 7.5×10^{-5} M

Figure 3. CD spectra of cytoplasmic GOT (subforms not separated; $A_{430\ m\mu}/A_{340\ m\mu} = 1.2$ at pH 4.8) in 0.01 M acetate, pH 4.8, and in 0.001 M triethanolamine HCl, pH 8.3. At pH 4.8, $\Delta A/A = 3.2 \times 10^{-3}$ at 430 mμ; at pH 8.3, $\Delta A/A = 2.0 \times 10^{-3}$ at 362 mμ. Protein concentration is 1.3×10^{-4} M


0.6
0.3
ABSORBANCE
pH 8.33
pH 5.20
300
400
500
WAVELENGTH, mμ

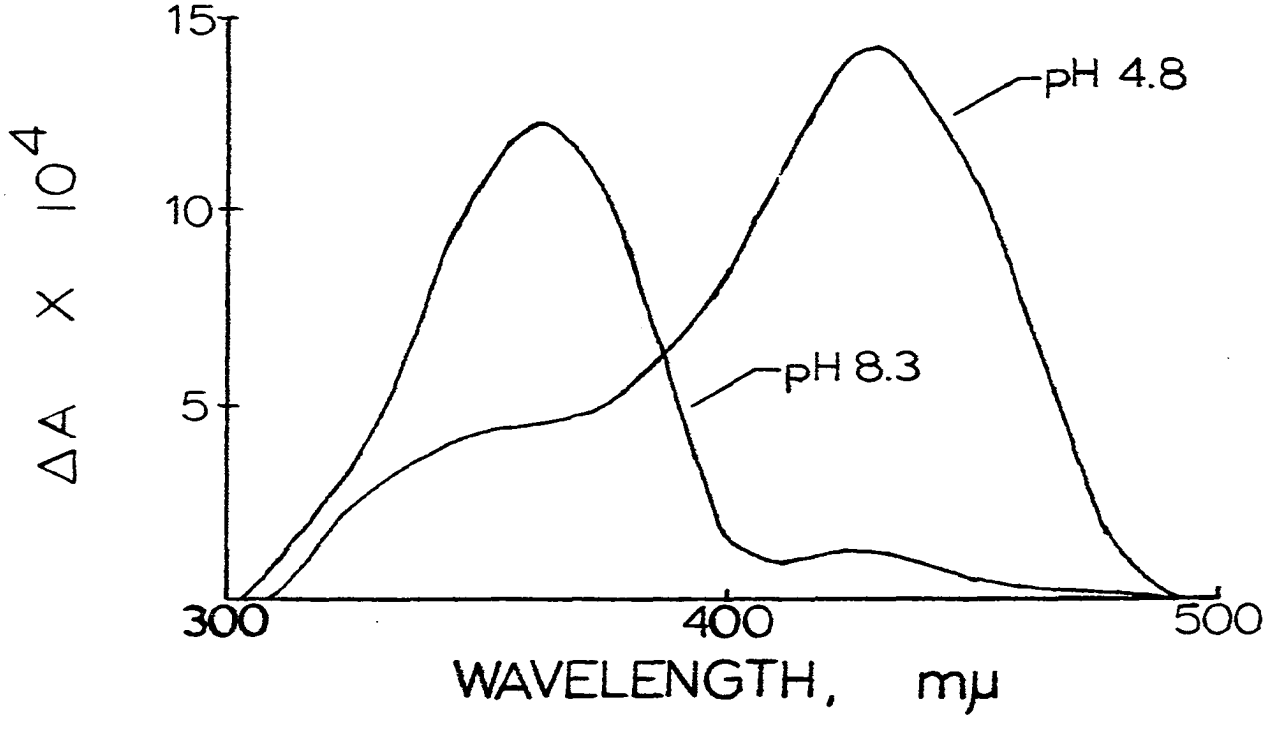
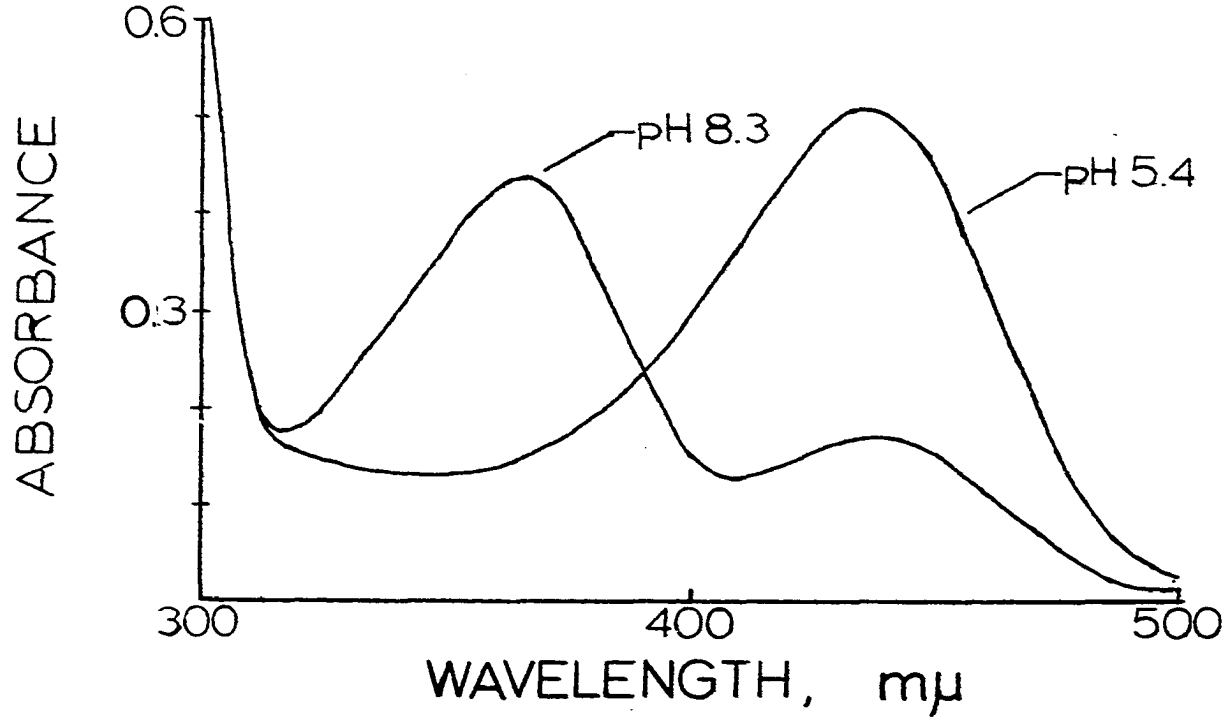

15
10
5
ΔA X 10^4
pH 4.8
pH 8.3
300
400
500
WAVELENGTH, mμ

Figure 4. Absorption spectra of α-GOT-α-ketoglutarate complex in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, ph 8.3, α-ketoglutarate concentration is 5.0×10^{-3} M. Protein concentration is 7.2×10^{-5} M

Figure 5. CD spectra of α-GOT-α-ketoglutarate complex in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, ph 8.3. At pH 5.4, $\Delta A/A = 2.5 \times 10^{-3}$ at 435 mμ; at pH 8.3, $\Delta A/A = 1.9 \times 10^{-3}$ at 365 mμ. α-ketoglutarate concentration is 5.0×10^{-3} M. Protein concentration is 7.2×10^{-5} M


0.6
0.3
ABSORBANCE
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ

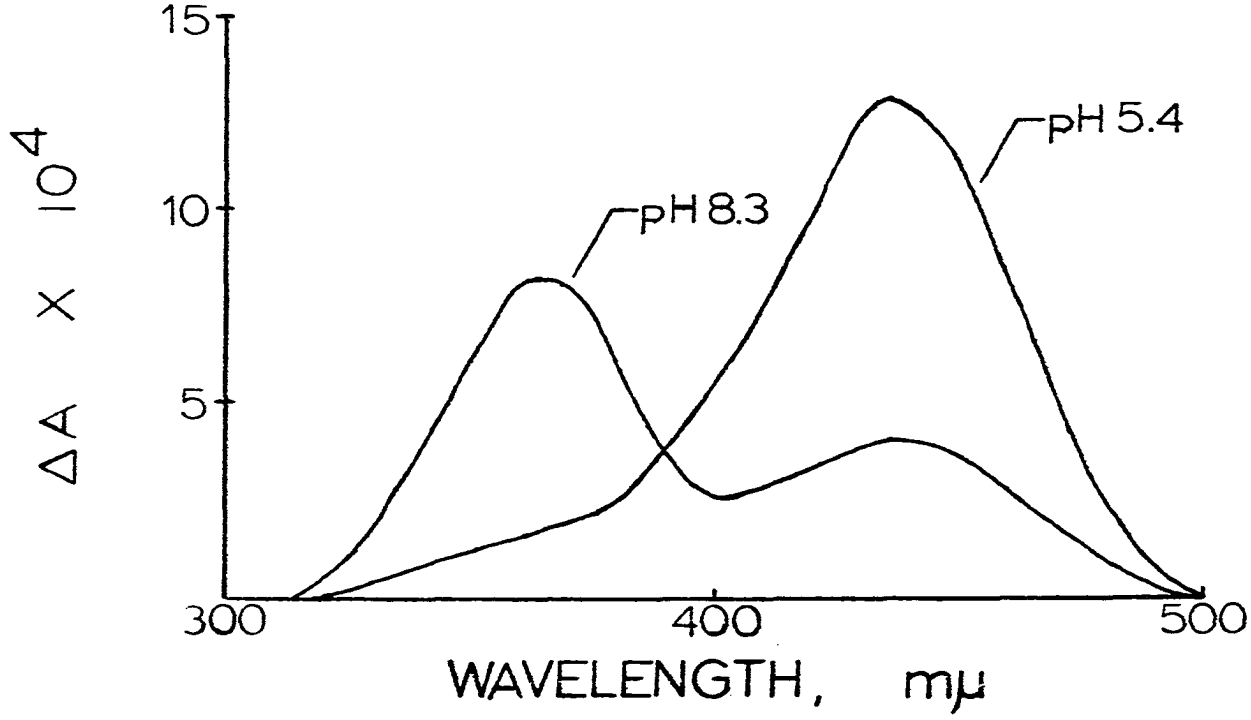
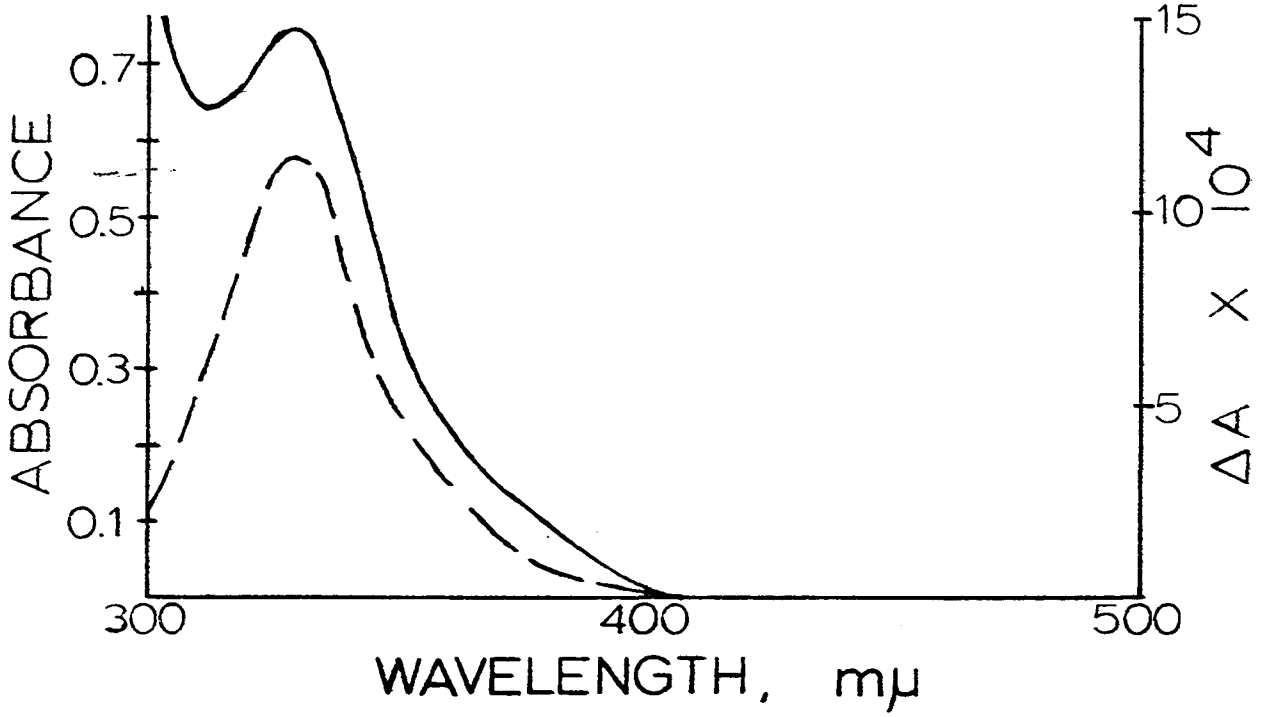
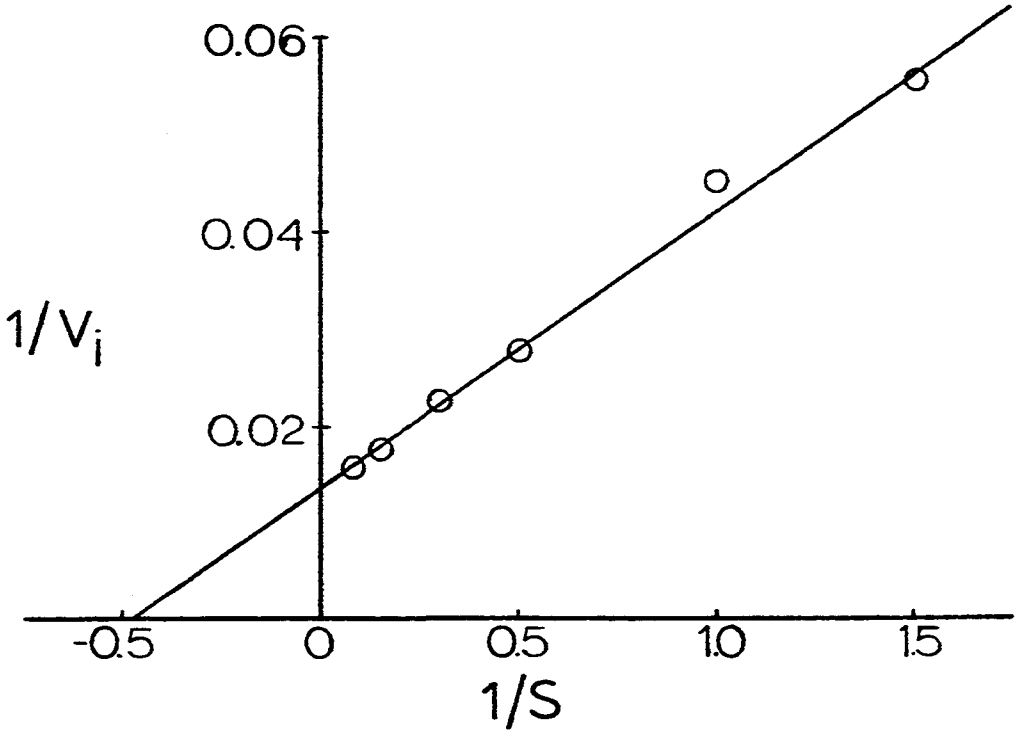

15
10
5
ΔA X 10^4
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ

Figure 6. Absorption spectrum (solid line) and CD spectrum (broken line) of the PMP form of cytoplasmic GOT (see legend for Figure 3, page 28) in 0.001 M triethanolamine HCl, pH 8.3. Concentration of L-glutamate is 0.01 M. $\Delta A/A = 1.5 \times 10^{-3}$ at 330 mμ. Protein concentration is 1.3×10^{-4} M

Figure 7. Lineweaver-Burke plot of α-GOT where S is the concentration of L-glutamate in mM and V_i is the initial velocity in mM/min/mg. Concentration of oxaloacetate is 0.667 mM


ABSORBANCE
0.7
0.5
0.3
0.1
300
400
500
WAVELENGTH, mμ
15
10
5
ΔA X 10⁴


0.06
0.04
0.02
1/Vi
-0.5
0
0.5
1.0
1.5
1/S

Vavra (1962) who utilized a GOT preparation containing all subforms.

apoGOT-I

PLP was bound very rapidly by apoGOT to form a complex whose absorption spectra closely approximated that of the native enzyme. However, the absorption at 410-420 mμ was higher for the reactivated apoGOT than it was for the native GOT. The apoGOT-I possessed a specific activity of 92-99% of the specific activity of native GOT.

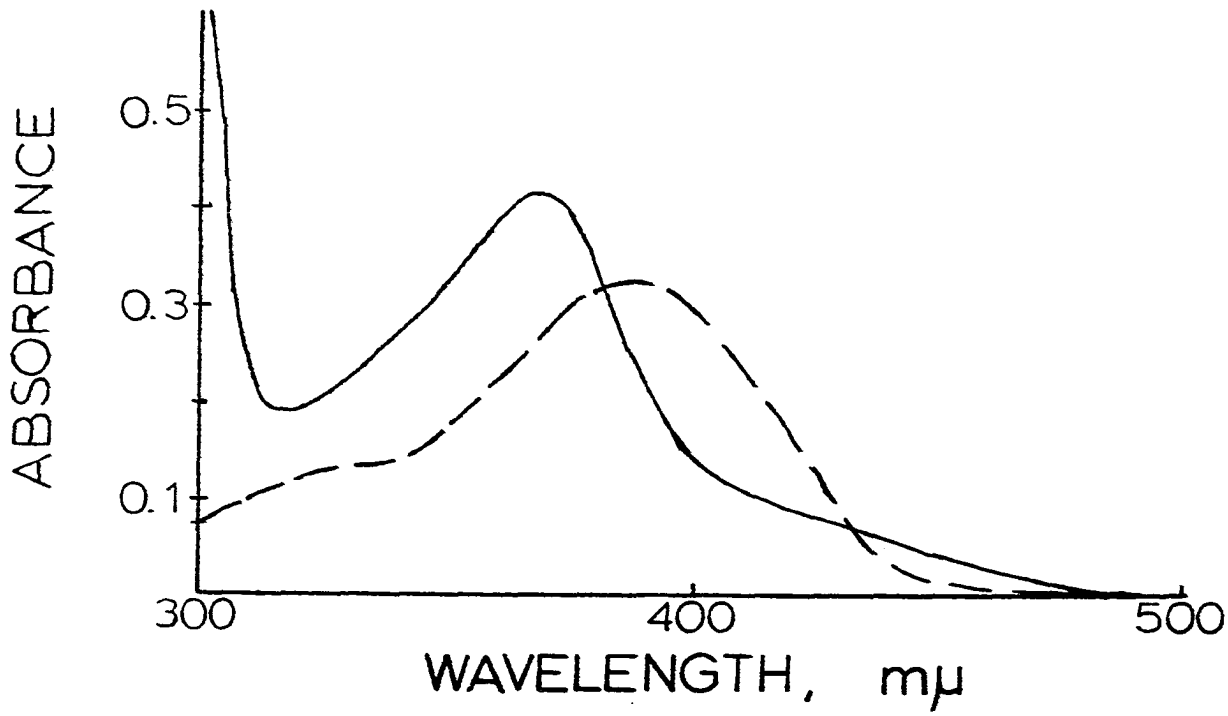
III Carboxymethyl-deoxypyridoxal

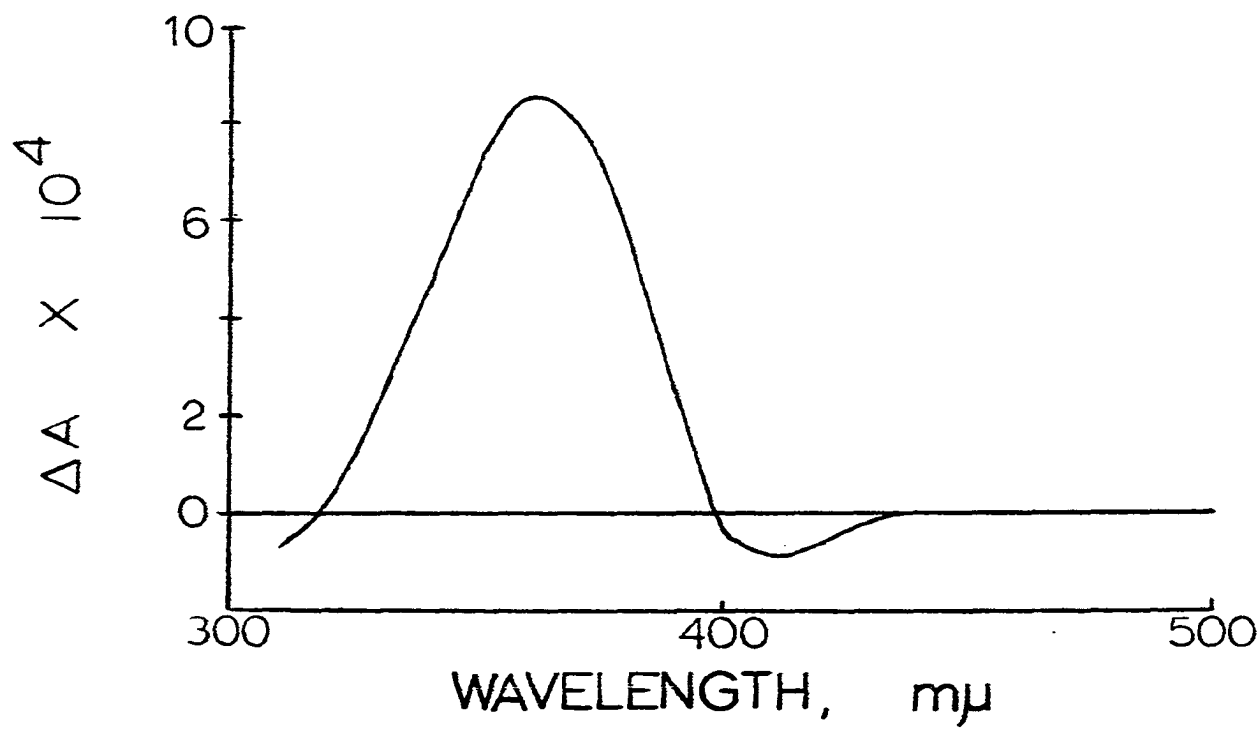
Binding

ApoGOT at a concentration of 8.9×10^{-5} M was allowed to react with an excess of III (2.0×10^{-4} M) and was then passed through a short column (1.5 x 15 cm) of Sephadex G-25. Two distinct yellow bands were formed, one corresponding to the protein fraction and the other to free III. The protein-bound III exhibited an absorbance maximum at 370 mμ, a shift of 20 mμ from that of the unbound compound. The absorbance ratio, $A_{280\ m\mu}/A_{370\ m\mu}$, of apoGOT-III was 10.5 from which it was assumed that amount of III bound was about the same as that of PLP in the native enzyme. To confirm this, a stoichiometric amount of III was added to apoGOT and the same spectrum was obtained. Thereafter, apoGOT-III was prepared by allowing an approximately equal molar amount of III to combine with apoGOT (Figure 8). ApoGOT-III displays circular dichroism as shown in Figure 9. The bound III gave two peaks, a positive one at 362 mμ with a Kuhn dissymmetry factor ($\Delta A/A$) of 1.6×10^{-3} and a negative one at 412 mμ. The amount of absorbance and CD at 412 mμ varied in the

