STUDIES ON THE ACTIVE SITE MODIFICATION OF PYRIDOXAL AND FLAVIN DEPENDENT ENZYMES WITH ACETYLENIC AND OLEFINIC SUBSTRATE ANALOGUES

by

PATRICK ALLEN MARCOTTE

S.B., Massachusetts Instiute of Technology (1973)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May 23, 1977

Signature of Author.. Signature redacted .....
Departmentof Chemistry, 1977

Certified by..... Signature redacted .....
Thesis Supervisor

Accepted by..... Signature redacted .....
Chairman, Department Committee

This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

Professor George M. Whitesides — Signature redacted — Chairman

Professor Christopher T. Walsh — Signature redacted — Thesis Supervisor

Professor Ellen J. Henderson — Signature redacted

Studies on the Active Site Modification of Pyridoxal and Flavin Dependent Enzymes with Acetylenic and Olefinic Substrate Analogues

by Patrick Allen Marcotte

Submitted to the Department of Chemistry of May 23, 1977 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

ABSTRACT

The reactions of the olefinic amino acid vinylglycine (2-amino-3-butenoate) and the acetylenic amino acid propargylglycine (2-amino-4-pentynoate) with four enzymes have been examined. These amino acids incorporate a reactive functionality, and, upon reaction with these enzymes, inactivation of the enzyme due to covalent modification may result.

The reaction of these potential inactivators with two pyridoxal-phosphate dependent transaminases was studied. L-alanine aminotransferase suffers loss of activity upon reaction with L-propargylglycine, covalent modification of the enzyme occuring in one of every two reactions of enzyme and the "suicide substrate." No inactivation is observed as the result of the transamination of L-vinylglycine by this enzyme.

D-amino acid transaminases isolated from two species of Bacillus were found to undergo covalent modification upon their reaction with D-vinylglycine. The enzymes were observed to catalyze 450-800 transaminations before suffering loss of activity. These enzymes demonstrated little or no sensitivity towards inactivation in the course of their reaction with propargylglycine.

L-amino acid oxidase from Crotalus adamanteus venom suffers inactivation upon its oxidation of L-vinylglycine. Loss of activity is the probable result of alkylation of an amino acid residue of the apoprotein in one of every 2000 catalytic oxidations of the amino acid. D-amino acid oxidase from pig kidney is insensitive towards inactivation as a consequience of its oxidation of D-vinylglycine.

The study of the reactions of the amino acid oxidases with propargylglycine is divided into three parts:

1. After enzymatic oxidation of propargylglycine, a complex series of non-enzymatic reactions is observed. Three species produced in the incubation have been characterized. Two of the molecules (2-aminopent-2-en-4-ynoate and 2-amino-4-keto-2-pentenoate) are powerful competitive inhibitors of D-amino acid oxidase and induce intense charge transfer bands upon binding to the enzyme. The major accumulated product of the reaction is a cyclic eneamine lactone, 2-amino-4-hydroxy-2,4-pentadiene-γ-lactone.

2. D-amino acid oxidase undergoes covalent modification upon its oxidation of D-propargylglycine. The modified enzyme has been characterized as to its spectral and catalytic properties, and has been found to have

markedly changed substrate specificity and kinetic parameters from the native enzyme. L-amino acid oxidase has been found to be insensitive to covalent modification as a result of its oxidation of L-propargylglycine.

3. The process by which D-amino acid oxidase undergoes covalent modification upon its oxidation of D-propargylglycine has been investigated. A hypothesis for the mechanisms of the alkylation process is proposed.

Thesis advisor: Christopher T. Walsh
Title: Associate Professor of Chemistry and Biology

ACKNOWLEDGEMENTS

There are a number of persons who must be recognized for their contributions to this work. Chris Walsh, although occasionally being in too great a hurry to publish preliminary work, has been a great advisor and has been unceasingly helpful in the development of this work. Rob Spencer, in the midst of commentary of a wide variety of topics, made a number of suggestions which turned out to be of great value. And Paula Olsiewski deserves a special note, for trying to care about me even when I wasn't caring about anyone else.

Every present and past member of Chris Walsh's group has at some time or another been helpful in this reasearch. Yak Cheung, Tom Cromartie, Jed Fisher, Jorge Goldstein, Greg Kaczorowski, and Pat Shannon made helpful suggestions along the way. I also want to thank Sue Coppersmith, Carol Cummings, T. Y. Dang, Joanne Donovan, Fred Jacobson, Tim Jones , Laurie Shaw, Mark Snyder, and Elizabeth Wang for their kindnesses. I must also acknowledge Lorne Murphy, without whom I might not have made it this far.

I have also had the same two roommates for the last four years (must be some kind of record). So I must thank Lance Antrim and Mike Portnoff for putting up with me.

Finally, I want to thank my parents for their constant encouragement.