Figure 8. Absorption spectra of III (broken line) and apoGOT-III (solid line) in 0.01 M triethanolamine HCl, pH 8.3. Concentration of III is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M

Figure 9. CD spectrum of apoGOT-III in 0.01 M triethanolamine HCl, pH 8.3. Concentration of III is 1.0×10^{-4} M. Protein concentration is 9.3×10^{-5} M. $\Delta A/A = 1.6 \times 10^{-3}$ at 362 mμ


ABSORBANCE
0.5
0.3
0.1
300
400
500
WAVELENGTH, mμ


ΔA X 10^4
10
6
2
0
300
400
500
WAVELENGTH, mμ

different preparations of apoGOT-III. ApoGOT-III did not show any pH dependent change in the absorption or CD spectra over the range of pH 5.4 to 8.3.

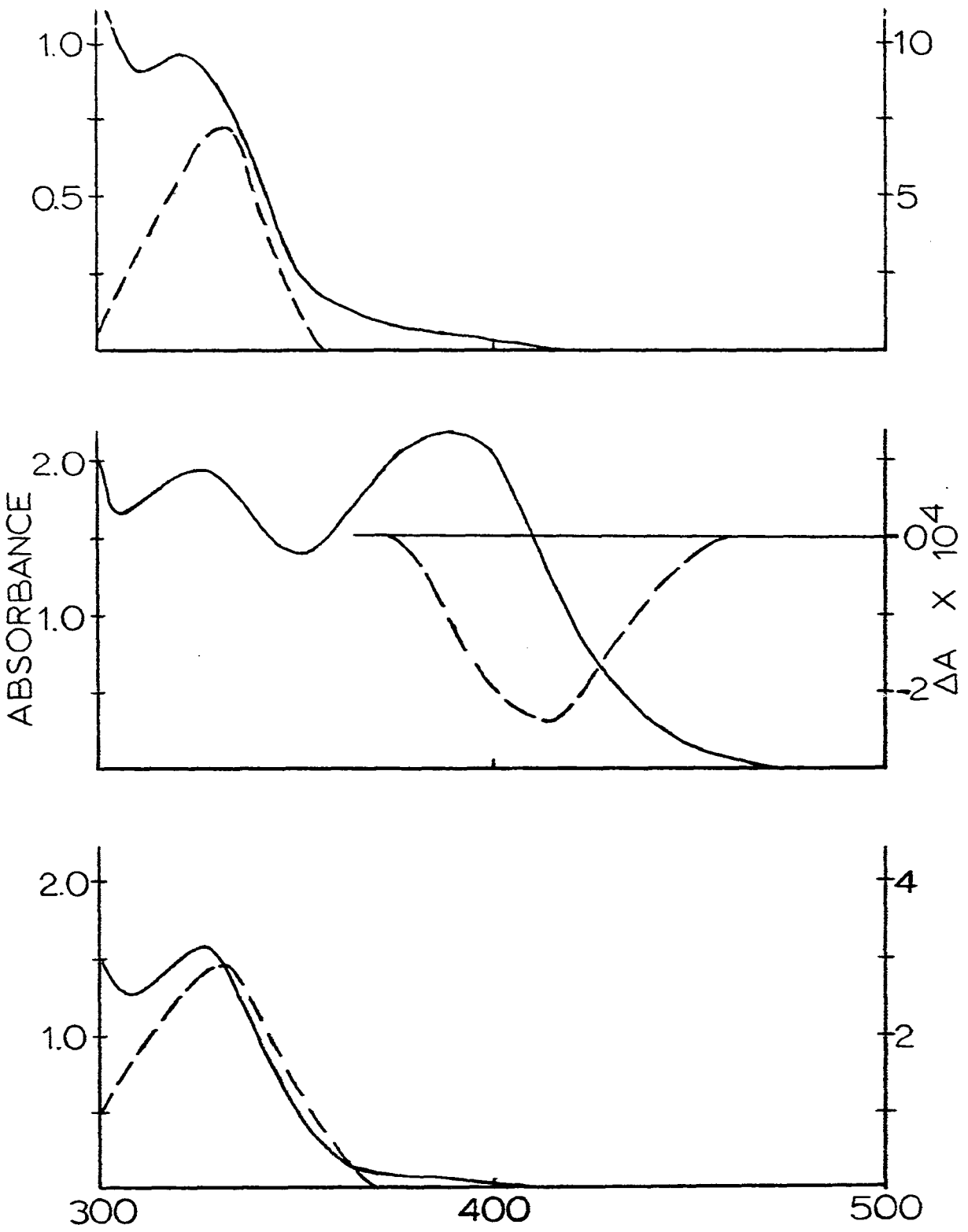
The apoGOT-III complex may be reduced with sodium borohydride giving rise to a maximum at 332 mμ in the absorption and CD spectra ($\Delta A/A$ was 7.4×10^{-4} and the $A_{280\ m\mu}/A_{332\ m\mu}$ ratio was 5.03; Figure 10a). Incubation with an excess of PLP followed by dialysis reactivated the reduced apoGOT-III slightly with a specific activity of 0.16 mM/min/mg, a result which was expected since the borohydride reduction is usually accompanied by some resolution of the enzyme. Under the same conditions a control of apoGOT was activated by PLP to an activity of 8.8 mM/min/mg. The spectral characteristics of reduced apoGOT-III in the presence of PLP are interesting. A ten-fold excess of PLP was added to three ml. of reduced apoGOT-III (3.27 mg/ml) and the solution was dialyzed against 0.001 M triethanolamine HCl overnight. Under these conditions, the CD at 332 mμ was destroyed and a small negative peak ($\Delta A/A = 1.67 \times 10^{-4}$) at 414 mμ appeared (Figure 10b) while the absorption spectrum showed two peaks with maxima at 325 mμ and 390 mμ. The complex had no additional enzymic activity (0.17 mM/min/mg). Reduction of this complex with sodium borohydride followed by dialysis gave absorption and CD peaks at 325 mμ and 329 mμ respectfully (Figure 10c).

Information regarding the binding constant of III relative to that of PLP and the rate constant of the dissociation of III from the enzyme was obtained by observing the displacement of III by PLP. For the

Figure 10a. Absorption and CD spectra of reduced apoGOT-III in 0.001 M triethanolamine HCl, pH 8.3, $\Delta A/A = 7.4 \times 10^{-4}$ at 332 mμ. Protein concentration is 8.2×10^{-5} M

Figure 10b. Absorption and CD spectra of reduced apoGOT-III after addition of PLP in 0.001 M triethanolamine HCl, pH 8.3. $\Delta A/A = 1.7 \times 10^{-4}$ at 414 mμ. Protein concentration is 8.2×-0^{-5} M

Figure 10c. Absorption and CD spectra of complex shown in Figure 10b after reduction with sodium borohydride in 0.001 M triethanolamine HCl, pH 8.3. $\Delta A/A_5 = 1.9 \times 10^{-4}$ at 329 mμ. Protein concentration is 5.3×10^{-5} M


1.0
0.5
10
5
2.0
1.0
ABSORBANCE
0
-2
ΔA x 10⁴
2.0
1.0
4
2
300
400
500
WAVELENGTH, mμ

relative binding constant data, a stoichiometric amount of PLP was added to apoGOT-III (the final concentration of each was 2.0×10^{-6} M) and the subsequent reactivation of the enzyme at 25° followed by activity assays (Figure 11). The relationship between the dissociation constants and the activity may be derived as follows.

From

$$EI \rightleftharpoons E + I \quad \text{and} \quad EIII \rightleftharpoons E + III \tag{1}$$

the two dissociation constants are defined as

$$K_I = [E][I]/[EI] \text{ and } K_{III} = [E][III]/[EIII] \tag{2}$$

Dividing K_I by K_{III},

$$\frac{K_I}{K_{III}} = \frac{[EIII][I]}{[EI][III]} \tag{3}$$

Since the total amount of enzyme $[E_T]$ is equal to the sum of all forms of E and assuming that there is no E that is not combined with one of the cofactors, then

$$[EIII] = [E_T] - [EI] \tag{4}$$

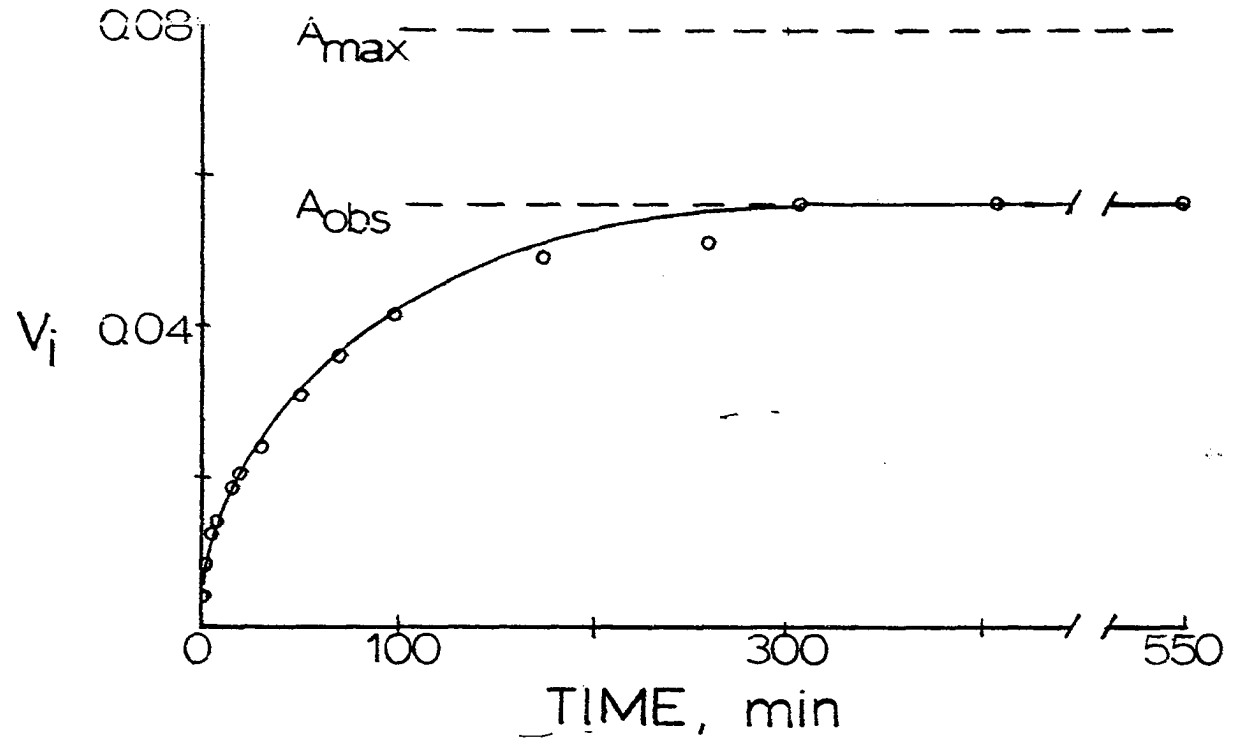
Substituting (4) into (3) and rearranging gives

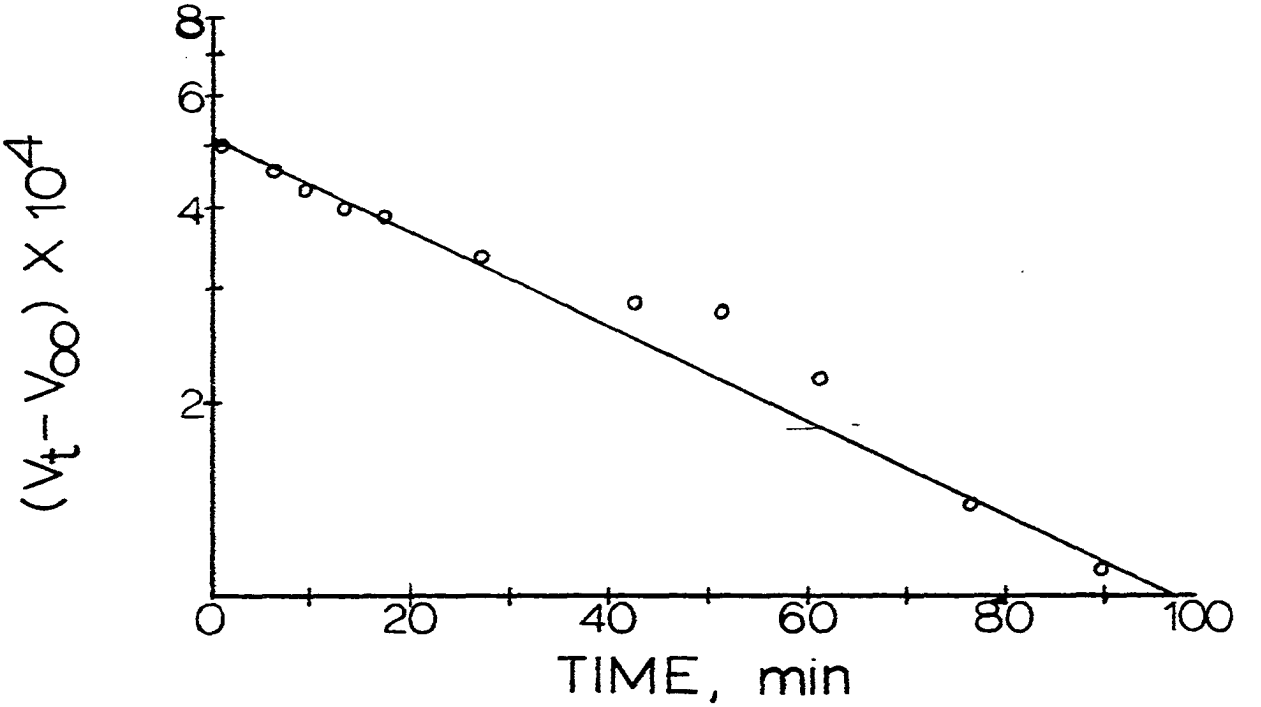
$$\frac{[E_T]}{[E_I]} = \frac{K_I}{K_{III}} \times \frac{[III]}{[I]} + 1 \tag{5}$$

The maximum activity possible (A_{max}) is proportional to $[E_T]$ and the observed activity at equilibrium (A_{obs}) is proportional to [EI] since EIII is virtually inactive at the low concentration used. Hence,

Figure 11. Plot of reactivation of apoGOT-III by PLP. V_i is the initial velocity in mM/min. Concentrations of III and PLP are 2.0×10^{-6} M each. Protein concentration is 1.8×10^{-6} M. A_{obs} is the initial velocity of apoGOT-III plus PLP at equilibrium and A_{max} is the initial velocity of an equal amount of apoGOT plus PLP only

Figure 12. First order plot of reactivation of apoGOT-III by an excess of PLP. V_t, the initial velocity at time t in absorbance units/sec., minus V_∞, the initial velocity at equilibrium, is plotted on a $\log_{10}$ scale. Concentrations of III, PLP, and protein are 1.0×10^{-6} M, 2.5×10^{-5} M, and 9.4×10^{-7} M respectively


0.08
A_{max}
A_{obs}
V_i
0.04
0
100
300
550
TIME, min


8
6
4
2
$(V_t - V_\infty) \times 10^4$
0
20
40
60
80
100
TIME, min

$$\frac{A_{max}}{A_{obs}} = \frac{K_I}{K_{III}} \times \frac{[III]}{[I]} + 1 \qquad (6)$$

In this experiment A_{max} = 0.079 mM/min. which is the activity of a control solution of apoGOT plus PLP at a concentration of 2.0×10^{-6} M and A_{obs} is the competition equilibrium activity of 0.056 mM/min. [III] is equal to 2.0×10^{-6} M - [EIII] and [I] is equal to 2.0×10^{-6} M - [EI]. [EI] and [EIII] were determined from

$$[EI] = A_{obs}/A_{max} \times [E_T] = 1.28 \times 10^{-6} \text{ M} \qquad (7)$$

$$[EIII] = [E_T] - [EI] = 0.52 \times 10^{-6} \text{ M} \qquad (8)$$

Substitution into (6) gives

$$\frac{0.079}{0.056} \quad \frac{2.0 \times 10^{-6} - 0.52 \times 10^{-6}}{2.0 \times 10^{-6} - 1.28 \times 10^{-6}} + 1 \qquad (9)$$

$$K_I = 0.19\ K_{III}$$

Therefore, III is bound approximately one-fifth as tightly as PLP. While this result is subject to several errors (denaturation of protein, determination of protein and cofactor concentrations, and activity measurements), the tighter binding of PLP compared to that of III is confirmed by the fact that III caused no loss of enzymic activity when incubated with native GOT at 5° during periods of up to ten days.

The rate constant of the dissociation of III from apoGOT-III at 25° was evaluated by reactivating the enzyme with a large excess of PLP (Figure 12).

For the reactions

$$EIII \underset{k_2}{\overset{k_1}{\rightleftharpoons}} III + E \qquad (10)$$

$$E + I \xrightarrow{k_3} EI$$

the following conditions hold

$$[E_T] = [EIII] + [E] + [EI] \qquad (11)$$

$$\frac{d[E_T]}{dt} = \frac{d[EIII]}{dt} + \frac{d[E]}{dt} + \frac{d[EI]}{dt} = 0 \qquad (12)$$

Assuming a steady-state existence for E ($d[E]/dt = 0$),

$$\frac{d[EI]}{dt} = -\frac{d[EIII]}{dt} \qquad (13)$$

and, as a further consequence of the steady-state assumption,

$$[E] = \frac{k_1[EIII]}{k_2[III]+k_3[I]} \quad \text{and} \quad \frac{d[EI]}{dt} = k_3[I][E] \qquad (14)$$

substituting for [E]

$$\frac{d[EI]}{dt} \quad \frac{k_1k_3[I][EIII]}{k_2[III]+k_3[I]} \qquad (15)$$

From (13) and (15)

$$\frac{d[EI]}{dt} = -\frac{d[EIII]}{dt} = \frac{k_1k_3[I][EIII]}{k_2[III]+k_3[I]} \qquad (16)$$

When $k_3[I] >> k_2[III]$ (by making [I] >> [III])

$$\frac{d[EI]}{dt} = -\frac{d[EIII]}{dt} = \frac{k_1 k_3 [I][EIII]}{k_3[I]} = k_1[EIII] \qquad (17)$$

Therefore, when [I] >> [III], the slope of a first order plot ($\ln A_t/A_\infty$ vs. time) is equal to k_1, the rate constant for the dissociation of apoGOT-III. From Figure 12, k_1 = 2.303 x slope = 2.83 x 10^{-4} sec^{-1}. The half-life, $t_{1/2}$, of the dissociation is given by

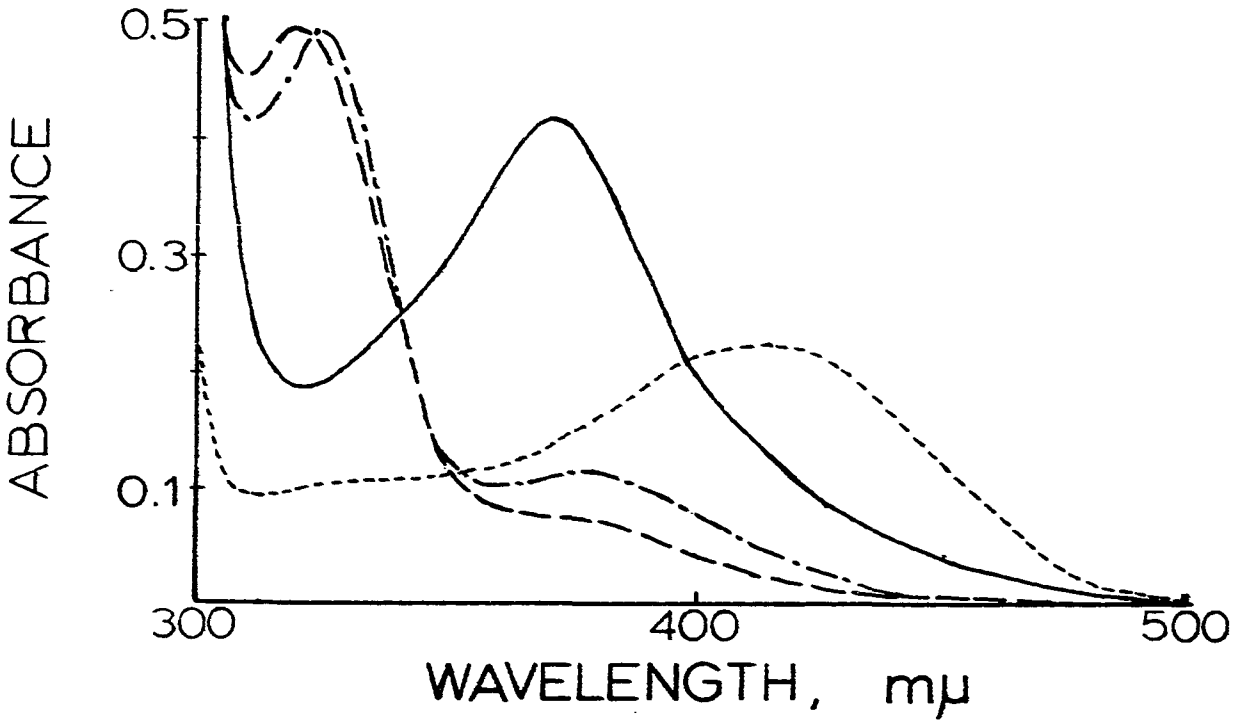
$$t_{1/2} = \frac{\ln 2}{k_1} \quad \frac{0.69}{2.83 \times 10^{-4}} = 2.44 \times 10^3 \text{ sec} = 41 \text{ min.} \qquad (18)$$

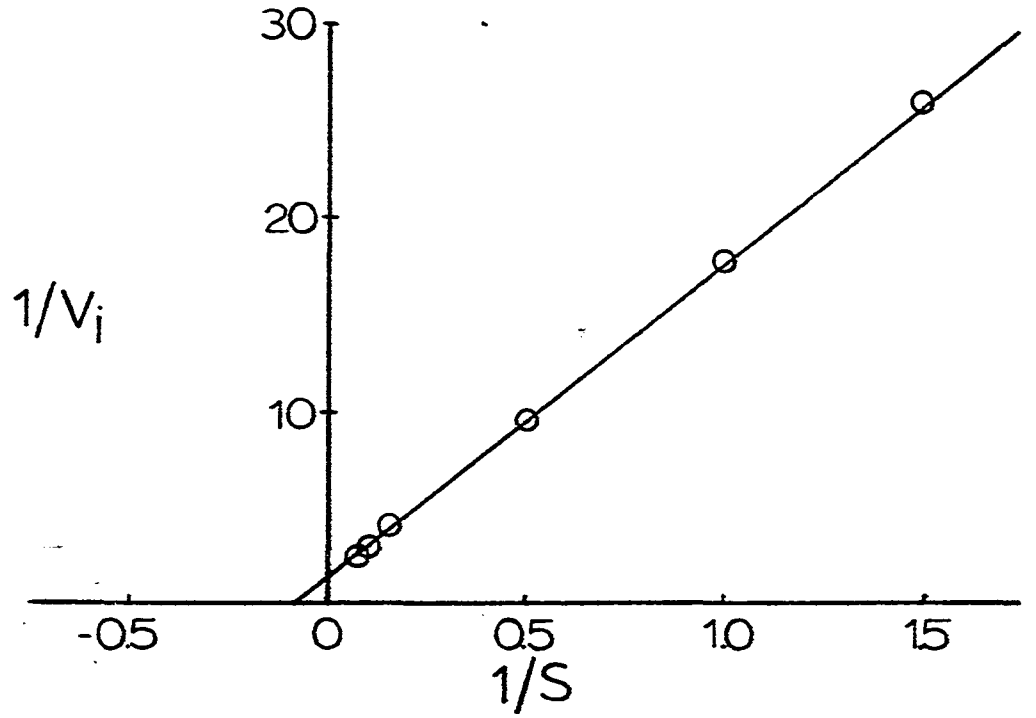
Reaction with substrates

ApoGOT-III showed a small amount of catalytic activity in the usual assay (20 μmoles each of L-aspartate and α-ketoglutarate, 200 μmoles Tris, pH 8.3; 3 ml.); at 5.5 x 10^{-5} M the activity was 0.095 mM/min/mg which is only 0.23% of the activity (41.0 mM/min/mg) exhibited by PLP-reactivated apoGOT at a concentration of 5.5 x 10^{-7} M. However, the addition of 0.1 ml of 0.2 M L-glutamate to 1.0 ml of 6.0 x 10^{-5} M apoGOT-III in a cuvette caused a rapid decrease in the 370 mμ peak (Figure 13) and the formation of a peak at 320 mμ. About 94% of the 370 mμ peak disappeared within two minutes followed by a slower approach to the equilibrium position shown in Figure 13. The absorption at 412 mμ decreased also but twice as slowly as the 370 mμ absorption. After 28 minutes, 0.1 ml of 0.1 M α-ketoglutarate, pH 4.8, was added to the cuvette which caused the 320 mμ peak to rapidly shift to 323 mμ and slowly decrease to form a peak at 375 mμ. The absorption spectrum shown in Figure 13 was obtained 20 minutes after the addition of α-ketoglutarate,

Figure 13. Absorption spectra of apoGOT-III and III after reaction with substrate.
———— apoGOT-III, pH 8.3 (no substrate)
— — — — apoGOT-III in 1.82 x 10^{-2} M L-glutamate, pH 8.3
—·—·— apoGOT-III in 1.66 x 10^{-2} M L-glutamate and 8.3 x 10^{-3} M α-ketoglutarate, pH 6.5
– – – – – III in 1.82 x 10^{-2} M L-glutamate, pH 8.3
Concentration of III is 6.0 x 10^{-5} M. Protein concentration is 5.3 x 10^{-5} M. All pH 8.3 solutions are in 0.01 M triethanolamine HCl

Figure 14. Lineweaver-Burke plot of apoGOT-III where S is the concentration of L-glutamate in mM and V_i is the initial velocity in mM/min/mg. Concentration of oxaloacetate is 0.667 mM


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ


30
20
10
1/Vi
-0.5
0
0.5
1.0
1.5
1/S

and 30 hours of incubation at room temperature resulted in a $A_{323\ m\mu}/A_{370\ m\mu}$ ratio of 1.2. The final pH of the solution was 6.5 L-aspartate also caused the absorption to shift from 370 mμ to 320 mμ and this change could be reversed by α-ketoglutarate or oxaloacetate.

The species absorbing at 320 mμ showed very little CD and was not bound firmly to the protein. When aspartate-treated apoGOT-III containing this species was passed through a short column of Sephadex G-25, the $A_{280\ m\mu}/A_{320\ m\mu}$ ratio increased from 4.5 to 13.2, which indicated a substantial loss of chomophore. Since the above results suggested that apoGOT-III undergoes transamination--in a manner analogous to that of the native GOT--to give carboxymethyl-deoxypyridoxamine, a solution containing apoGOT (6.9×10^{-5} M) and synthetic carboxymethyl-deoxypyridoxamine (6.3×10^{-5} M) in 10^{-3} M triethanolamine HCl, pH 8.3, was prepared. This preparation, after standing at room temperature for 24 hours, demonstrated the same characteristics (absorption spectrum, CD, looseness of binding) as that of the aspartate- or glutarate-treated apoGOT-III and was converted by α-ketoglutarate and oxaloacetate to a form absorbing at 370 mμ.

Since III appeared to be bound at the active site in a specific manner, the low rate of apoGOT-III-catalyzed transamination was of interest. In order to determine whether the amino acid substrate was allowed to bind properly or not, the reactivity toward L-glutamate at a saturating level of oxaloacetate was investigated and a Lineweaver-Burke plot constructed (Figure 14). While V_1, the maximum velocity, was one hundredth of that of α-GOT (0.735 mM/min/mg for apoGOT-III, 74 mM/min/mg for α-GOT), the Michaelis constant for glutamate, K_{Glu}, was in-

creased only 5.7 times (K_{Glu} = 12.2 ($\pm$ 1.8) mM for apoGOT-III, 2.15 mM for α-GOT). Because of the low initial velocities and inhibition by α-ketoglutarate (see below), the L-aspartate--α-ketoglutarate substrate pair could not be utilized for such a study.

The reactivity of apoGOT-III toward some amino acids and some common inhibitors of GOT was investigated and compared with that of α-GOT as shown in Table 2. Substrate inhibition of apoGOT-III by α-ketoglutarate was very pronounced even at a high level of L-aspartate (Table 3) and is much higher than that shown by GOT (Velick and Vavra, 1962). The complex formed between apoGOT-III and α-ketoglutarate absorbed light at 380 mμ and possessed CD ($\Delta A/A = 7.1 \times 10^{-4}$) at 372 mμ at pH 8.3 (Figures 15 and 16). Furthermore, the complex's CD spectrum was pH dependent with a peak at about 450 mμ appearing at pH 5.4. Unfortunately, the complex was quite unstable at the lower pH and the absorption spectrum at pH 5.4 does not yield much information on the pH dependence.

The behavior of III with amino acids was briefly investigated. III in the presence of L-glutamate absorbed light at 415 mμ (Figure 13); no absorption at 320 mμ appeared in a five hour period. With 1.0×10^{-4} M III in 0.01 M L-aspartate in 0.01 M triethanolamine HCl, pH 8.3, no significant amount of 320 mμ absorbing species was produced unless incubated for at least 72 hours at room temperature.

Table 2. Substrate and inhibitor specificity of apoGOT-III

Substrate (s) or Inhibitor (i)	α-GOT[a]		apo-GOT-III[b]	
	Sp. Activity (mM/min/mg)	% of maximum activity	Sp. Activity (mM/min/mg)	% of maximum activity
L-glutamate[c](s)	66.7	(100)	0.46	(100)
D-glutamate[c](s)	0	0	0	0
L-alanine[c](s)	0	0	0	0
L-valine[c](s)	4.0	6.0	0.17	37.0
L-serine[c](s)	0	0	0	0
L-methionine[c](s)	0	0	0	0
α-Methyl-D,L-aspartate[d,e](i)	28.5	42.7	0.17	37.0
glutarate[e](i)	45.7	68.6	0.23	50.0
adipate[e](i)	54.3	81.5	0.20	43.5
succinate[e](i)	54.3	81.5	0.20	43.5
L-aspartate[f](s)	40.5	-	0.095	-
D-aspartate[f](s)	0	0	0	0
α-Methyl-D,L-aspartate[d,f](s)	0	0	0	0

[a] 0.0035 mg used in each assay.

[b] 0.035 mg used in each assay.

[c] 20 μmoles amino acid, 2 μmoles oxaloacetate, 200 μmoles Tris, pH 8.3, volume 3 ml.

[d] 40 μmoles inhibitor (20 μmoles each isomer).

[e] 20 μmoles inhibitor, 20 μmoles L-glutamate, 2 μmoles oxaloacetate, 200 μmoles Tris, pH 8.3, volume 3 ml.

[f] 20 μmoles amino acid, 20 μmoles α- ketoglutarate, 200 μmoles Tris, pH 8.3, volume 3 ml.

Table 3. Inhibition of apoGOT-III by α-ketoglutarate[a]

α-ketoglutarate (mM)	Specific Activity (mM/min/mg)
6.67	0.093
3.33	0.133
2.00	0.158
0.667	0.200
0.333	0.225
0.200	0.268

[a] 0.035 mg of apoGOT-III used in each assay. All assays were 6.67 mM in L-aspartate and 66.7 mM in Tris, pH 8.3; volume 3 ml.

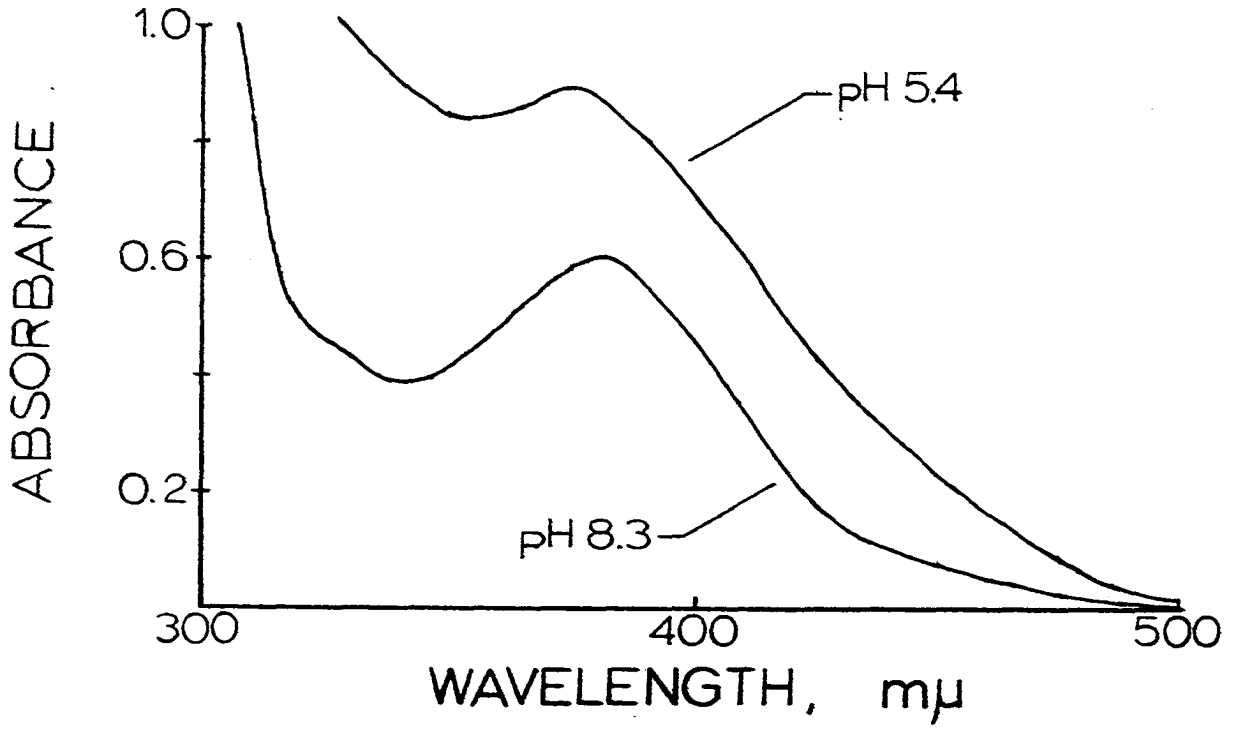
IV Deoxypyridoxalyl Phosphonic Acid

Binding

ApoGOT (9.3×10^{-5} M) was combined with IV (1.0×10^{-4} M) in a cuvette at pH 8.3; within two minutes the absorption maxima of the unbound IV at 369 and 325 mμ were destroyed and absorption maxima at 420 and 359 mμ appeared (Figure 17). Addition of 1.0 M acetate buffer, pH 5.4, (to a final concentration of 0.1 M) caused a decrease in the absorption at 359 mμ and an increase at 430 mμ. The corresponding CD spectra are shown in Figure 18; at pH 8.3, the peak is at 355 mμ ($\Delta A/A = 1.0 \times 10^{-3}$) with a small negative peak in the 395 to 425 mμ region and, at pH 5.4, a peak at 440 mμ appeared ($\Delta A/A = 2.0 \times 10^{-3}$).

Figure 15. Absorption spectra of apoGOT-III--α-ketoglutarate complex in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3. The absorbance at 510 mμ (0.46) was subtracted from the pH 5.4 spectrum as a partial correction for turbidity. Concentrations of α-ketoglutarate, III, and protein are 5.0×10^{-3} M, 1.0×10^{-4} M, and 9.3×10^{-5} M respectively

Figure 16. CD spectra of apoGOT-III--α-ketoglutarate complex in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3. At pH 8.3, $\Delta A/A = 7.1 \times 10^{-4}$ at 372 mμ. Concentrations are the same as Figure 15