May 19, 1977

TABLE OF CONTENTS

LIST OF TABLES

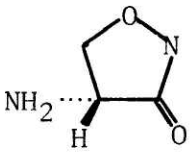
LIST OF FIGURES

page

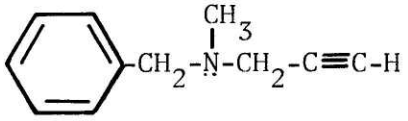
CHAPTER I

INTRODUCTION

There are a number of reasons for pursuing research into the modification of enzymes using active site directed substrate analogues. A rationale frequently cited is the potential pharmacological importance of such agents[1,2,3,4]; that by designing a molecule which will inactivate a single enzyme, and therefore affect a specific metabolic process, useful theraputic agents might be developed. Two clinical drugs which are known to be active site directed enzyme inactivators are in use: the antibiotic D-cycloserine[5](D-4-amino-3-isoxolidone), which blocks bacterial synthesis of D-alanine, an essential metabolite in bacterial cell wall biosynthesis; and pargyline[6] (N-methyl-N-propynylbenzylamine) an inactivator of monoamine oxidase which has been used as an anti-hypertensive drug.



D-cycloserine



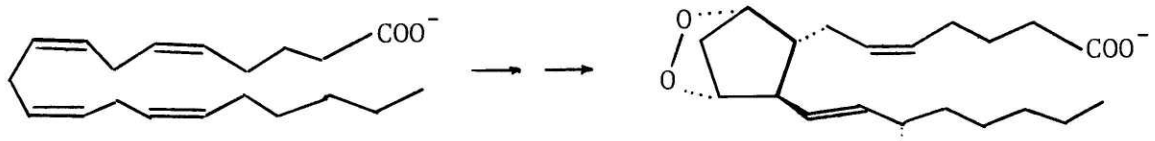
pargyline

However, for both of these chemicals, the molecular basis of their action was studied after promising clinical effects had been discovered. Thus, work such as will be described in this paper did not lead to the development of these agents. Furthermore, although thought to be relatively specific in their action, both of these drugs lead to serious side effects, and the use of other substances is preferred in most cases.[5,6]

As more information is developed on the molecular basis of the action

of drugs, other substances currently in use may be found to be active site directed enzyme inactivators. For examble, the action of the penicillins and cephalosporins has been theorized to be due to covalent modification of the enzyme which catalyzes the final step in bacterial cell wall biosynthesis.[7]

The most common of all drugs, aspirin, is known to inhibit the synthesis of protaglandin G_2 in platelets.[8] It has recently been shown that the mechanism of this inhibition is through the acetylation of a susceptible nucleophile of the enzyme which catalyzes the cyclooxygenation of arachidonic acid to protaglandin G_2.[9]



arachidonic acid PGG_2 OOH

The search for pharmacologically active chemicals cannot be presented as the purpose of this work. Present knowledge of the molecular basis of disease does not, for the most part, allow the investigator to target enzymes for inactivation which are known to be involved in a disorder. The approach of this work has been the matching of previously isolated enzymes in amino acid metabolism with non-physiological substrate analogues which might cause their inactivation. Although the two amino acids used in this work, vinylglycine and propargylglycine, probably affect relatively few enzymes, it is

doubtful they have potential as clinical agents, since they have serious effects on mammalian enzyme systems. Therefore, rationales other than the synthesis of useful drugs form the basis of this work.

A second pharmacological application of active site directed substrate analogues is the use of these agents to inactivate in vivo a target enzyme, thereby producing in an experimental animal a condition which might resemble that caused by a particular genetic defect. In this way, information as to the metabolic function of a specific enzyme can be obtained. Approaches to the treatment of a disorder caused by the deficiency of an enzyme might also be a development of these investigations.[2,3]

A third reason for the study of active site directed enzyme inactivation derives from the specific modification of one (or a few) residues of an enzyme induced by these agents. The enzyme thus covalently modified can then be degraded and the site of alkylation be determined. Together with studies of the chemical and physical properties of the enzyme, such as subunit structure, amino acid composition and sequence, and in favorable cases, the three dimensional structure of the enzyme as determined by X-ray diffraction analysis, information on the geometry of the active site and on the factors important in binding and catalysis can be obtained. The use of active site directed alkylating agents has led to the identification of three residues of catalytic importance at (or near) the active site of chymotrypsin.[10]

However, work to date has solved the primary structure and three dimensional configuration of relatively few proteins. In addition, the technological difficulties of X-ray diffraction analysis makes this technique

usually applicable only to relatively small proteins. Therefore, information on enzyme structure that can be gained from studies using modification agents is usually limited to the identification of residues near the site of alkylation in the amino acid sequence of the protein.

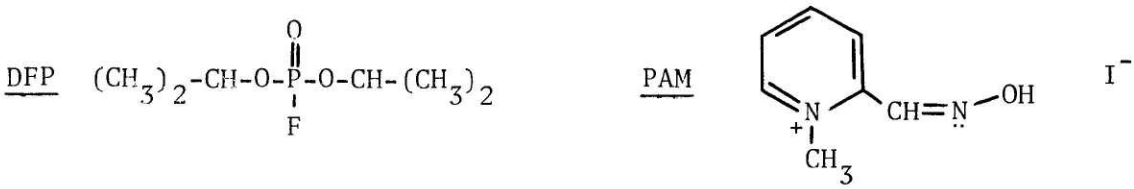
A fourth manner in which the use of active site directed enzyme inactivators can be valuable is in the study of enzyme mechanisms. A number of enzymes which catalyze a similar transformation show striking differential susceptibility towards inactivation by a substrate analogue. An example is the action of vinylglycine on several pyridoxal phosphate dependent transaminases. Pig heart L-aspartate aminotransferase was recently shown to be inactivated efficiently by vinylglycine -- no catalytic action before inactivation can be observed[11]. On the other hand, this work will show that the bacterial D-amino acid transaminases are inactivated, but only after carrying out several hundred catalytic transaminations. Finally, pig heart L-alanine aminotransferase has been found to have no susceptibility to inactivation while carrying out the transamination. The bases for these effects are hidden in the detailed geometry of the active site; e.g., the ability of the enzyme to carry out general acid or base catalysis on the bound molecule or a nucleophilic group of the enzyme placed in a susceptible location. This work will demonstrate several instances of such differential inactivation.

Evidence on the mechanism of enzymatic catalysis can also be obtained by cases in which alkylation by an active site directed reagent results in the formation of a catalyst which still retains a fraction of its initial activity[12]. This allows the identification of an amino acid resi-

due the alkylation of which changes, but does not destroy, the catalytic properties of the enzyme. The modified enzyme may have greatly changed kinetic properties, exhibiting different binding affinities for substrates and a different rate determining step, from the native enzyme.[13] From these cases of modified enzyme formation, information regarding the mechanism of enzyme catalysis can be drawn.

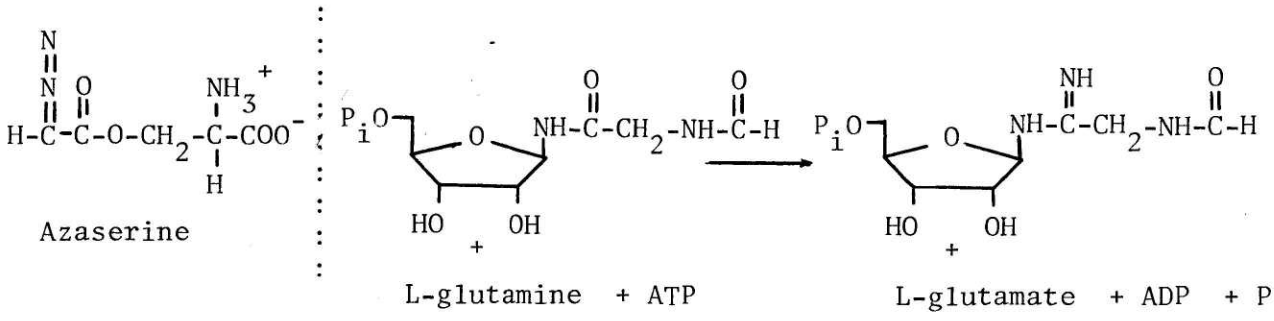
A BRIEF HISTORY OF THE USE OF ACTIVE SITE DIRECTED ENZYME INACTIVATORS

The beginning of the study of active site enzyme modification was the discovery, in 1947, that the cause of organophosphorous poisonings by compounds such as diisopropylfluorophosphate (DFP) was by the specific phosphorylation of an essential serine residue of certain serine proteases and esterases, most notably acetylcholinesterase.[14] An understanding



of the mechanism of such poisonings led to the formulation of antidotes to the poisons, one of which is pralidoxime iodide (PAM).[15] [16] It was many years (until 1964) before it was established that these reagents, which do not resemble in any obvious way the substrate of the enzyme, form a reversible complex with the enzyme before alkylation takes place.[17]

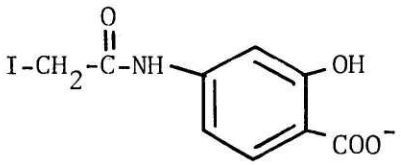
In 1957, J. Buchanan at M.I.T., seeking to understand the mechanism by which azaserine, an antibiotic isolated from Streptomycetes,[18] interfered with purine biosynthesis,[19] determined that this molecule caused rapid, irreversible inactivation of formyl-glycinamine ribonucleotide-L-glutamine aminotransferase.[20] The alkylation was shown to occur at the glutamine



binding site of the enzyme. Buchanan et al later determined that a single

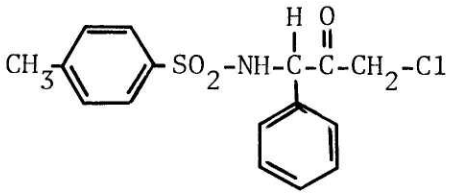
cysteine residue was susceptible to alkylation by azaserine.[21]