1.0
0.6
0.2
ABSORBANCE
pH 5.4
pH 8.3
300
400
500
WAVELENGTH, mμ

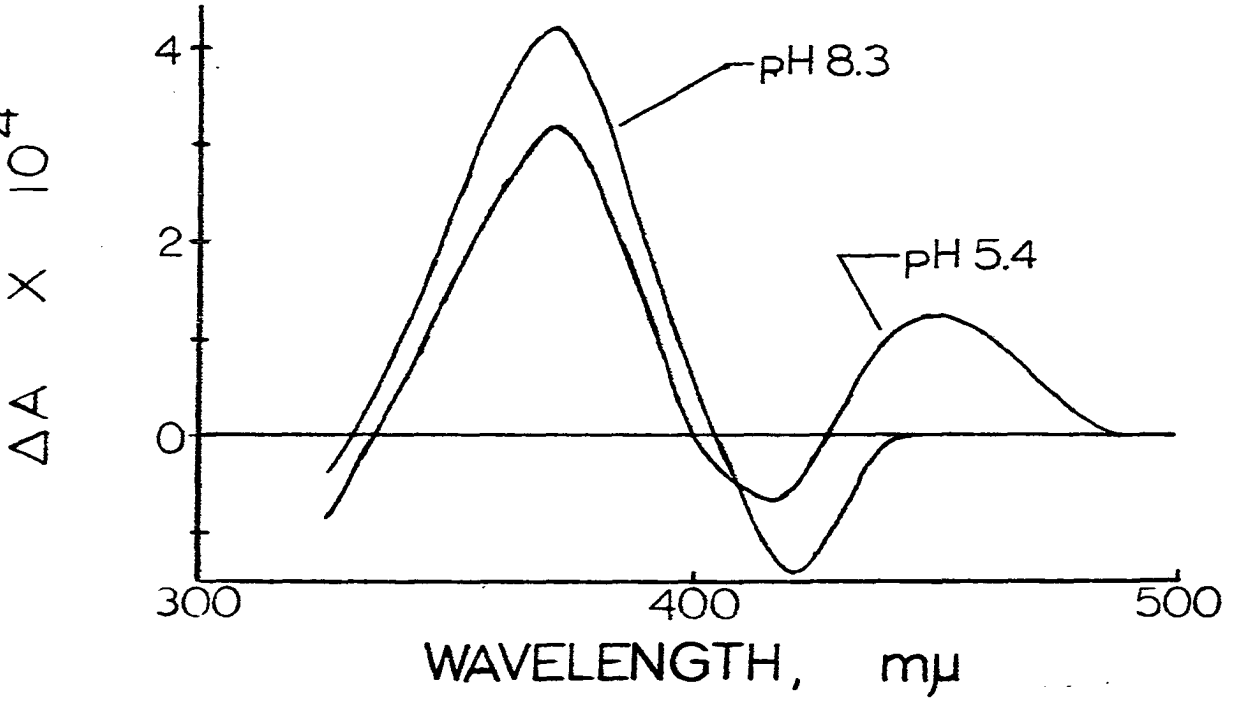
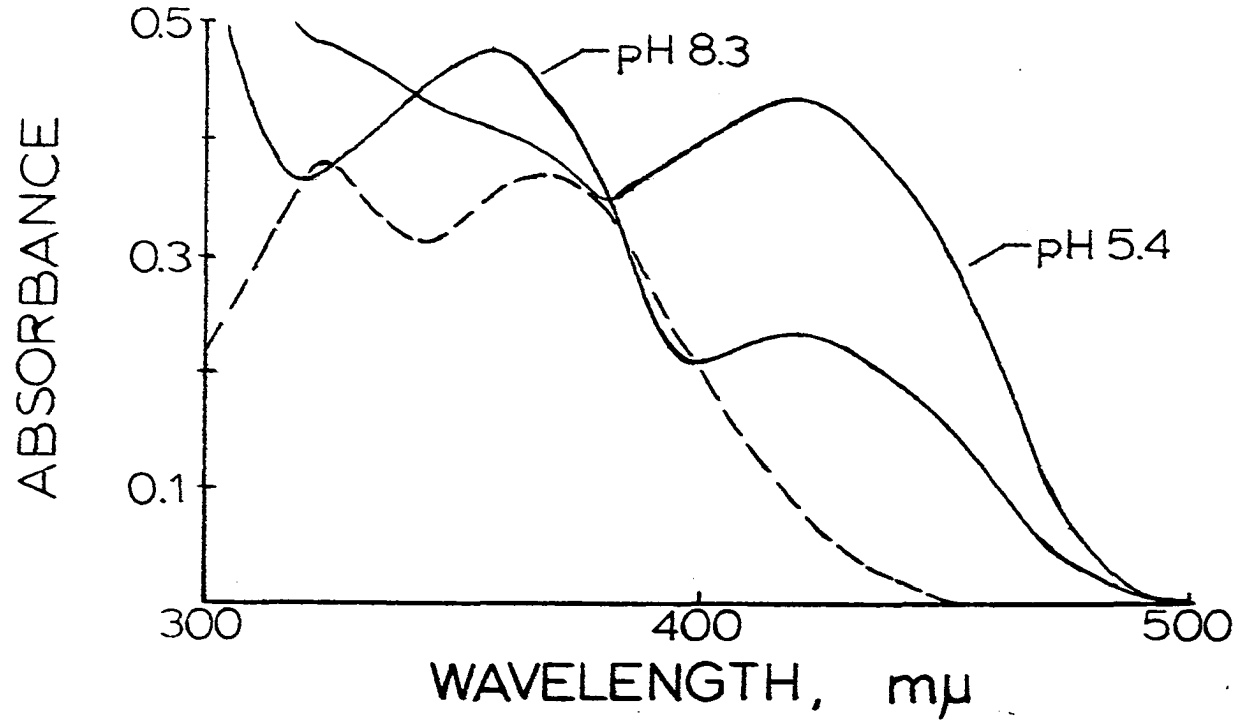
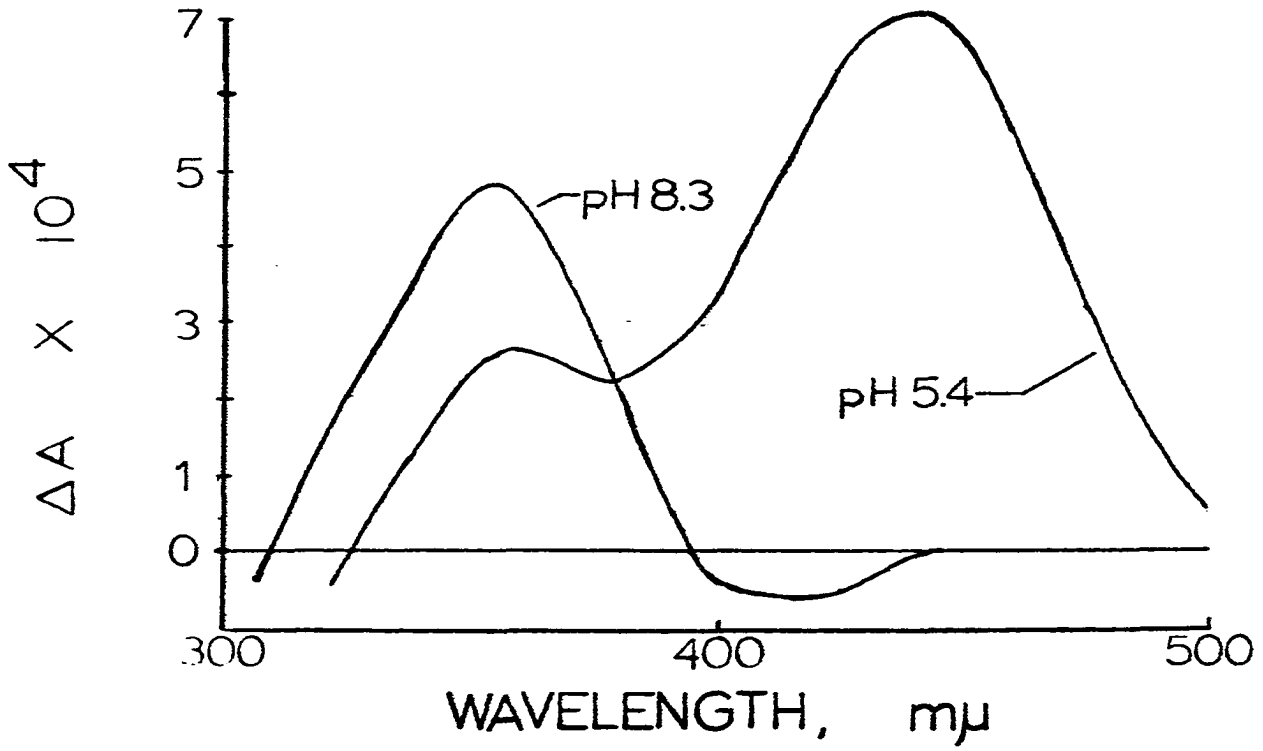

4
2
0
ΔA X 10⁴
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ

Figure 17. Absorption spectra of apoGOT-IV in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl pH 8.3, (solid lines); and of IV in 0.01 M triethanolamine HCl, pH 8.3, (broken line). Concentrations of IV and protein are 1.0×10^{-4} M and 9.3×10^{-5} M respectively

Figure 18. CD spectra of apoGOT-IV in 0.1 M acetate, pH 5.4, and in 0.1 M triethanolamine HCl, pH 8.3. At pH 5.4 $\Delta A/A = 2.0 \times 10^{-3}$ at 440 mμ; at pH 8.3, $\Delta A/A = 1.0 \times 10^{-3}$ at 335 mμ. Concentrations are the same as Figure 17


0.5
0.3
0.1
ABSORBANCE
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ


7
5
3
1
0
ΔA X 10⁴
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ

Using 6.0×10^{-5} M IV and 5.3×10^{-5} M apoGOT the above absorption data were confirmed in a second experiment at pH 8.3, but in a third preparation of apoGOT-IV (also at 6.0×10^{-5} M) an additional absorption maximum at 330 mμ was obtained. This preparation utilized apoGOT that had not been freshly prepared.

Like the case of III, IV could be displaced from the protein by PLP and the rate constant, k_1, of the dissociation of IV from apoGOT-IV in the presence of an excess of PLP (see page 31 for method) was evaluated. From the slope of a first order plot (Figure 19), a value of 3.44×10^{-4} sec^{-1} was calculated which gave a half-life, $t_{1/2}$ of 33 minutes for the dissociation.

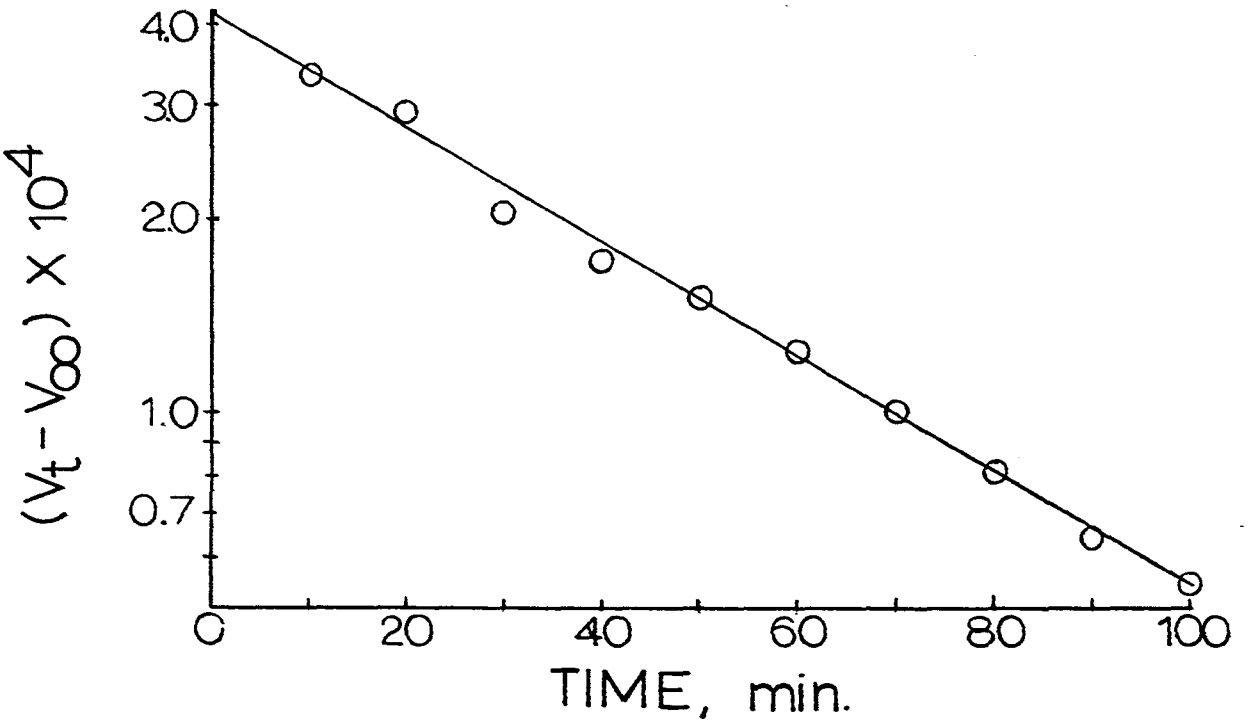
Reaction with substrates

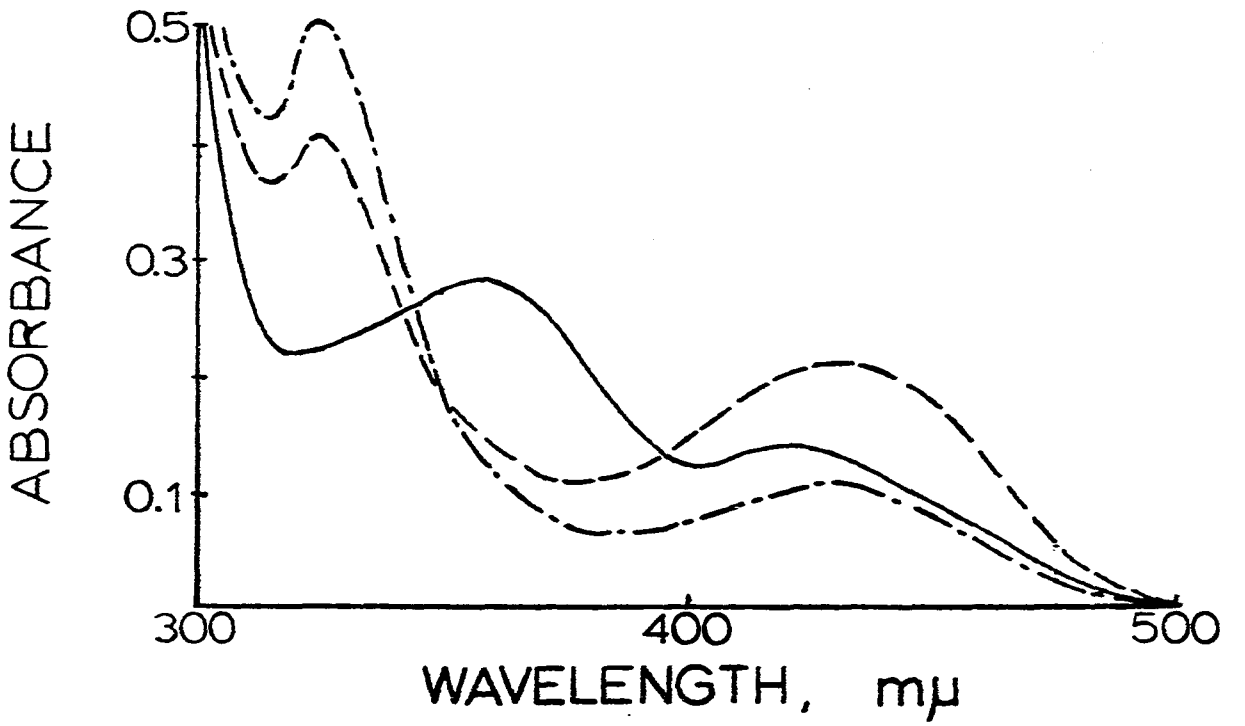
With L-aspartate and α-ketoglutarate (20 μmoles each, 200 μmoles Tris, pH 8.3; volume 3 ml) apoGOT-IV showed about the same amount of catalytic activity as that of apoGOT-III; at 5.5×10^{-5} M the activity of apoGOT-IV was 0.10 mM/min/mg which is 0.24% of the activity (41.0 mM/min/mg) of apoGOT-PLP at a concentration of 5.5×10^{-7} M. The same activity was exhibited by a second preparation of apoGOT-IV.

The addition of 0.1 ml of 0.2 M L-glutamate to 1.0 ml of 6.0×10^{-5} M apoGOT-IV in a cuvette caused a rapid decrease in the absorption at 359 mμ and the appearance of a peak at 325 mμ (Figure 20). An absorption peak at 430 mμ initially appeared (maximum absorbance occurred at about two minutes after addition of L-glutamate) and then slowly decreased; as the 430 mμ absorption declined, the absorbance at 325 mμ increased. The absorption changes ceased in 40 minutes; at this point 0.1 ml of 0.1 M

Figure 19. First order plot of reactivation of apoGOT-IV by an excess of PLP. V_t, the initial velocity at time t in absorbance units/sec., minus V_∞, the initial velocity at equilibrium, is plotted on a $\log_{10}$ scale. Concentrations of IV, PLP, and protein are 1.0×10^{-6} M, 2.5×10^{-5} M, and 914×10^{-7} M respectively

Figure 20. Absorption spectra of apoGOT-IV after reaction with L-glutamate.
———— apoGOT-IV (no substrate)
— — — apoGOT-IV in 1.82×10^{-2} M L-glutamate (2 minutes)
—.—.— apoGOT-IV in 1.82×10^{-2} M L-glutamate (15 minutes)
Concentration of IV is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M. All solutions are in 0.01 M triethanolamine HCl, pH 8.3


4.0
3.0
2.0
1.0
0.7
$(V_t - V_\infty) \times 10^4$
0
20
40
60
80
100
TIME, min.


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ

α-ketoglutarate, pH 4.8, was added which caused no initial change in the absorption spectrum. After 21 hours at room temperature, the absorption at 430 mμ had increased very slightly (0.06 absorbance units) while the absorption at 325 mμ had decreased by half as much (0.03). The final pH of the solution was 6.5. The preparation of apoGOT-IV with an absorption peak at 330 mμ exhibited this same behavior toward L-glutamate.

In 1.82×10^{-2} M L-glutamate, IV at concentration of 9.0×10^{-5} M exhibited two absorption maxima in 0.01 M triethanolamine HCl, pH 8.3; one at 419 mμ ($\varepsilon = 3.7\ mM^{-1}\ cm^{-1}$) and the other at 292 mμ ($\varepsilon = 5.8\ mM^{-1}\ cm^{-1}$).

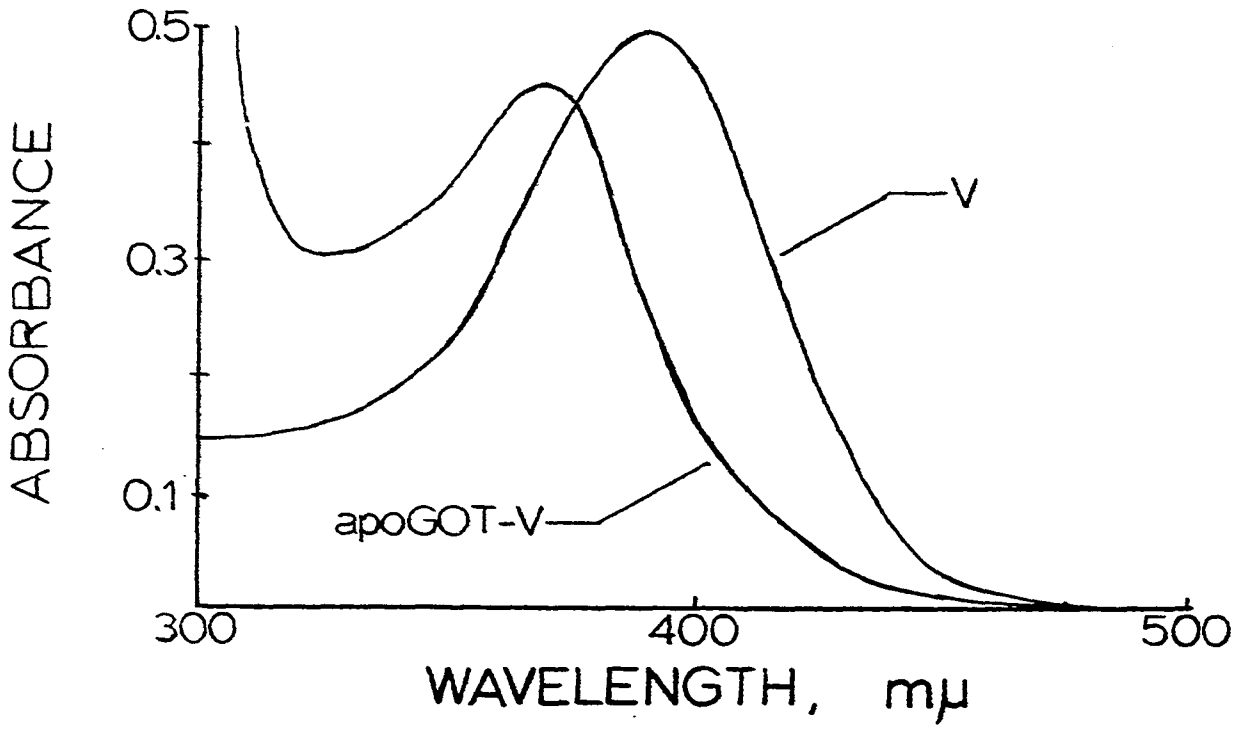
V Methyl Phosphonic Acid of Pyridoxal Phosphate

Binding

ApoGOT (9.3×10^{-5} M) was combined with V (1.0×10^{-4} M) in 0.01 M triethanolamine HCl, pH 8.3. The bound V exhibited an absorption peak at 370 mμ, a change of 20 mμ from the absorption maximum of the unbound V, (Figure 21) and a CD spectrum with a maximum at 365 mμ ($\Delta A/A = 1.5 \times 10^{-3}$) as shown in Figure 22. Lowering the pH of the solution to 5.4 by addition of 1.0 M acetate (final concentration was 0.1 M) did not change the absorption or CD spectra. (6.0×10^{-5} M V and 5.3×10^{-5} M apoGOT) gave a much higher absorbance at 370 mμ then expected (relative to its concentration) as shown in Figure 24. The reason for this discrepancy is not known but it is assumed to be the

Figure 21. Absorption spectra of apoGOT-V and V in 0.01 M triethanolamine HCl, pH 8.3. Concentration of V is 1.0×10^{-4} M. Protein concentration is 9.3×10^{-5} M

Figure 22. CD spectrum of apoGOT-V in 0.01 M triethanolamine HCl, pH 8.3. $\Delta A/A = 1.5 \times 10^{-3}$ at 365 mμ. Concentrations are the same as Figure 21