B. R. Baker, at University of California at Santa Barbara, was the first (in 1961) to use a synthetic molecule to intentionally inactivate a target enzyme. Making use of the known, reversible, inhibition of lactic dehydrogenase and glutamic dehydrogenase by substituted salicylic acid derivatives, Baker produced irreversible inactivation of these enzymes by 4-(iodoacetamido)-salicylic acid.[22] Other substituted salicylates were

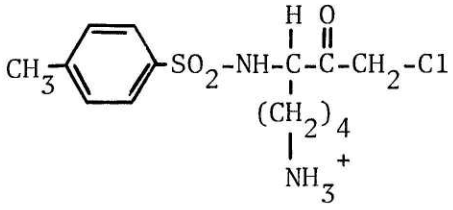


synthesized in efforts to find more specific inhibitors of these enzymes.[1]

After the work of Baker, other examples of active site modification rapidly followed. The most intensively studied enzymes were the mammalian proteases, chymotrypsin and trypsin, which were shown by E. Shaw to be inactivated by tosylphenylalanylchloromethylketone[23] (TPCK) and tosyllysyl-chloromethylketone[24] (TLCK). These molecules resemble synthetic substrates



TPCK

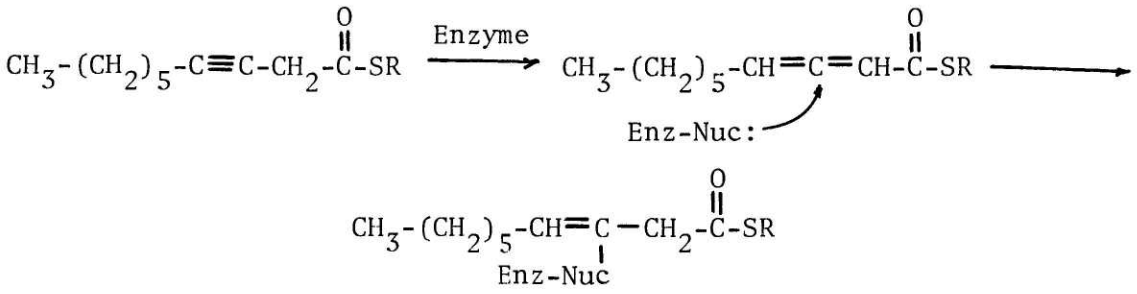


TLCK

of the enzyme but incorporate the reactive chloromethyl ketone functionality in place of a hydrolyzable ester or amide. Both TPCK (for chymotrypsin)[25] and TLCK (for trypsin)[26] cause the alkylation of a histidine residue, later shown in the three dimensional structure of these enzymes to be acting as a general base at the active site.[27,28]

The inactivators thus far discussed derive their specificity from the binding of the inhibitor at the active site of the enzyme followed by attack by an enzyme nucleophile on the reactive portion of the inactivator. Other examples of this type of inactivator, often called "affinity inactivators," have been found; the most commonly used reactive functionalities are the α-haloketone, α-diazoketone, and epoxide groups. These investigations have been well reviewed and I will not discuss them here.[1,4,10,29]

The use of inhibitors which contain reactive groups suffers from the inherent chemical reactivity of these species, a property which could both decrease their lifetime in solution and also reduce their specificity. A potentially more specific class of active site directed inactivators was first discovered by Bloch in 1968 when it was found in his laboratory that β-hydroxythiolester dehydrase is irreversibly inactivated upon incubation with 3-decynoyl-N-acetylcysteamine.[30]



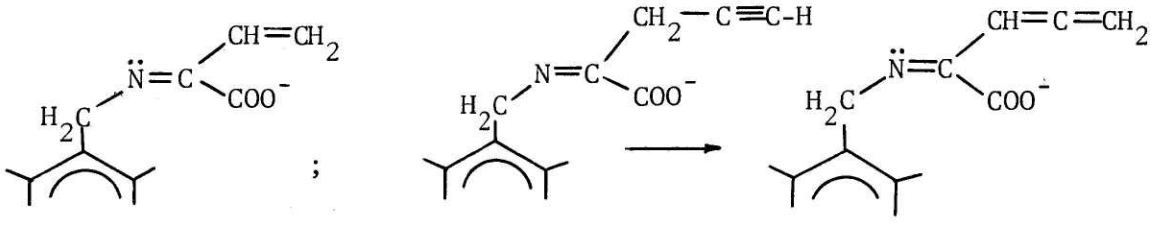
The mechanism of this inactivation is the enzyme catalyzed rearrangement of the acetylenic thiolester to the electrophilic conjugated allenic thiolester, followed by attack at C_3 by an enzyme nucleophile, resulting in irreversible loss of enzyme activity. Thus the enzyme has, by its own catalytic properties, produced the reactive species which leads to inactivation.

Many further examples of enzyme inactivators of this type, termed "k_{cat} inhibitors"[2] or "suicide substrates"[3], have been found. Suicide substrates have the advantage that they should show specificity for enzymes which catalyze a specific transformation. The inactivators are inert in solution and should have no irreversible effect on enzymes which bind them but do not catalyze the appropriate reaction.

Recent work with these inhibitors has been the subject of numerous recent reviews;[2,3,4,29] therefore I will limit the general discussion of this work to the following brief treatment. Almost all of these inactivators can be grouped into a few categories.

1. Inactivation of isomerases by acetylenic substrate analogues. Similar to the original work of Bloch, these inactivators incorporate an acetylenic group which is converted to a conjugated allene by action of the enzyme.[31,32]

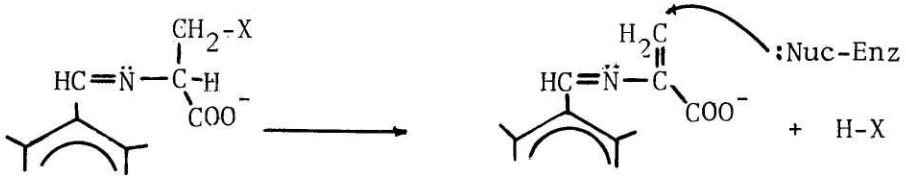
2. Inactivation of pyridoxal-phosphate dependent enzymes by olefinic or acetylenic amino acids.[33] The enzyme converts the suicide substrate into a bound conjugated olefin or conjugated allene which is attacked by an enzyme nucleophile.



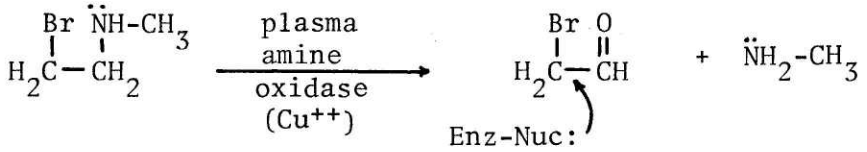
3. Inactivation of flavoprotein dehydrogenases and oxidases with acetylenic and olefinic substrate analogues.[34] Upon incubation of flavoproteins with acetylenic substrates, inactivation with modification of the bound coenzyme often occurs. Other flavoproteins are inactivated by attack of enzyme

nucleophiles on bound conjugated olefins or conjugated allenes similar to the mechanism previously described for pyridoxal enzymes and isomerases. The inactivation of flavoproteins is discussed in more detail in Chapter III.

4. Inactivations induced by halide containing substrate analogues. Reaction of pyridoxal-phosphate dependent enzymes with β-halo-α-amino acids has been found to result in inactivation.[35] The inactivation most likely procedes from attack of an enzyme nucleophile on the conjugated olefin produced by elimination of HX.



In other cases of enzyme inactivation induced by halide containing substrate analogues, it is probable that the enzyme converts the suicide substrate into an α-halo carbonyl or α-halo imine species which is then attacked by an enzyme nucleophile.[36]



5. Inactivations produced by nitriles. In a manner analogous to that described for acetylene induced inactivation, alkylation results from enzymatic conversion of the nitrile to a ketenimine, followed by attack of an enzyme nucleophile.[37] [38]

By a large measure, the most prominent use of suicide substrates has been in the inactivation of pyridoxal and flavin dependent enzymes which catalyze reactions of amino acids, amines, and hydroxyacids. The current work is a continuation of that trend.

GENERAL PRINCIPLES OF ACTIVE SITE ENZYME MODIFICATION

The simplest model of the action of an active site directed enzyme inactivator is given by (1).[10 39]

$$(1) \quad E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,I \xrightarrow{k_{inactivation}} E\text{-}I$$