0.5
0.3
0.1
ABSORBANCE
V
apoGOT-V
300
400
500
WAVELENGTH, mμ

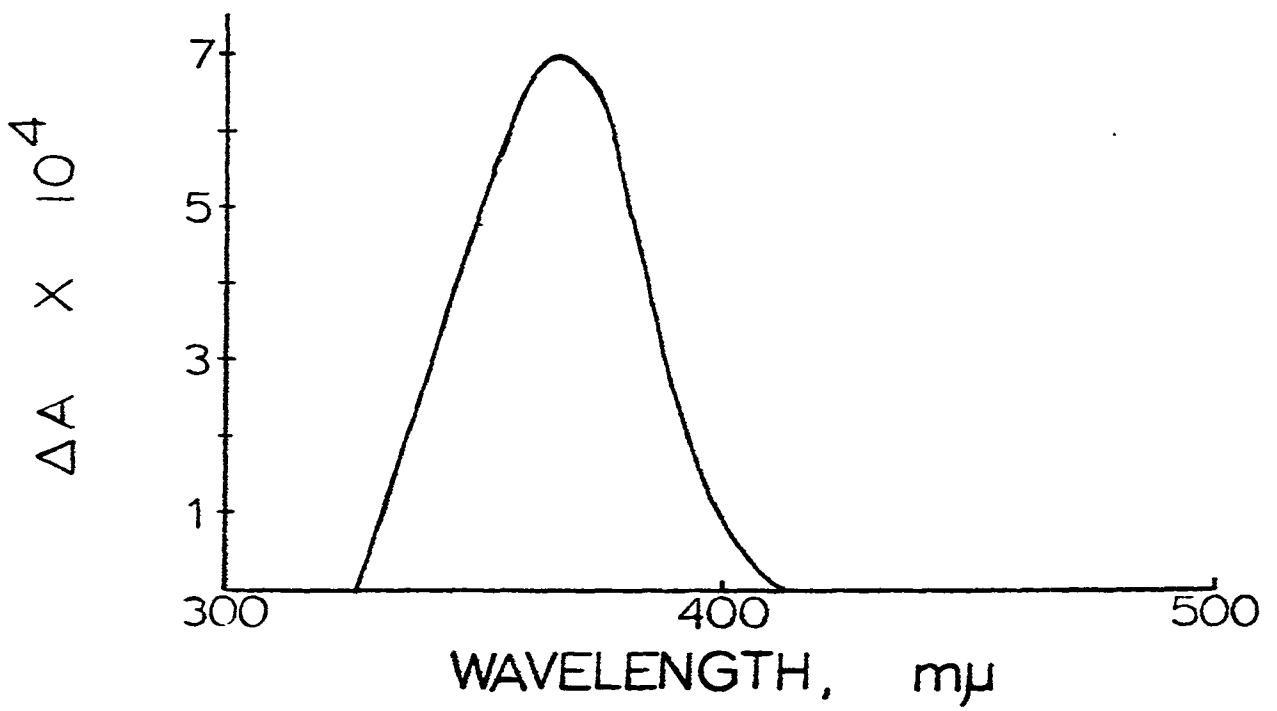
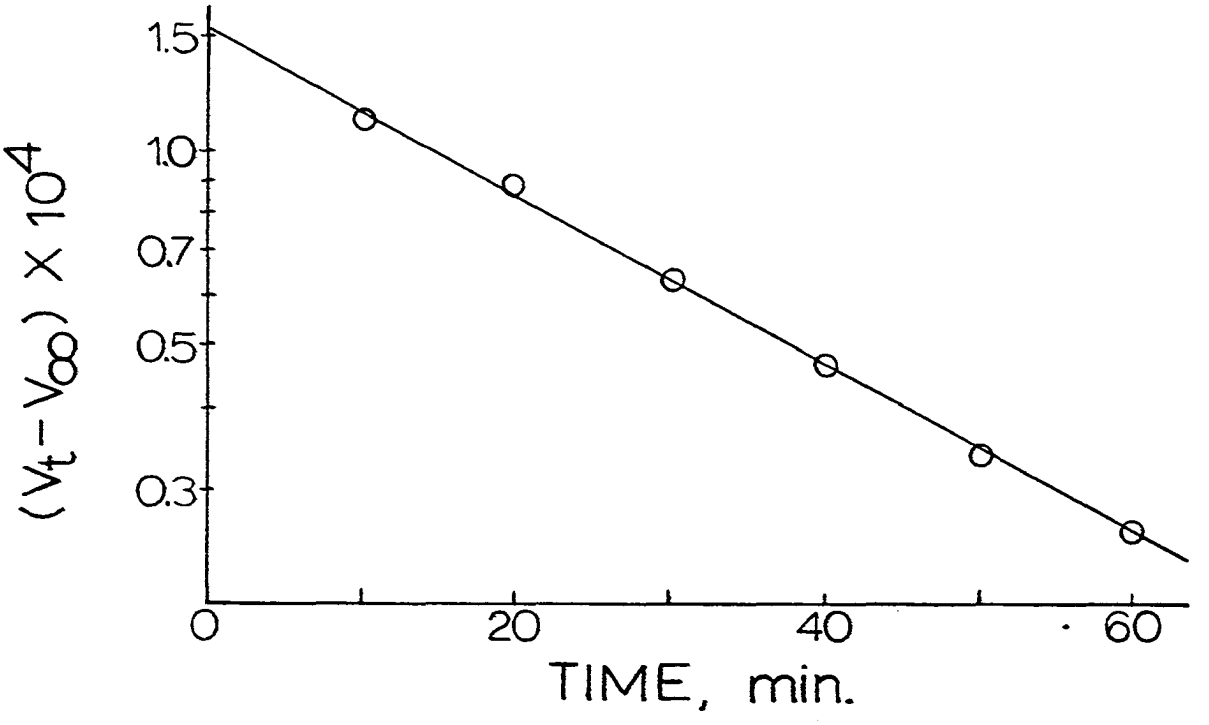
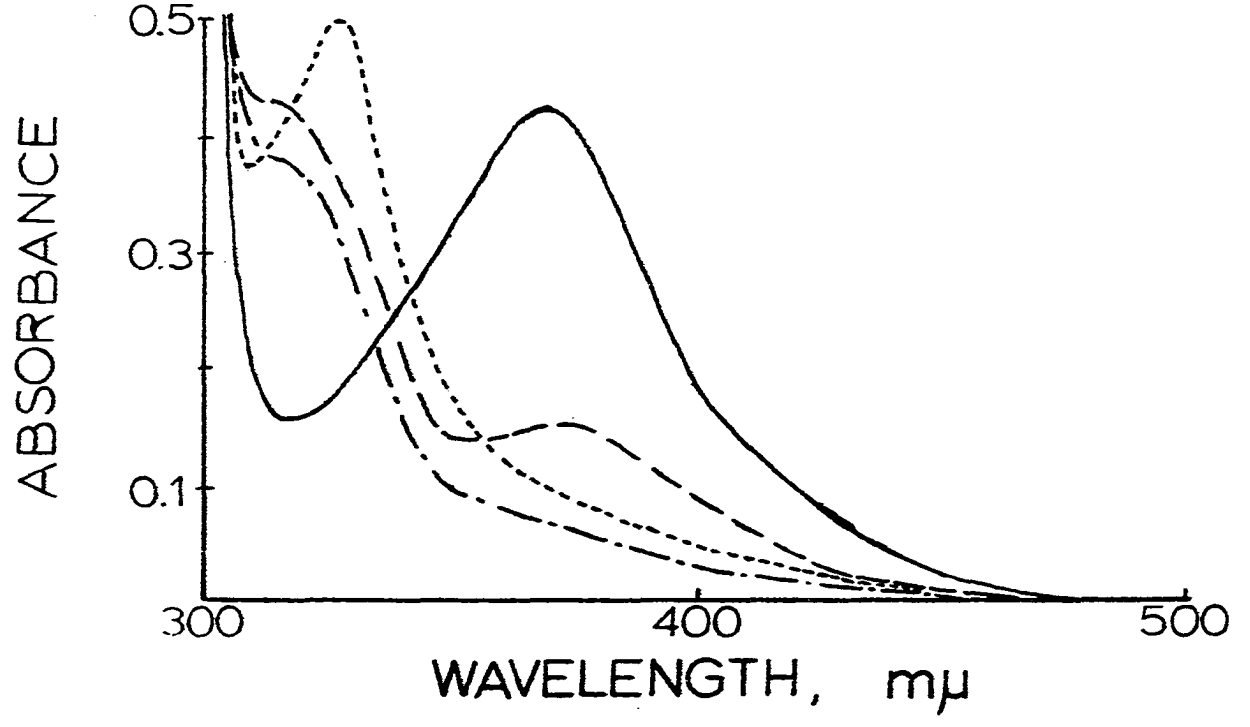

7
5
3
1
ΔA X 10^4
300
400
500
WAVELENGTH, mμ

Figure 23. First order plot of reactivation of apoGOT-V by an excess of PLP. V_t, the initial velocity at time t in absorbance units/sec., minus V_∞, the initial velocity at equilibrium, is plotted on a $\log_{10}$ scale. Concentrations of V, PLP, and protein are 1.0×10^{-6} M, 2.5×10^{-5} M, and 9.4×10^{-7} M respectively

Figure 24. Absorption spectra of apoGOT-V after reaction with substrates.
———apoGOT-V, pH 8.3 (no substrate)
— —apoGOT-V in 1.82×10^{-2} M L-glutamate, pH 8.3, (2 minutes)
—·—·apoGOT-V in 1.82×10^{-2} M L-glutamate, pH 8.3, (20 minutes)
– – – –,apoGOT-V in 1.66×10^{-2} M L-glutamate and 8.3×10^{-3} M α-ketoglutarate, pH 6.5
Concentration of V is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M. All pH 8.3 solutions are in 0.01 M triethanolamine HCl


1.5
1.0
0.7
0.5
0.3
$(V_t - V_\infty) \times 10^4$
0
20
40
60
TIME, min.


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ

result of an experimental error. In this second preparation the 370 mμ absorption was 94% complete within 3 minutes after mixing and the maximum absorbance occurred after 15 minutes; during this time the absorbance in the 400 to 430 mμ region decreased.

Since V could be displaced from apoGOT-V by PLP, the rate constant, k_1, of the dissociation of apoGOT-V was determined in the same manner as were III and IV (see page 31 for method). The first order plot shown in Figure 23 gave a value of 5.04×10^{-4} sec^{-1} for k_1 and a half-life, $t_{1/2}$, of 23 minutes for the dissociation.

Reaction with substrate

With L-aspartate and α-ketoglutarate (20 μmoles each, 200 μmoles Tris, pH 8.3; volume 3 ml.) apoGOT-V showed a small amount of enzymic activity; at 5.5×10^{-5} M the activity was 0.056 mM/min/mg which is 0.14% of the activity (41.0 mM/min/mg) of apoGOT-PLP at a concentration of 5.5×10^{-7} M.

The addition of 0.1 ml of 0.2 M L-glutamate, pH 8.3, to 1.0 ml of 6.0×10^{-5} M apoGOT-V in a cuvette resulted in the conversion of the 370 mμ species to a form absorbing at 315 mμ (Figure 24). After 2 minutes 35% of the 370 mμ absorption remained which subsequently decreased to 14% during an 18 minute period (see Figure 24) and ceased. At this point 0.1 ml of 0.1 M α-k ..oglutarate, pH 4.8, was added; the absorption maximum was rapidly changed to 327 mμ and a small increase at 370 mμ was noted. The absorption at 370 mμ increased at a very slow rate (about 0.01 absorbance units in 30 minutes). The final pH of the solution was 6.5.

V (9.0×10^{-5} M) in 1.82×10^{-2} M L-glutamate exhibited two absorption maxima in 0.01 M triethanolamine HCl, pH 8.3: 405 mμ ($\varepsilon = 3.85$ mM^{-1} cm^{-1}) and 278 mμ ($\varepsilon = 4.0$ mM^{-1} cm^{-1}).

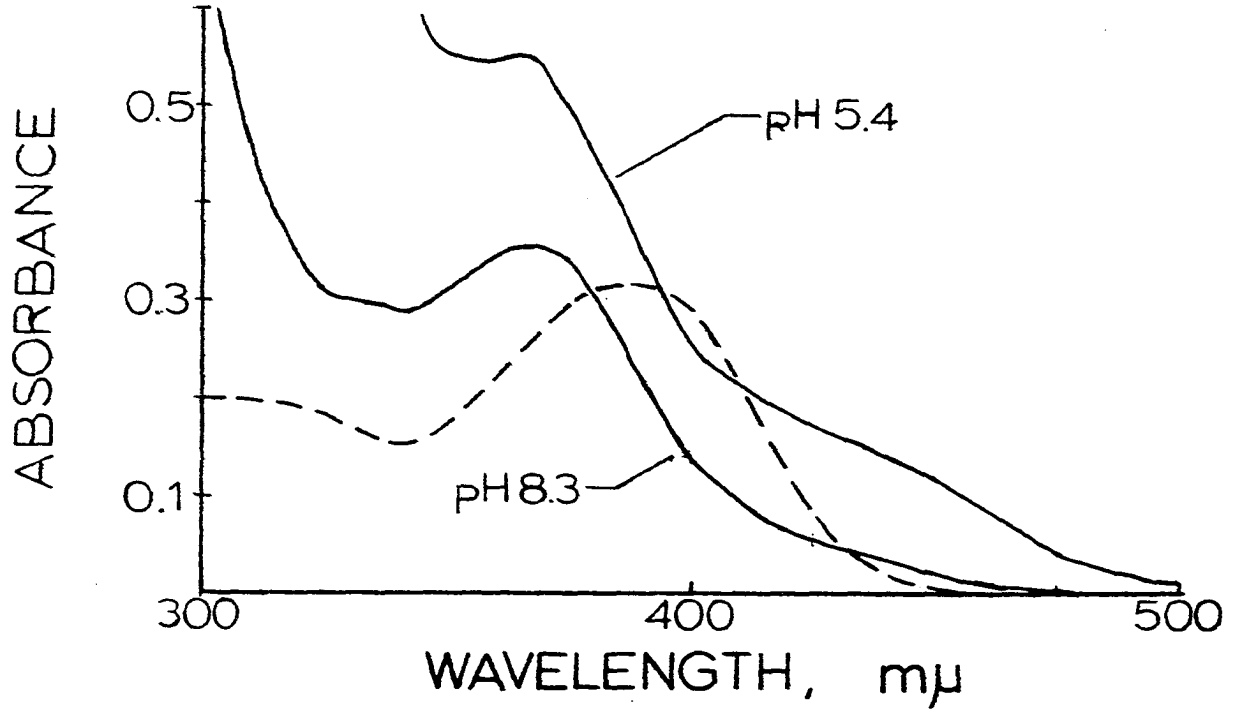
VI Cyanoethyl Ester of Pyridoxal Phosphate

Binding

ApoGOT (9.3×10^{-5} M) and VI (1.0×10^{-4} M) were allowed to react in 0.01 M triethanolamine HCl, pH 8.3; a shift of 20 mμ from the absorbance maximum of the free compound (390 mμ) to 370 mμ was observed (Figure 25). 1.0 M acetate, pH 5.4, when added to a final concentration of 0.1 M, caused an increase in absorption in the 430 mμ region and also resulted in the complex becoming unstable. The corresponding CD spectra (Figure 26) showed a single peak at 369 mμ ($\Delta A/A = 1.3 \times 10^{-3}$) at pH 8.3; at pH 5.4, a low broad peak in the 430 mμ region appeared while the major CD peak shifted slightly to 363 mμ. A second preparation of apoGOT-VI (6.0×10^{-5} M VI and 5.3×10^{-5} M apoGOT) exhibited a higher absorbance at 370 mμ than expected for its concentration (see Figure 28); the same observation that occurred with a preparation of apoGOT-V. In this second preparation the absorption at 370 mμ was about 67% complete 2 minutes after mixing, with the remainder being added over a 20 minute period. As the absorption at 370 mμ increased, the absorbance in the 390 mμ to 430 mμ region decreased.

Figure 25. Absorption spectra of apoGOT-VI in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3 (solid lines); and of VI in 0.01 M triethanolamine HCl, pH 8.3, (broken line). Concentrations of VI and protein are 1.0×10^{-4} M and 9.3×10^{-5} M respectively

Figure 26. CD spectra of apoGOT-VI in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3. At pH 8.3, $\Delta A/A = 1.3 \times 10^{-3}$ at 369 mμ. Concentrations are the same as Figure 25


ABSORBANCE
0.5
0.3
0.1
pH 5.4
pH 8.3
300
400
500
WAVELENGTH, mμ

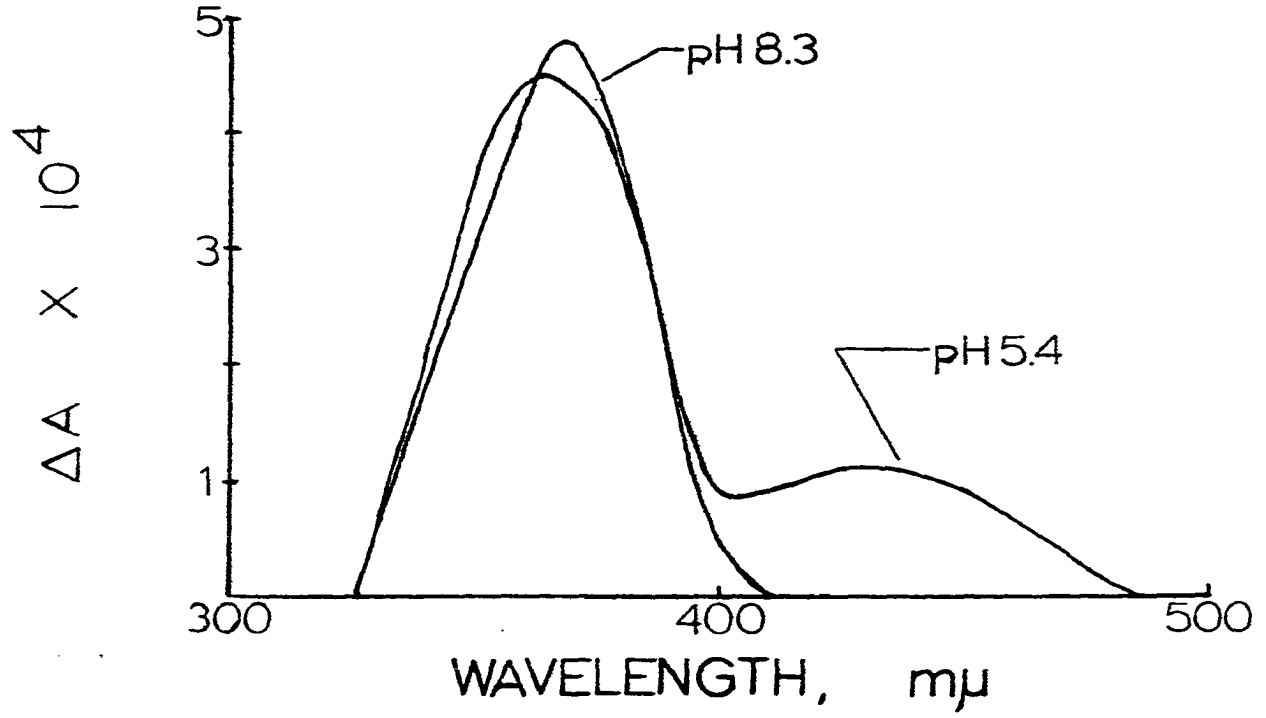
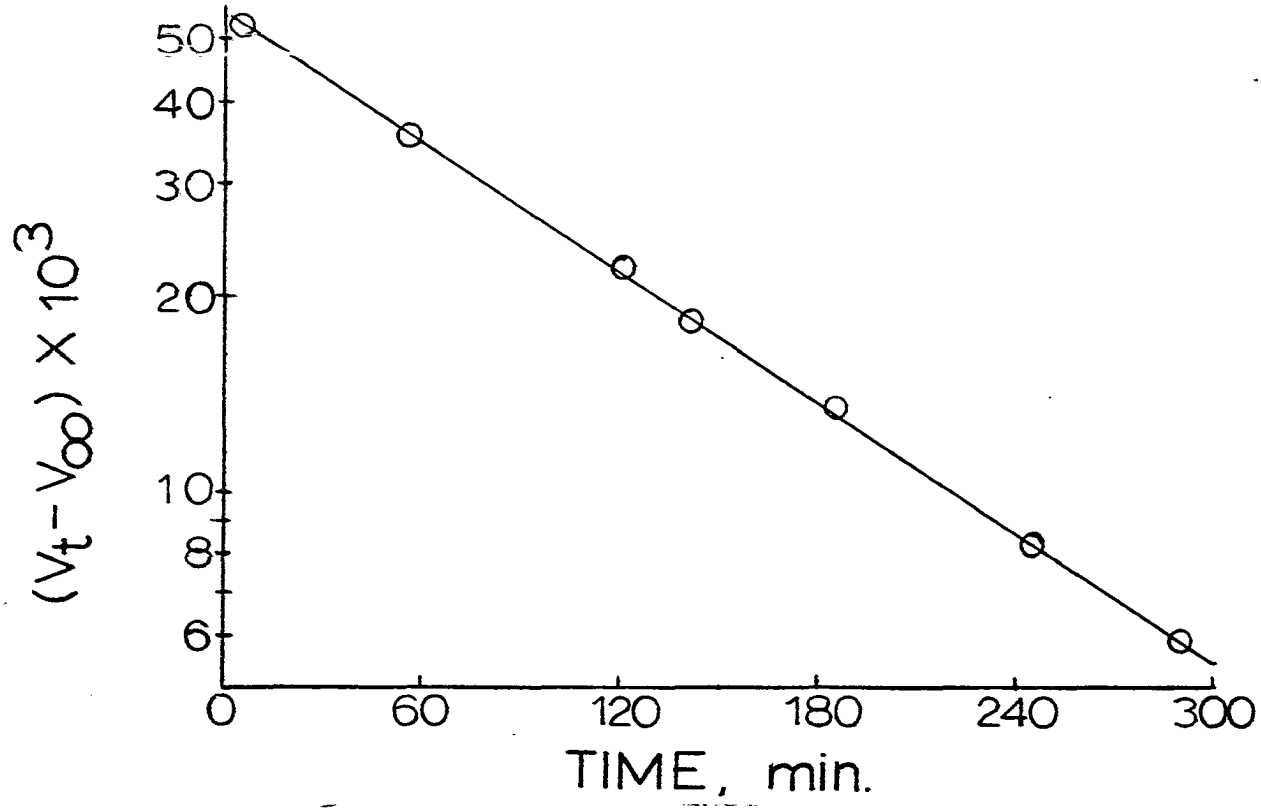
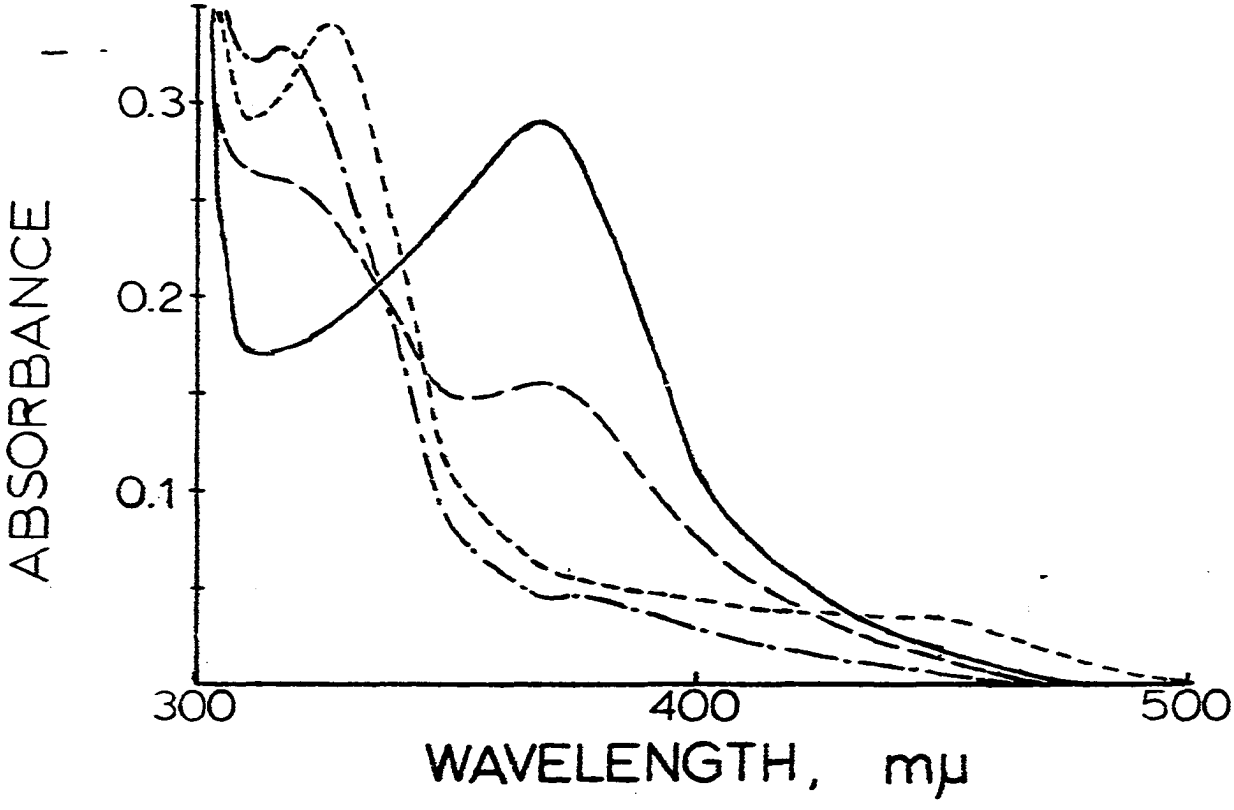