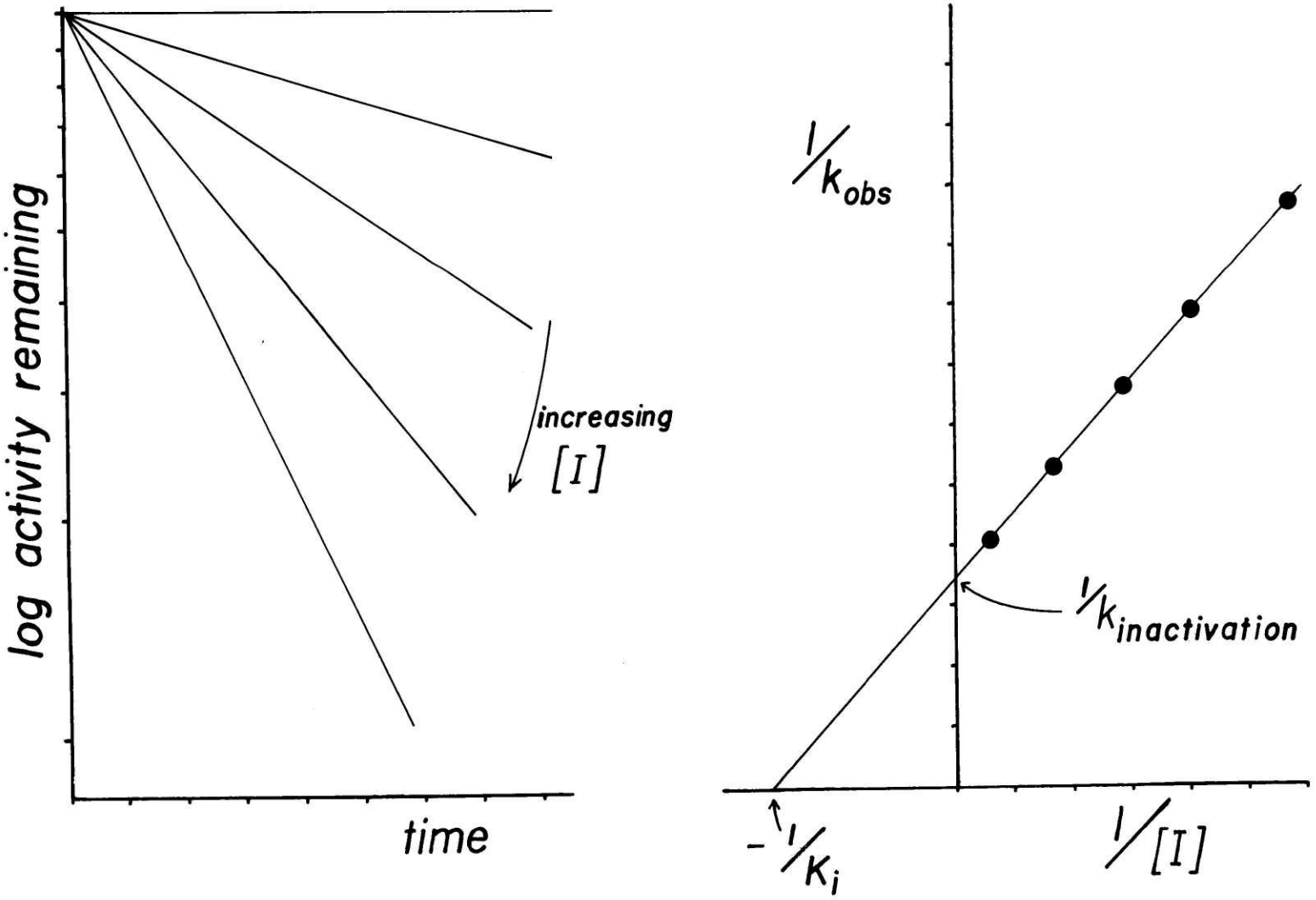
Initial reversible formation of an active site complex is followed by formation of a covalent bond between the enzyme and inactivator, resulting in irreversible inactivation of the enzyme. The observed loss of enzyme activity should be first order in enzyme, and is related to the kinetic constants by the following expressions (2).[39]

$$(2) \quad \frac{d[E]}{dt} = -k_{obs}[E] \quad ; \quad k_{obs} = \frac{k_{inac}[E][I]}{K_i + [I]} \quad ; \quad K_i = \frac{k_{inac} + k_{-1}}{k_1}$$

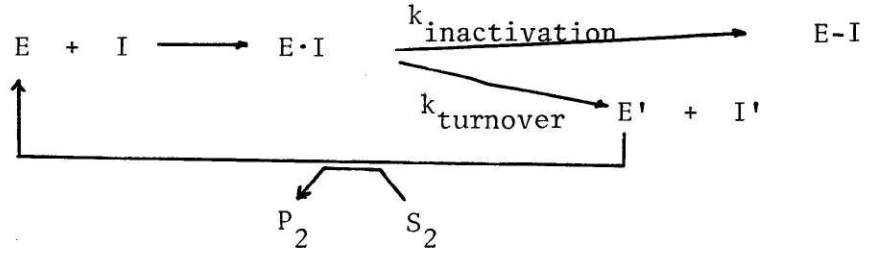
The expression for k_{obs} has the same form as the Michaelis-Menten equation describing the steady state rate of product formation by enzymatic catalysis. In the same manner as a Lineweaver-Burk plot, a plot of $1/k_{obs}$ versus $1/[I]$ yields a graph (Figure 1) with vertical intercept of $1/k_{inac}$ and horizontal intercept $(-1/K_i)$. This model works well in the case of inhibitors which do not undergo catalysis (affinity inactivators), such as the chloroketone inactivators of trypsin and chymotrypsin.[10]

The inactivation of certain enzymes by a "suicide substrate" is also described adequately by (1); $k_{inactivation}$ now being a complex rate constant encompassing both the enzymatic transformation of the substrate to the reactive species and the alkylation process. However, in many cases, the reac-

FIGURE 1


log activity remaining
time
increasing
[I]
1/k_obs
1/k_inactivation
-1/K_i
1/[I]

tion of the enzyme and the suicide substrate is complicated by the catalytic action of the enzyme on the inactivator, followed by release of a product without enzyme modification having taken place. Therefore, the simple model for the inactivation process (1) needs to be expanded; a more general scheme is given by (3).[2]



(3)

The above scheme demonstrates the partition between the catalytic reaction (referred to as "turnover") and enzyme inactivation. The ratio of the rate constants $k_{turnover}/k_{inactivation}$ is determined by the number of catalytic events which occur before inactivation is observed, and is a measure of the efficiency of the suicide substrate in producing inactivation. The number of turnovers before inactivation found in the present work has varied from one (50% efficiency) to several thousand (< 0.1% efficiency).

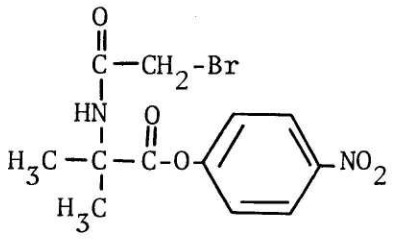
After reaction of an enzyme with a suicide substrate, and release of a product, it is common that the enzyme, although not covalently modified, has been converted to a form which is not competent to react with another molecule of the potential inactivator. This enzyme form is designated by E' in (3). The simplest case of this occurrence is in the reaction of a flavoprotein oxidase with an amino acid. The enzyme is converted by the catalytic reaction to its two electron reduced form, and must be reoxidized by the oxygen in solution before it can react with another molecule of amino

acid.[40] A requirement for oxygen will be seen in the reactions of the two flavoprotein oxidases studied in this work. In the reaction of pyridoxal phosphate dependent transaminases with a potential amino acid inactivator, catalytic reaction without alkylation leaves the enzyme in the pyridoxamine form, which must be reoxidized by a molecule of keto acid (usually α-ketoglutarate) to complete the cycle.[41] In (3), the general case is illustrated; the second substrate is designated by S_2, E' is converted to E as a consequence of its catalysis of the reaction $S_2 \rightarrow P_2$.

Several problems are found when a large number of catalytic events occur before inactivation is observed. The first is the kinetic problem which arises when a large percentage of the potential inactivator is consumed before inactivation, making analysis of rates more difficult. A more serious complication occurs if the released product is itself an electrophile and has the capacity of alkylating nucleophiles not at the active site of the enzyme. Then the observed inactivation may be the result of (one or more) non-specific alkylations rather than specific modification at the active site. This phenomenon was found during oxidation of L-vinylglycolate (2-hydroxy-3-butenoate) by rat kidney L-hydroxy acid oxidase.[42] The enzyme was inactivated upon carrying out the oxidation but studies with [^{14}C]vinylglycolate revealed numerous alkylations per enzyme molecule. Furthermore, the inactivation was prevented by addition of dithiothreitol as a scavenger, proving the inactivation was proceding from the products species, 2-keto-3-butenoate, released into solution.

The simple model of the inactivation of an enzyme given by (1)

also proves insufficient when the alkylated enzyme, although inherently less efficient a catalyst, still retains considerable activity. The first example of an active site directed modification producing a "wounded enzyme" was found upon the incubation of chymotrypsin with p-nitrophenyl-N-bromoacetyl-α-aminoisobutyrate (NBAIB). Unlike the inactivation produced by tosylphenylalanylchloromethylketone (TPCK), incubation of the enzyme with NBAIB causes alkylation of methionine-192 and was observed to result in the formation of a catalyst with "20% residual activity."[43] More precisely, it was found that the K_m of the synthetic substrate used to measure activity (N-acetyltyrosine ethyl ester) had been increased by a factor of 10. The V_{max} of hydrolysis had actually <u>increased</u> by 50% upon modification.[12] Cases such as this one lend ambiguity to the simple descriptions of what is meant by an "active site direacted inactivation."