ΔA X 10⁴
5
3
1
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ

Figure 27. First order plot of reactivation of apoGOT-VI (no PLP added). V_t, the initial velocity at time t in absorbance units/min., minus V_∞, the initial velocity of apoGOT-PLP at the same concentrations, is plotted on a $\log_{10}$ scale. Concentrations of VI and protein are 1.0×10^{-6} M and 9.4×10^{-7} M respectively

Figure 28. Absorption spectra of apoGOT-VI after reaction with substrates
——— apoGOT-VI, pH 8.3 (no substrate)
— — apoGOT-VI in 1.82×10^{-2} M L-glutamate, pH 8.3, (2 minutes)
—.—.apoGOT-VI in 1.82×10^{-2} M L-glutamate, pH 8.3, (30 minutes)
– – – – apoGOT-VI in 1.66×10^{-2} M L-glutamate and 8.3×10^{-3} M α-ketoglutarate, pH 6.4, (60 minutes after addition of α-ketoglutarate)
Concentration of VI is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M. All pH 8.3 solutions are in 0.01 M triethanolamine HCl


50
40
30
20
10
8
6
$(V_t - V_\infty) \times 10^3$
0
60
120
180
240
300
TIME, min.


0.3
0.2
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ

Reaction with substrate

Assays of apoGOT-VI gave unexpected results; the activity of the complex increased with time as shown in Table 4.

Table 4. Reactivation of apoGOT-VI

Time (minutes)	Activity[a] (mM/min)
5	0.0040
55	0.0180
120	0.0314
140	0.0354
185	0.0401
245	0.0456
290	0.0480
320	0.0490

[a]20 μmoles L-glutamate, 2 μmoles oxaloacetate, 200 μmoles Tris, pH 8.3; volume 3 ml. 0.0035 mg of apoGOT-VI used for each assay.

Using the activity of PLP-reactivated apoGOT of the same concentration as a value of V_∞, the data in Table 4 gave a straight line on a first order plot (Figure 27). From this plot the value of the rate constant was calculated as 1.3×10^{-4} sec^{-1} which is equivalent to a half-life of 88 minutes for the reactivation.

VI does not display this self-reactivating behavior when bound to either L-glutamic acid decarboxylase[1] or to glycogen phosphorylase b[2].

A spectral examination of apoGOT-VI's behavior toward substrate was performed. 0.1 ml of 0.2 M L-glutamate, pH 8.3, was added to 1.0 ml of 6.0×10^{-5} M apoGOT-VI in 0.01 M triethanolamine HCl, pH 8.3, about 20 minutes after VI was mixed with apoGOT. The addition of L-glutamate caused a slow decrease in the 370 mμ absorption (Figure 28) and the appearance of a shoulder at about 317 mμ. About 30 minutes were required for the absorbance changes to cease; at this point 0.1 ml of 0.1 M α-ketoglutarate, pH 4.8, was added. The α-ketoglutarate caused an immediate formation of a peak at 326 mμ (see Figure 28) and the absorption along a plateau from 370 mμ to 450 mμ was very slowly increased. The final pH of the solution was 6.4.

VI (9.0×10^{-5} M) in 1.82×10^{-2} M L-glutamate has (in 0.01 M triethanolamine HCl, pH 8.3) two absorption maxima: 399 mμ ($\varepsilon = 2.5\ mM^{-1}\ cm^{-1}$) and 284 mμ ($\varepsilon = 3.2\ mM^{-1}\ cm^{-1}$).

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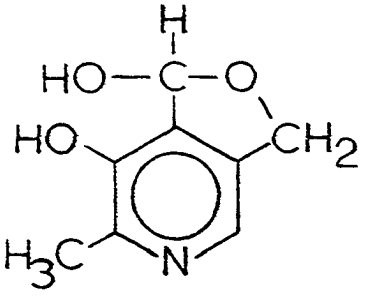
[1]Dr. Margaret L. Fonda, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Private communication. 1968.

[2]George F. Johnson, Ames, Iowa, Iowa State University of Science and Technology, Department of Biochemistry and Biophysics. Private communication. 1968.

VII and VIII Pyridoxal and Deoxypyridoxal

Pyridoxal (VII) predominately exists in acidic, neutral, and alkaline solutions as a hemiacetal (VIIa); although the proportion of



VIIa

free aldehyde increases at high pH (Martell, 1963). The absorption band at 390 mμ which is assigned to the dipolar carbonyl form is very weak, while the band assigned to the dipolar hemiacetal form (at 317 mμ) is quite intense. VII at a concentration of 1.0×10^{-4} M was combined with apoGOT (9.3×10^{-5} M); absorbance in the region above 350 mμ increased only slightly, and possessed no enzymic activity. Therefore, VII was not utilized for further study.

Deoxypyridoxal (VIII) at a concentration of 5.0×10^{-4} M was allowed to combine with apoGOT (1.1×10^{-4} M) which had been prepared from cytoplasmic GOT (subforms not separated). The CD of this solution showed two peaks of very low intensity: one in the 350 to 375 mμ region (maximum CD $= -1.9 (\pm 0.5) \times 10^{-4}$). The solution was dialyzed

against buffer for one hour; the CD band above 400 mμ was reduced approximately in half (maximum CD = 0.7 ($\pm$ 0.5) x 10^{-4}) while the CD below 400 mμ remained the same. Dialysis was not complete as the absorption spectrum exhibited free VIII. Of interest is the fact that the CD above 400 mμ was reduced during a short period of dialysis while CD below 400 mμ is not and that the intensity of apoGOT-VIII's CD is very low.

VIII (6.25×10^{-5} M) was combined with apoGOT (9.0×10^{-5} M; prepared from α-GOT); in 20 minutes the absorption maximum shifted from 390 mμ (the maximum for the free compound) to 380 mμ (Figure 29)[1]. Also the absorption around 430 mμ was quite high relative to that of the unbound compound. Addition of 0.1 ml of 0.2 M L-glutamate (total volume was then 0.9 ml) resulted in the disappearance of the 380 mμ peak and the formation of a peak at 320 mμ. Absorption about 425 mμ reduced more slowly than that at 380 mμ. The absorbance changes ceased in 4 minutes. 0.1 ml of 0.1 M α-ketoglutarate was then added; the absorbance in the 375 mμ region increased very slowly (about 0.02 in 10 minutes).

All experiments with VII and VIII were done in 0.01 M triethanolamine HCl, pH 8.3.

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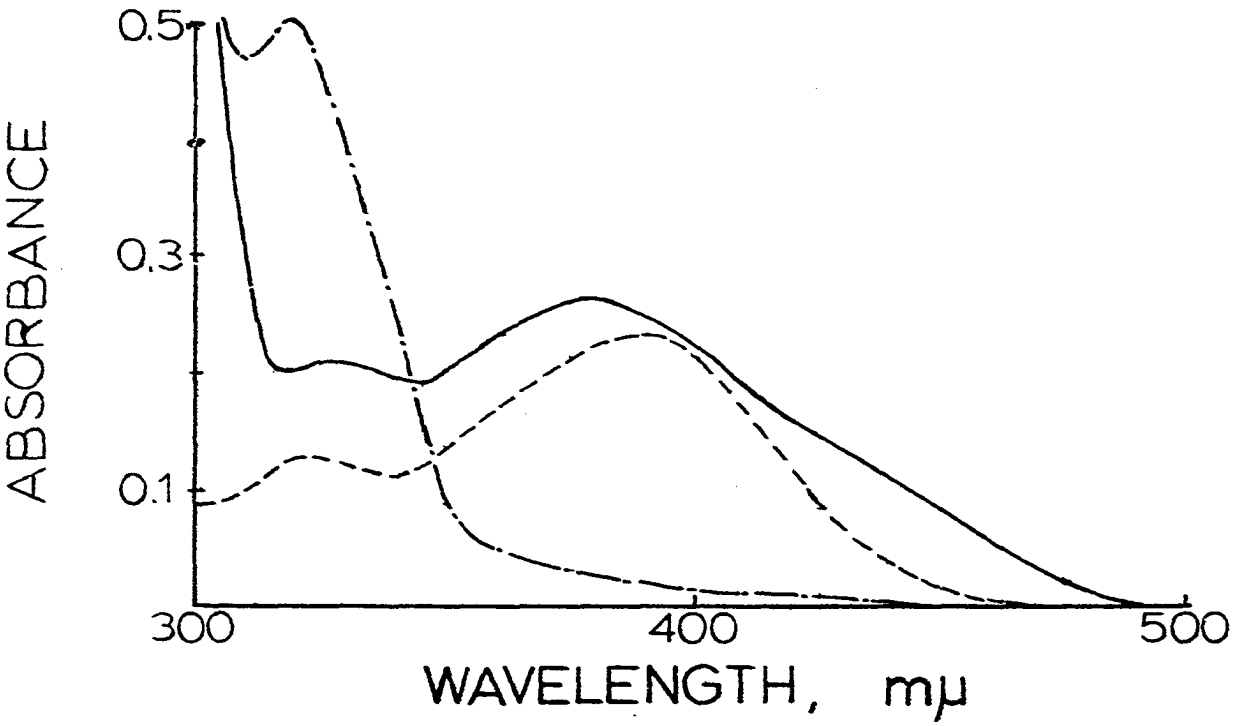
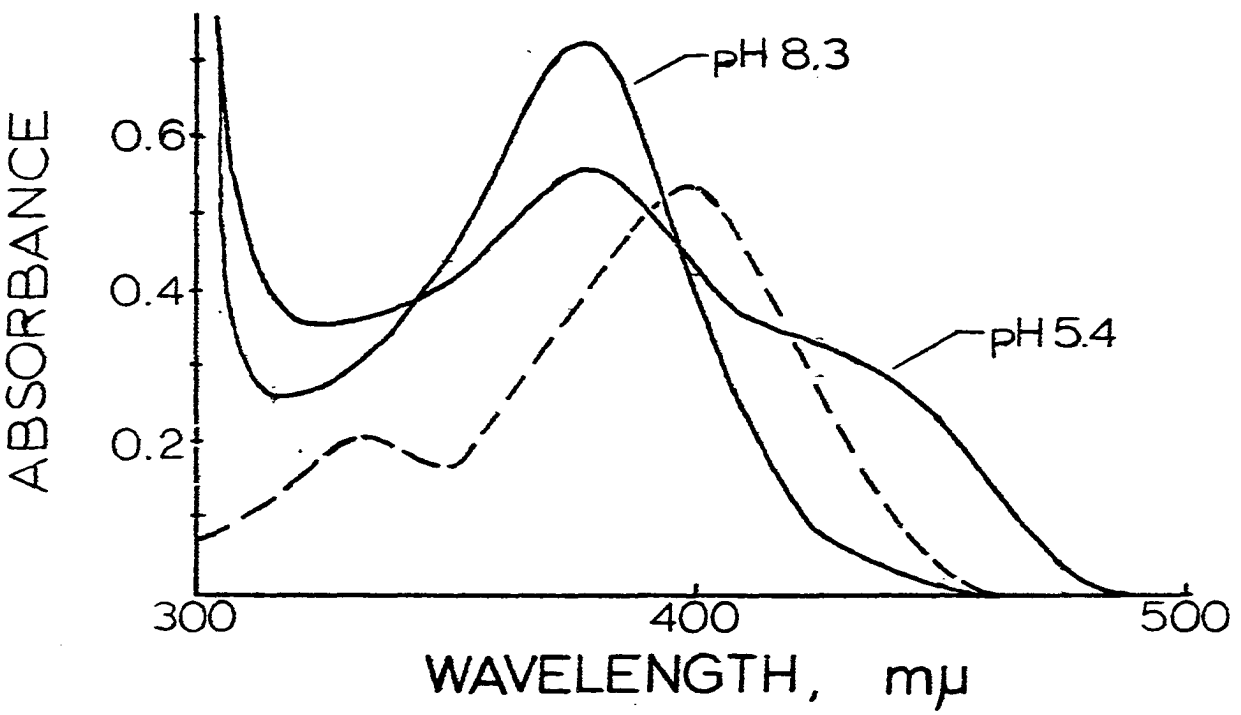
[1] This experiment was performed by Dr. Margaret L. Fonda of the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa.

Figure 29. Absorption spectra of VIII and of apoGOT-VIII before and after reaction with L-glutamate.
———apoGOT-VIII (no substrate)
—·—·—apoGOT-VIII in 2.22×10^{-2} M L-glutamate (10 minutes)
– – – – – –VIII
Concentration of VIII is 6.25×10^{-5} M. Protein concentration is 9.0×10^{-5} M. All solutions are in 0.01 M triethanolamine HCl, pH 8.3

Figure 30. Absorption spectra of apoGOT-IX in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3, (solid lines); and of IX in 0.01 M triethanolamine HCl, pH 8.3, (broken lines). Concentration of IX is 1.0×10^{-4} M. Protein concentration is 9.3×10^{-5} M


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ


pH 8.3
pH 5.4
0.6
0.4
0.2
ABSORBANCE
300
400
500
WAVELENGTH, mμ

IX N-Methyl Pryidroxal Phosphate

Binding

IX (1.0×10^{-4} M) was combined with 9.3×10^{-5} M apoGOT in 0.01 M triethanolamine HCl, pH 8.3; the absorption maximum was shifted about 20 mμ (from 398 mμ to 377 mμ) after binding (Figure 30). An additional maximum was formed at 425 mμ upon addition of 1.0 M acetate, pH 5.4, to a final concentration of 0.1 M in acetate. The CD spectra also exhibited pH-dependent behavior (at 375 mμ, $\Delta A/A = 2.0 \times 10^{-3}$ at both pH values) as shown in Figure 31. The absorption characteristics of apoGOT-IX at pH 8.3 was confirmed in a second preparation using concentrations of 6.0×10^{-5} M IX and 5.3×10^{-5} M apo-GOT (see Figure 32). This latter preparation was utilized for activity studies.

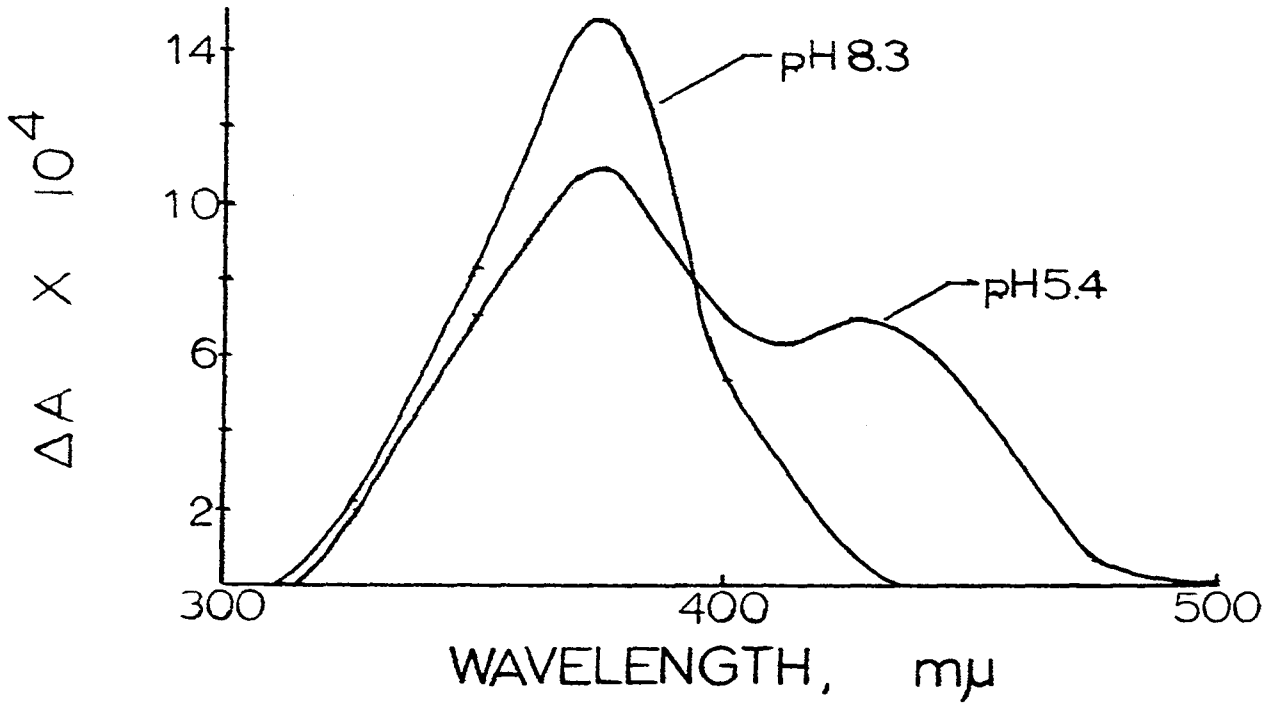
Reaction with substrate

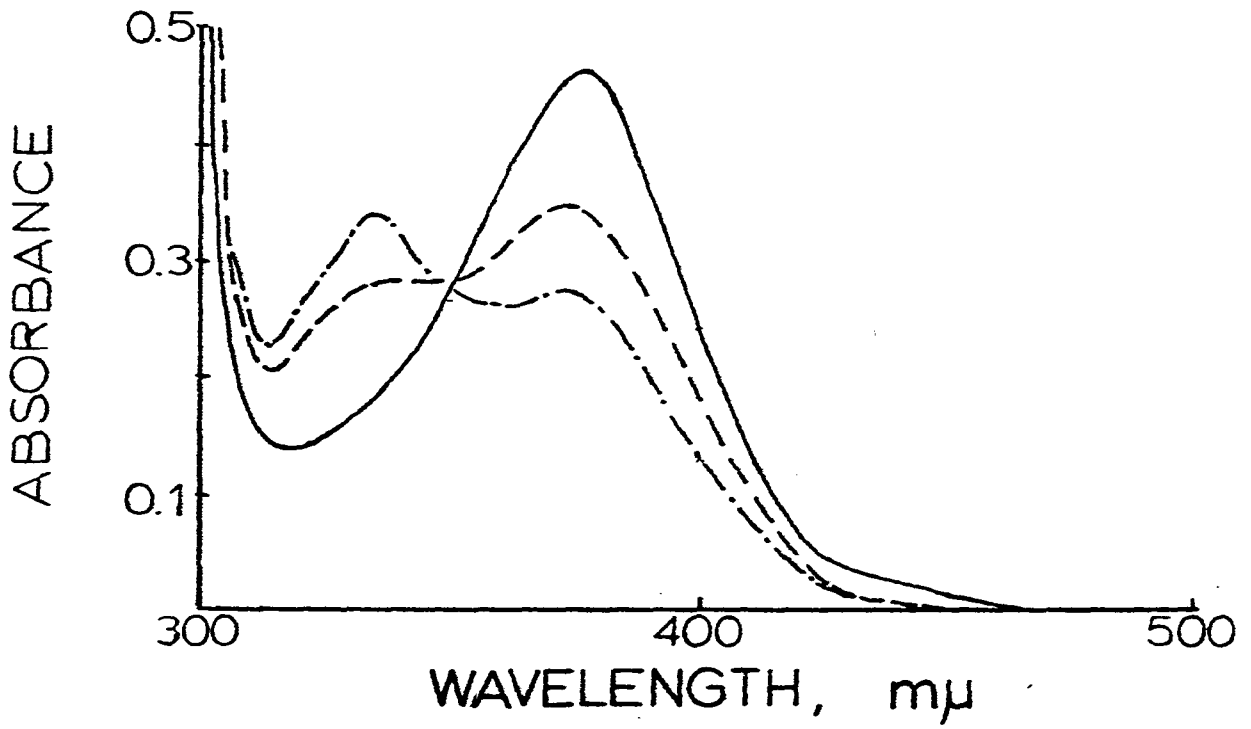
The addition of 0.1 ml of 0.2 M L-glutamate, pH 8.3, to 6.0×10^{-5} M apoGOT-IX caused the slow reduction of the 377 mμ peak and the formation of a maximum at 335 mμ (Figure 32). After 25 minutes the 377 mμ peak was reduced by only 40%. The solution was incubated overnight at room temperature and 0.1 ml of 0.1 M α-ketoglutarate, pH 4.8, was added. The α-ketoglutarate, did not cause any change in the absorption during a 15 minute period. The final pH of the solution was 6.4.

At 5.5×10^{-5} M, apoGOT-IX showed a very small amount of enzymic activity (0.05 mM/min/mg) with L-aspartate and α-ketoglutarate (20 μmoles each, 200 μmoles Tris, pH 8.3; volume 3 ml). This activity is only 0.12% of the activity of apoGOT-PLP at 5.5×10^{-7} M. The low activity was

Figure 31. CD spectra of apoGOT-IX in 0.1 M acetate, pH 5.4, and in 0.01 M triethanolamine HCl, pH 8.3. At 377 mμ, $\Delta A/A = 2.0 \times 10^{-3}$ at both pH 5.4 and 8.3. Concentrations are the same as Figure 32

Figure 32. Absorption spectra of apoGOT-IX after reaction with L-glutamate.
——— apoGOT-IX (no substrate)
— — apoGOT-IX in 1.82×10^{-2} M L-glutamate (2 minutes)
—·—·apoGOT-IX in 1.82×10^{-2} M L-glutamate (25 minutes)
Concentration of IX is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M. All solutions are in 0.01 M triethanolamine HCl, pH 8.3


ΔA X 10^4
14
10
6
2
pH 8.3
pH 5.4
300
400
500
WAVELENGTH, mμ


ABSORBANCE
0.5
0.3
0.1
300
400
500
WAVELENGTH, mμ

confirmed by another preparation of apoGOT-IX.

Addition of L-glutamate to IX did not cause any change in the absorption behavior of IX.

X Pyridoxal Phosphate N-oxide

Binding and reaction with substrate

X at a concentration of 6.0×10^{-5} M was combined with apoGOT (5.3×10^{-5} M) in 0.01 M triethanolamine, HCl pH 8.3. The binding caused a reduction in the absorption maximum (392 mμ) of the free X and the formation of a broad band from 375 mμ to 410 mμ (Figure 33). The absorption at 430 mμ was increased and a shoulder at 320 mμ was present.

The addition of 0.1 ml of 0.2 M L-glutamate, pH 8.3, (total volume was then 1.1 ml) caused the rapid destruction of the absorbance above 350 mμ except for a slower reduction at 425 mμ (Figure 34). Also, a definite peak at 325 mμ was formed. The absorption changes stopped in about 14 minutes. At this point, 0.1 ml of α-ketoglutarate, pH 4.8, was added; a peak was quite rapidly formed at 425 mμ which slowly increased during the next hour (see Figure 34). In addition, α-ketoglutarate caused a slight reduction in absorbance at 325 mμ and a shift to 320 mμ. The final pH of the solution was 6.6.

At 5.5×10^{-7} M, apoGOT-X had an activity of 12.6 mM/min/mg in L-aspartate and α-ketoglutarate (20 μmoles each, 200 μmoles Tris, pH 8.3; volume 3 ml) which is 31% of the activity possessed by apoGOT-PLP at this concentration.

Figure 33. Absorption spectra of apoGOT-X (solid line) and of X (broken line) in 0.01 M triethanolamine HCl, pH 8.3. Concentration of X is 6.0×10^{-5} M. Protein concentration is 5.3×10^{-5} M

Figure 34. Absorption spectra of apoGOT-X after reaction with L-glutamate.

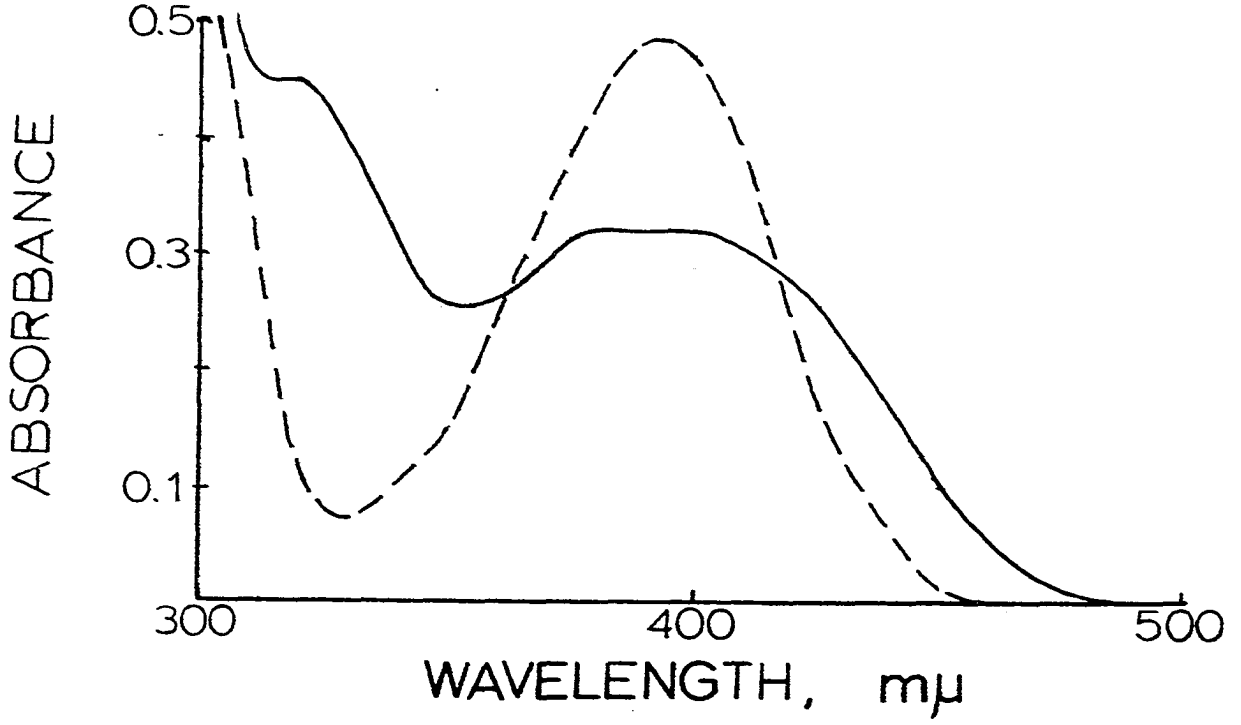
———— apoGOT-X in 1.82×10^{-2} M L-glutamate, pH 8.3 (2 minutes)

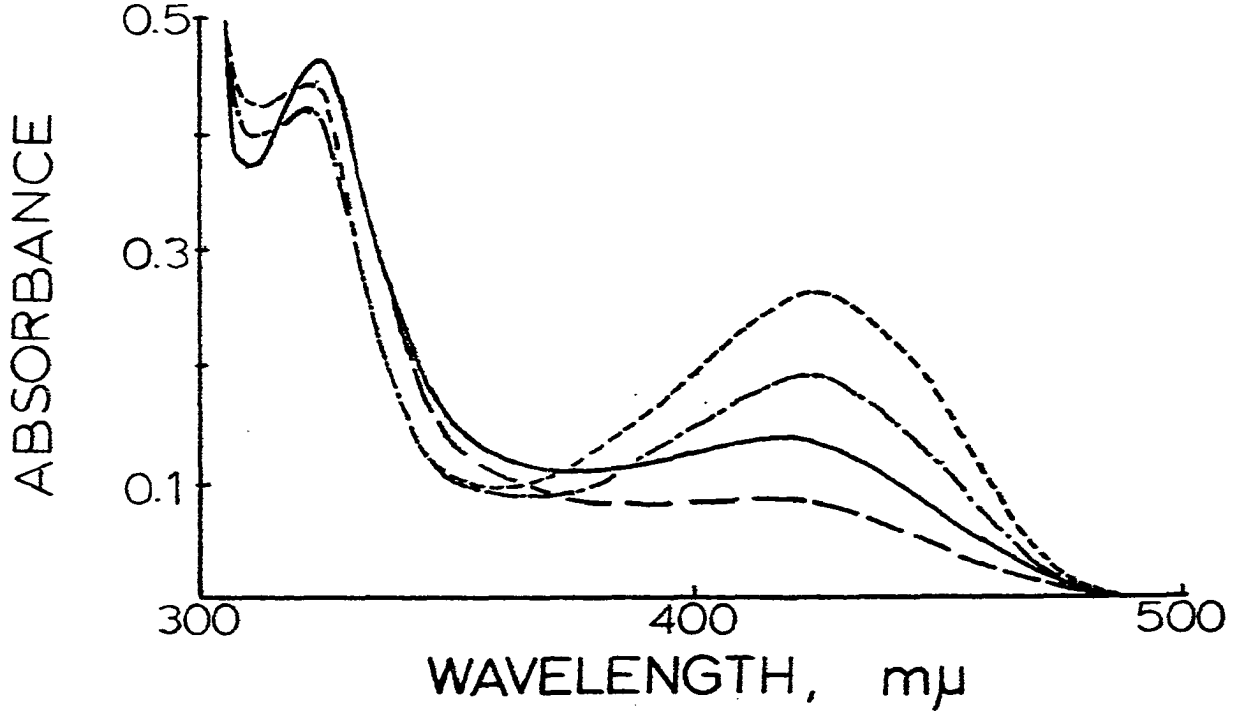
— — — apoGOT-X in 1.82×10^{-2} M L-glutamate, pH 8.3 (14 minutes)

—·—·— apoGOT-X in 1.66×10^{-2} M L-glutamate and 8.3×10^{-3} M α-ketoglutarate, pH 6.5 (2 minutes)

------ apoGOT-X in 1.66×10^{-2} M L-glutamate and 8.3×10^{-3} M α-ketoglutarate, pH 6.5 (75 minutes)

Concentrations of X and protein are the same as Figure 33. All pH 8.3 solutions are in 0.01 M triethanolamine HCl


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ


0.5
0.3
0.1
ABSORBANCE
300
400
500
WAVELENGTH, mμ

These results are in agreement with those of Ohishi, et al. (1968) who also reported that apoGOT-X exhibited CD at 420 mμ ($\Delta A/A = 1.3 \times 10^{-3}$ at pH 5.0) and that the absorption at 420 mμ is pH dependent.

X in the presence of L-glutamate shows a slight shift to 396 mμ.

Summary of Results

Spectral data of the various cofactors are summarized in Table 5 and their activities with apoGOT in Table 6.

Table 5. Summary of spectral data of compounds I - X[a]

Compound	Wavelength of maximum absorbance/CD, mμ			
	Free compound	Bound to apoGOT		
	pH 8.3	pH 8.3	pH 5.4	With L-glutamate	With L-glutamate and α-ketoglutarate
I	390	364/362 (2.0×10^{-3})	430/430[b] (3.2×10^{-3})	332/330 (1.5×10^{-3})	
III	390	368/362 (1.6×10^{-3})	same as pH 8.3	320	323
IV	369 325	359/355[c] 420/415 (1.0×10^{-3})	430/440 (2.0×10^{-3})	325[d]	325
V	390	370/365 (1.5×10^{-3})	same as pH 8.3	315	327

[a] CD maxima are shown following the absorbance maxima and are separated by a diagonal line (/). The values of ΔA/A are given in parenthesis. Compound II is not included because of its similarity with I. Compound VII is not included because of its lack of binding as a hemiacetal.

[b] Data obtained at pH 4.8.

[c] The single preparation of apoGOT-IV showing a 330 mμ peak is not included.

[d] In addition, an intermediate absorbing at 430 mμ was observed.

Table 5 (Continued)

Compound	Free compound pH 8.3	pH 8.3	pH 5.4	With L-glutamate	With L-glutamate and α-ketoglutarate
VI		370/369 (1.3×10^{-3})	same as[e] pH 8.3	317	326
VIII	391	380/∿365 ($\sim 2 \times 10^{-4}$)	-	320	320
IX	398	377/375 (2.0×10^{-3})	425/430 (2.4×10^{-3})	335	335
X	392	380-405/ 320/	∿420/420[f] (1.3×10^{-3})	325	320 425

[e]With the exception of the species absorbing at 430 mμ which is thought to be PLP (see Discussion).

[f]Source: Ohishi, et al., 1968. Data obtained at pH 5.

Table 6. Reactions of apoGOT-bound cofactors with substrates

Compound	Reaction with L-glutamate[a]	Reaction with α-ketoglutarate[a]	Assay activity[b] (mM/min/mg)
I	364 mμ to 332 mμ (<<2 minutes)	332 mμ to 364 mμ (<<2 minutes)	41.0
III	370 mμ to 320 mμ (∿2 minutes) / 412 mμ to 320 mμ (∿20 minutes)	320 mμ to 323 mμ (<2 minutes) / 323 mμ to 375 mμ (slow)	0.095
IV	359 mμ to 325 mμ (<2 minutes) / 359 mμ to 430 mμ (∿2 minutes) / 430 mμ to 325 mμ (slow)	325 mμ to 430 mμ (very slow)	0.10
V	370 mμ to 315 mμ (>2 minutes)	315 mμ to 327 mμ (<2 minutes) / 327 mμ to 370 mμ (very slow)	0.056
VI	370 mμ to 317 mμ (>20 minutes)	317 mμ to 326 mμ (>2 minutes) / 326 mμ to 370 mμ (very slow)	varying[c]
VIII	380 mμ to 320 mμ (∿2 minutes) / 425 mμ to 320 mμ (∿4 minutes)	320 mμ to 375 mμ (very slow)	-
IX	377 mμ to 335 mμ (>20 minutes)	335 mμ to 377 mμ (very slow)	0.05
X	380-405 mμ to 325 mμ (<2 minutes) / 425 mμ to 325 mμ (<20 minutes)	325 mμ to 320 mμ (< 2 minutes) / 320 mμ to 425 mμ (∿2 minutes)	12.6

[a] For reaction conditions, see text of Results.

[b] 20 μmoles each of L-aspartate and α-ketoglutarate, 200 μmoles of Tris, pH 8.3; volume 3 ml.

[c] Activity increased with age of preparation.

DISCUSSION

Analogs with varied substituents in the 5'-position will be considered with regard to their binding to apoGOT and their subsequent reactions with substrate before the pyridine ring nitrogen substituents are discussed.

5'-Analogs (III-VIII)

Binding characteristics

With the exception of VII (pyridoxal), all of the 5'-analogs are bound by apoGOT. In the case of VII, the lack of activity and the absence of absorption in the 360 to 380 mμ region may be ascribed to the non-availability of a free 4'-aldehyde group resulting from hemiacetal formation. While it cannot be claimed that the hemiacetal does not bind to apoGOT at all, the uselessness of VII as a coenzyme is apparent. At substrate-levels of concentration, however, VII can be transaminated into pyridoxamine by apoGOT (Wada and Snell, 1962) at less than 0.1% of the rate of normal transamination. The ease of dissociation of pyridoxamine (which, of course, cannot be in a hemiacetal configuration) from the enzyme reported by these authors demonstrates the small contribution of the 5'-hydroxy group to the compound's binding ability. Interestingly, this transamination was inhibited by inorganic phosphate, which presumably acted by occupying the site normally occupied by the 5'-phosphate of PLP and preventing pyridoxal or pyridoxamine from binding.

The evidence for the binding of the remaining 5'-analogs (III-VI,

VIII) to apoGOT is conclusive: all demonstrated a characteristic hypsochromic shift of 10 to 25 mμ from the electronic absorption maxima of the free compounds, all were rigidly located in an asymmetric environment as shown by their circular dichroism, and all showed some enzymic activity (although poorly). The optical properties of the bound cofactors are interesting. Like PLP, these analogs exhibited a decrease in the wavelengths of their absorption maxima upon binding as imines at the active site of GOT (10-11 mμ for IV and VIII; 20-26 mμ for PLP, III, V, and VI). In addition, at pH 8.3 the absorption spectra of all cofactors showed the presence of a species absorbing in the 410 to 430 mμ region. Except for analog VIII (deoxypyridoxal), the absorption above 400 mμ decreased as the maxima in the 370 mμ region increased. This was particularly evident for those analogs which bound more slowly to the protein (V and VI). It is proposed that the absorption above 400 mμ results from a non-specific binding of the cofactors to the protein; that is, the analogs may bind to sites other than the active site before binding to the active site. Martinez-Carrion and coworkers (1967) reported twenty lysyl residues present in the protein per mole of PLP; consequently, there should be several sites available for Schiff base formation between the protein and the coenzyme. Rearrangement of the co-factor from the easily-attacked non-specific sites to the active site would give the observed absorption changes. An alternative explanation may be advanced: the binding of the cofactors may occur at the active site, but in such a manner as to give rise to absorption maxima above 400 mμ. The bound cofactors may then rearrange to a conformation giving

absorption maxima about 370 mμ without leaving this site. Either case may be considered as a non-specific binding since PLP bound in an enzymically active configuration at the active site has an absorption maximum at 364 mμ. Further evidence for the existence of non-specific binding (without regard to which site is occupied) is given by the higher > 400 mμ absorption of apoGOT-PLP relative to that of the native enzyme, and it is known that an excess of PLP inhibits GOT's activity. Thus, PLP can bind to the protein in modes other than the active one; it is reasonable to think that the other cofactors can also.

The situation with VIII was the reverse of the above binding behavior: the absorption at 430 mμ increased while the maximum at 380 mμ decreased. This behavior of apoGOT-VIII would seem to indicate that VIII has a slightly higher affinity for a non-specific type of binding than for the active mode; however, the CD data do not confirm this conclusion (discussed below).

A further consequence of binding by these analogs (III-VI, VIII) was the appearance of a Cotton effect at their major absorbing frequencies. While small in magnitude, the circular dichroism exhibited by the bound analogs existed and was easily measurable. Since the unbound analogs are optically inactive, and become optically active upon binding, the rotational strength is a measure of these chromophores' interaction with their asymmetric environments. In other words, an averaging out of the configurations of the analog through free rotation is prevented by steric restraint. In addition, the rotational strength reflects the asymmetry induced in the electron distribution within the

chomophore. Thus, the rotational strength of a bound cofactor is dependent upon the steric hinderance imposed by the protein and upon the charge perturbation of the chromophore.

In this study, CD results are reported as the ration of observed CD (ΔA) to the normal absorption (A). Since the observed CD and the absorption were measured on the same sample with equal path length (1 cm.), then $\Delta A/A$ is equal to $\frac{\varepsilon_L - \varepsilon_R}{\varepsilon}$, where ε_L and ε_R are the molar extinction coefficients for left- and right-handed circularly polarized light respectively and ε is the usual molar extinction coefficient. This value is equivalent to the dissymmetry factor (or anistropy factor) of Kuhn (1958). Although "the anisotropy factor of a given absorption band is a unique function of the spatial and angular distribution of the corresponding vibrating momentum" (Kuhn, 1958, p. 425), it is not utilized here in any theoretical sense. CD results are reported as $\Delta A/A$ because this value provides a convenient means of expressing CD that is independent of concentration and, hence, gives a method for comparing the amount of CD shown by the different cofactors. CD measurements were made on the apoGOT-cofactors to demonstrate unequivocally that the analogs were bound. At best, values for the observed CD can be used only as an indication of asymmetry produced by binding.

With reference to the values reported for $\Delta A/A$, it should be noted that ΔA and A values were taken at the peak position of the CD spectra in those cases where the absorption and CD maxima did not occur at the same wavelength. While most CD and absorption spectra were quite closely

aligned, some were not. Furthermore, $\Delta A/A$ values are not constant over the total transition band contrary to previous claims for single transitions (Kuhn, 1958). This anomaly frequently occurs and the usual explanation offered assumes the existence of two electronic transitions which overlap, and that one of these transitions is optically inactive (Velluz et al., 1965, p. 206). This explanation seems reasonable for the case of a protein-bound cofactor where the absorption band may consist of several vibrational transitions.

The observed $\Delta A/A$ values, at pH 8.3, ranged from $\sim 2 \times 10^{-3}$ for PLP. With the exception of apoGOT-VIII, all of the bound cofactors possessed moderately high amounts of CD at their major absorption peaks (see Figures 3, 6, 9, 18, 22, and 26). When bound to apoGOT analogs III (carboxymethyl-deoxypyridoxal) and IV (deoxypyridoxalyl phosphonic acid) exhibit a small amount of negative CD above 400 mμ (Figures 9 and 18). THis is thought to arise from a non-specific type of binding at a site where asymmetry is imposed.

With regard to non-specific types of binding, the experiment illustrated in Figure 10 is pertinent. ApoGOT-III was reduced with sodium borohydride; as with PLP after reduction, the absorption and CD maxima shifted to 332 mμ (Figure 10a). Addition of a ten-fold excess of PLP (followed by dialysis to remove the free PLP) destroyed the CD at 332 mμ and established a small negative CD peak at 414 mμ; the absorption spectrum showed maxima at 390 and 325 mμ (Figure 10b). While the absorption of the apoGOT-III reduced complex remained, the asymmetry of its site was destroyed by PLP in the same region. Since the absorption

maximum at 390 mμ is characteristic of free PLP, it is possible that the PLP is bound by noncovalent interaction and not by imine formation. After reduction of the PLP-reduced apoGOT-III complex, the absorption and CD maxima were at 329 mμ (Figure 10c). This result would be obtained if PLP was bound covalently as an imine and reduced to a secondary amine; or if bound noncovalently, PLP would be reduced to the alcohol, pyridoxol phosphate, which absorbs at 330 mμ. The latter possibility does not explain the appearance of CD at 329 mμ, since it is hard to see why the reduction of a free aldehyde group to a free hydroxyl group would induce asymmetry. However, it is quite possible that the pyridoxol phosphate is removed during the borohydride reduction, which would restore the asymmetry of the site. At this point, it is not known which kind of binding is involved.

An experiment conducted with apoGOT-VIII with excess VIII present had two peaks of very low intensity with one peak located below 400 mμ and the other above. A short (and not complete dialysis reduced the CD above 400 mμ. Since the dialysis was very gentle (no dilution of protein occurred), the asymmetry of the binding should not be affected; therefore, the reduced CD above 400 mμ reflects the ease of dissociation of VIII from this type of binding as contrasted with the binding which absorbs in the 370 mμ region. This result contradicts the binding characteristics of VIII as shown by an absorption change from 380 mμ to 430 mμ (discussed above). It should be noted that two different apoGOT preparations were used; the subforms were not separated in the

preparation utilized for the CD experiment and the additional non-specific binding of VIII with apo-α-GOT may just occur with this sub-form. In either case, VIII does not seem to bind as rigidly as do the other analogs, and it shows a higher tendency toward non-specific binding.

Examination of the effect of pH upon the behavior of the absorption and CD spectra of the bound analogs, III-VI, yielded two unexpected and important conclusions. Only IV showed a pH dependence similar to that of PLP in the pH range of 5.4 to 8.3 while III, V (the methyl phosphonic acid of PLP), and VI (the cyanoethyl ester of PLP) did not. Therefore, the pH dependent spectral shift is subject to the presence of two negative charges (or at least the potential of two negative charges by ionization) on the 5'-substituent. Furthermore, in order to influence the pK of the imine nitrogen in such a manner, the 5'-substituent must fold back and lie over the pyridine ring. The small amount of absorption at 430 mμ (pH 5.4) shown by VI (Figure 26) is thought to be caused by PLP (see below).

Information regarding the relative ease of dissociation of III, IV, and V from apoGOT was obtained by reactivating apoGOT-III, -IV, and -V with PLP in excess (Figures 12, 19, and 23). The half-lives of the dissociation of III, IV, and V were 41, 34, and 23 minutes respectively. That the methyl phosphonic acid derivative is the most easily dissociated is not surprising since it is the most bulky of the three analogs. To be sure, more steric hindrance is imposed upon IV than III; that is,

there are fewer conformations allowed for IV, but it was thought that the ionic attraction between the protein and 5'-substituent would be greater for IV than for III. Nevertheless, III is bound more firmly than IV. The rates of binding of these cofactors parallel this series: III and IV are bound (as shown by absorption changes) within two minutes, while V requires a short while longer. Data obtained from reactivating apoGOT-III with a stoichiometric amount of PLP (Figure 11), while subject to error, gives a value for III's affinity for apoGOT as about 20% that of PLP.

The longer time required by VI for binding to apoGOT (about twenty minutes) demonstrates the difficulty of inserting a bulky cyanoethyl group into the binding site.

In brief, these analogs show that the spatial arrangement and the charge distribution of the 5'-substituent is of crucial importance in binding to the active site of GOP.

Reaction with substrates

Assays of the bound 5'-substituent cofactors did not provide much information as to their reaction characteristics with substrates except to demonstrate their poor catalytic abilities. By examining their behavior with substrates at spectroscopic levels of concentration, however, a somewhat clearer picture emerges.

ApoGOT-III reacts quite rapidly with L-glutamate as shown by a decrease in the absorption at 370 mμ and the appearance of the amine form which absorbs at 320 mμ (Figure 13). The absorption resulting from non-specific binding (at about 412 mμ) decreased also, but more slowly than

the major peak. The reaction with L-glutamate by the non-specifically bound form is interesting and three possible situations exist: a) the non-specific binding of III occurs at the active site, and the formation of a substrate-analog imine forces this aldimine intermediate into a conformation favorable for catalytic action by the enzyme; b) the non-specific binding of III occurs at a site other than the active site, and for catalysis to take place, the cofactor is removed from this site by Schiff base formation with the amino acid and is then bound at the active site either by binding as the substrate aldimine or by transaldimination with the ε-amino group at the active site; and c) the non-specifically bound III has some catalytic ability without a required rearrangement. While it is not possible to absolutely distinguish between these situations with the data available, (a) seems the more reasonable explanation. The situation described by (b) is thought to be the least likely to occur; and, while (c) is feasible, it is not as convenient as (a). The low enzymic activities of these analogs demonstrate the precise arrangement required at the active site for optimum catalytic ability. This would tend to discount the idea that non-specific binding of such a nature as to give an absorption maximum at a higher wavelength (and with a negative CD) could be similar enough to the active conformation to have catalytic ability. The process described by (b) would seem to be quite slow; therefore, (a) is the favored explanation.

The addition of α-ketoglutarate to the amine form of apoGOT-III

resulted in the rapid formation of an enzyme-substrate complex absorbing at 323 mμ which alowly transaminated to the aldehyde form absorbing at 370 mμ plus the amino acid product. That transamination is occurring is shown by the similar behavior of synthetic carboxymethyl-deosypyridoxamine when bound to apoGOT. While the concentration of L-glutamate exceeded that of α-ketoglutarate in the experiment illustrated in Figure 13, transamination of the keto acid was not prevented. Furthermore, the equilibrium lies strongly toward PLP and the amino acid in the native enzyme. Hence, while α-ketoglutarate is easily bound, it is not easily transaminated.

In order to determine whether the low rate of transamination of the amino acid substrate (compared to that of the native enzyme) was caused by hindered binding of substrate or not, the Michaelis constant for L-glutamate was obtained for both apoGOT-III (Figure 14) and native α-GOT (Figure 7). Since α-ketoglutarate caused severe substrate inhibition (see below) and because the initial velocities of apoGOT-III were so low, the oxaloacetate-L-glutamate assay system had to be employed. While V_{max} of apoGOT-III was one hundred times less than V_{max} for α-GOT, the K_{Glu} for apoGOT-III was 5.7 times the K_{Glu} for α-GOT. Thus, L-glutamate shows some decrease in affinity for apoGOT-III compared to native enzyme. However, the low rates of transamination of apoGOT-III are also reflected by the effect of the analog on V_{max}; hence, either the analog is bound in an improper conformation at the active site, or the 5'-phosphate of PLP plays a mechanistic role during the

reaction (or both of these may be combined).

In connection with conformational changes at the active site, the reactivity of apoGOT toward some substrates and inhibitors of native GOT was examined (Tables 2 and 3). Of interest are the observations: apoGOT-III showed more reactivity toward L-valine and more inhibition by glutarate, adipate, succinate, and α-ketoglutarate than did α-GOT; while the degree of inhibition by α-methyl-D, L-aspartate was about the same for both α-GOT and apoGOT-III. The reactivity toward L-valine demonstrates a decrease in specificity at the binding site of the hydrophobic chain by apo-GOT-III. The results for the inhibitors are interpreted to mean that III causes such distortion at the active site as to increase the affinity possessed by dicarboxylic acids. If the conclusions of Jenkins and D'Ari (1966b) regarding the binding of glutarate (ie. the second carboxyl group is bound at the protonated nitrogen of the aldimine linkage between the protein and the cofactor, and the dicarboxylic acid must undergo a conformational change after binding) are correct, then apoGOT-III's aldimine nitrogen may be placed so as to allow the dicarboxylic acids to bind without a conformational change (or with less required change).

The complex formed by apoGOT-III and α-ketoglutarate gave unexpected behavior at low pH. At pH 5.4, the CD of this complex exhibited a peak at about 450 mμ (Figure 16) similar to that of the α-GOT-α-ketoglutarate complex (Figure 5), but of much lower magnitude. Furthermore, the negative CD at about 415 mm of apoGOT-III was enhanced with

α-ketoglutarate. The effect on the pK by α-ketoglutarate lends support to the idea that a carboxylate group is attached to (or in the immediate vicinity of) the imine nitrogen. On the basis of the negative CD at 415 mμ, it is apparent that α-ketoglutarate can interact with the non-specifically bound III. Unlike the native enzyme, apoGOT-III's complex is unstable at pH 5.4 as shown by the absorption spectrum (Figure 15) compared with that of α-GOT (Figure 4).

An exact interpretation of the positions occupied by the interacting molecules at the active site of apoGOT-III is not possible without X-ray diffraction data, but it has been shown that analog III induces some distortion of GOT's active site.

The reaction of apoGOT-IV with L-glutamate was quite rapid (<2 minutes) with respect to the appearance of the amine form absorbing at 325 mμ. However, while the aldimine form of apoGOT-IV (359 mμ) was decreasing, a species absorbing at 430 mμ was produced. The 430 mμ species was produced relatively quickly (maximum absorbance at 430 mμ occurred in about two minutes), but its subsequent conversion to the amine was slow. Thus, there exists two conformations of apoGOT-IV which absorb at 359 mμ; one which is capable of forming an amino acid Schiff base that is tautomerized to the ketimine easily, and the other conformation forming an amino acid Schiff base that is not as reactive. The amine form produced by either conformation does not react with α-ketoglutarate to produce a complex with a different absorption maximum, but it does not transaminate with α-ketoglutarate either. Hence, it may be possible that an abortive type of enzyme-α-ketoglutarate complex exists which absorbs

at 325 mμ similar to that of apoGOT-III. The other possibility is that the affinity of α-ketoglutarate is lowered because the conformation of the active site of apoGOT-IV amine is not favorable for keto acid binding. Whatever the reason, the lack of an oxygen atom between the 5'-carbon and the phosphorus atom causes a large effect.

ApoGOT-V gives a slow reaction with L-glutamate to produce apoGOT-V amine which absorbs at 315 mμ. The methyl group attached to the phosphorous atom either hinders the binding of amino acid or inhibits the mechanistic role of the 5'-phosphate if the phosphate group plays such a role. Like apoGOT-III, the amine form of apoGOT-V shows the rapid formation of a complex with α-ketoglutarate by a very noticeable shift of the absorption maximum to 327 mμ. Subsequent transamination of this complex is very slow.

With respect to the other 5'-analogs, apoGOT-VI gives a slower reaction with the amino acid to give the amine form which absorbs at 317 mμ. The formation of a complex with α-ketoglutarate by the amine form of apoGOT-VI and the subsequent transamination of the complex was slower for this analog than for the others. Since compound V demonstrated a retarded behavior as a coenzyme, this effect of a large group (cyanoethyl) bonded to the 5'-phosphate is not surprising. However, the slow activation of apoGOT-VI (Table 4) to an activity equivalent to that of PLP was unexpected. The activation was shown to be a first order process (Figure 27). Two explanations are possible: a) analog VI is bound initially in an inactive mode and is slowly rearranged to an active conformation, and b) analog VI is bound as the cyanoethyl ester and is

then slowly hydrolyzed to PLP while on the enzyme surface. Either process would give first order kinetics, but the second explains the small amount of absorption and CD at 430 mμ exhibited at pH 5.4 (Figures 25 and 26) and, hence, is the favored explanation. While the hydrolysis of the cyanoethyl group of VI is thought to occur on the surface of GOT, this phenomenon is not exhibited by two other pyridoxal phosphate enzymes (see page 71).

ApoGOT-VIII was quite similar to apoGOT-III: rapid conversion of the aldimine (380 mμ) to the amine form (320 mμ), a slower conversion to the amine form by the non-specifically bound cofactor, and a slow transamination with α-ketoglutarate. While a complex with α-ketoglutarate and the amine for with a different absorption maximum did not appear, such a complex could absorb at 320 mμ.

In conclusion, the 5'-analogs examined in the study had a pronounced effect upon GOT's catalytic ability and upon the enzyme's spectral behavior. In particular, the formation of abortive complexes with α-ketoglutarate was more pronounced, and the proportion of non-specifically bound cofactor was higher with these analogs than for the native enzyme. Furthermore, the pK of the imine nitrogen was shown to depend upon the 5'-substituent.

Ring N-Analogs (IX and X)

Binding characteristics

Both of the pyridine ring nitrogen substituent analogs appeared to be bound firmly by apoGOT. N-methylpyridoxal phosphate (IX was bound more slowly than pyridoxal phosphate-N-oxide (X), but with a higher degree of asymmetry associated with the binding. The $\Delta A/A$ value for IX at pH 8.3 was the same as that possessed by the native enzyme. IX did not exhibit non-specific binding of the type to give absorption above 400 mμ.

The binding of X to apoGOT at pH 8.3 gave a broad absorption band from 380 mμ to 405 mμ, a shoulder at 320 mμ, and high absorption at 430 mμ. Hence, several species of apoGOT-X are present. The number and nature of these species are not known.

Since the 5'-substituent of both analogs is the normal phosphate ester, their spectra show a pH dependence in the range pH 5.0 to 8.3 Ohishi, et al. (1968) reported absorption and CD maxima at 420 mμ at pH 5.0 for GOT-X.

Reaction with substrate

With L-glutamate, apoGOT-IX reacted slowly (>20 minutes) to produce the amine form absorbing at 335 mμ, the reverse reaction with α-keto-glutarate was extremely slow. Only the two forms with absorption at 377 mμ and 335 mμ were apparent. This behavior has two explanations: a) IX is bound in an unfavorable conformation for catalysis, or b) the methyl substituent prevents the pyridine ring nitrogen from playing

its mechanistic role as an "electron sink."

ApoGOT-X reacted very rapidly to form the amine which absorbs at 325 mμ. While the absorption from 380 mμ to 405 mμ was destroyed, a species absorbing at 425 mμ reacted to form the amine at a slower rate. With α-ketoglutarate, the 325 mμ absorption rapidly shifted to 320 mμ which subsequently reduced as absorption at 425 mμ increased. No absorption increase was noted in the 380 mμ region. These spectral changes are very odd and do not explain the high catalytic activity (30% of the activity of native GOT) observed with this analog. While the transamination of amino acid seemed to proceed in a normal manner (although with several species of apoGOT-X present), the reaction of amine apoGOT-X with α-ketoglutarate did not produce an aldimine with absorption around 380 mμ. Apparently, a Schiff base intermediate of X and amino acid (absorbing at 425 mμ) is capable of hydrolyzing to release the amino acid product.

The same explanations for IX's behavior may be advanced for X. However, in neither IX or X, is it known whether improper binding or improper electron distribution in the cofactor is the correct explanation.

SUMMARY OF CONCLUSIONS

1) Carboxymethyl-deoxypyridoxal (III) is bound firmly to apoGOT. A non-specific type of binding with this cofactor was shown to occur at the active site of the enzyme. III affected both K_{Glu} and V_{max} in the catalyzed transamination of L-glutamate. The conformation of the active site was shown to be distorted by the binding of III which in-ed the affinity of dicarboxylic acids for this site. While apoGOT-III did not show pH dependence of its absorption and CD spectra, its complex with α-ketoglutarate did.

2) Deoxypyridoxalyl phosphonic acid (IV) binds to apoGOT to form two different conformations at the active site. One of these conformations reacts with amino acid substrate to form the amine of apoGOT-IV, while the other produces an intermediate Schiff base which absorbs at 430 mμ. α-ketoglutarate does not alter the absorption spectrum of apoGOT-IV amine, but does not transaminate rapidly either.

3) The methyl phosphonic acid of PLP (V) was bound to apoGOT at a slower rate than III or IV; furthermore, it was shown to be more easily dissociated from the protein than either III or IV. With substrate added, apoGOT gave a slow reaction with L-glutamate and a notable complex of the amine form with α-ketoglutarate.

4) The cyanoethyl ester of PLP (VI) was bound slowly to apoGOT and demonstrated a very slow reactivity with substrates which was caused by the steric hindrance imposed by the bulky cyanoethyl group. The slow activation of apoGOT-VI is thought to be due to PLP formed by the hydrolysis of VI on the enzyme surface.

5) Deoxypyridoxal (VIII) was bound to apoGOT with a lower asymmetry associated with the binding site than the other 5'-analogs. In addition, VIII shows a higher degree of non-specific binding. A complex of α-ketoglutarate and the amine form of apoGOT-VIII was not observed spectrophotometrically, although the transamination of α-ketoglutarate was very low.

6) The 5'-analogs all showed more of a tendency toward non-specific binding and a higher degree of keto acid abortive complex formation than the native enzyme did.

7) the pK of the aldimine formed between cofactor and protein was shown to be dependent upon the 5'-substituent.

8) The pyridine ring nitrogen substituents, N-methyl PLP (IX) and PLP-N-oxide (X), were bound to apoGOT and possessed pH dependent absorption and CD spectra. IX exhibited very low catalytic ability while X had 30% of the activity of native GOT. It is suggested that the difference in activity between IX and X may be caused either by improper binding or by the substituent's effect upon the ring nitrogen. It is not known which of these is the correct explanation.

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