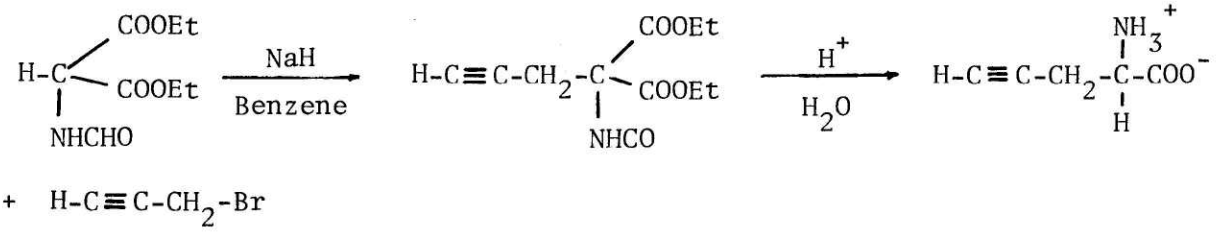


NBAIB

ON PROPARGYLGLYCINE AND VINYLGLYCINE

Two amino acids are used prominently in this work. A short discussion as to their origin and previous use is appropriate.

DL-propargylglycine was first synthesized as a potential antibiotic in 1949.[44] The procedure involved the acid hydrolysis of the condensation product of diethylformamidomalonate and propargyl bromide.



+ $H\text{-}C\equiv C\text{-}CH_2\text{-}Br$

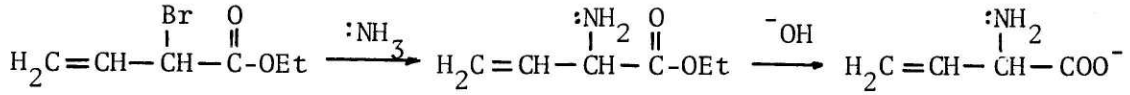
Jansen et al[45] published a detailed procedure for the synthesis of propargylglycine, including the resolution of the racemic amino acid by treatment of DL-N-acetylpropargylglycine with hog kidney acylase I. L-propargylglycine was isolated from a fermentation broth of a strain of Streptomycetes.[46]

The DL-amino acid was shown to be an effective growth inhibitor of a bacterium (E. coli) and yeast (S. cerevisiae)[47]. No studies on the potential pharmacological use or toxicity of this amino acid on higher organisms have been reported; however, injection of a rat with a small amount (0.5 mg) of DL-propargylglycine leads to the in vivo total destruction of the γ-cystathionase activity of its liver within 24 hours.[48]

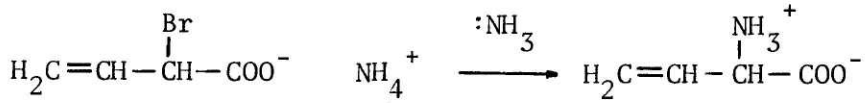
L-propargylglycine has been reported to cause the in vitro inactivation of three mammalian enzymes: pig heart L-alanine aminotransferase,[49] rat liver γ-cystathionase,[48] and (in the presence of formate) L-aspartate

aminotransferase.[50] L-propargylglycine is also an *in vitro* inactivator of *S. typhimurium* cystathionine-γ-synthase,[49] an enzyme in methionine biosynthesis. Becuase the growth inhibition of *B. subtilis* by L-propargylglycine can be reversed by addition of L-methionine,[46] it is probable that the major effect of the antibiotic *in vivo* on this microorganism is the inactivation of cystathionine-γ-synthase. Inactivation of hog kidney D-amino acid oxidase is observed upon its oxidation of D-propargylglycine.[51 52] DL-propargylglycine has also been found to inhibit amylase synthesis *in vitro* by slices of pigeon pancreas.[53]

DL-vinylglycine was first synthesized by two laboratories, independently, in 1974. Friis *et al* prepared vinylglycine in 6.6% yield by saponification of the ethyl ester of vinylglycine produced from reaction of concentrated ammonia with ethyl 2-bromo-3-butenoate.[54]



Rando prepared vinylglycine[11] by addition of 2-bromo-3-butenoic acid to concentrated ammonia and achieved a reported 50% synthesis of DL-vinylglycine.[55]



This olefinic amino acid has also been found to be a natural product,[56] isolated from a mushroom (Rhodophyllus nidorosus), as an enantiomeric mixture with an excess of the D-isomer. L-vinylglycine has been reported to inactivate *in vitro* L-aspartate aminotransferase[11] and *Crotalus adamanteus* venom

L-amino acid oxidase.[52] D-Vinylglycine has been reported to inactivate two bacterial D-amino acid transaminases.[57] A number of pyridoxal phosphate dependent isomerases have been reported to catalyze the conversion of L-vinylglycine to 2-ketobuyrate and ammonium ion, among them E. coli tryptophan synthase,[58] sheep liver threonine deaminase,[59] S. typhyimurium cystathionine γ-synthase[60] and rat liver γ-cystathionase.[61] No pharmacological or toxicological properties of vinylglycine have yet been reported.

CHAPTER II

ACTIVE SITE DIRECTED INACTIVATION OF PYRIDOXAL DEPENDENT TRANSAMINASES WITH ACETYLENIC AND OLEFINIC SUBSTRATE ANALOGUES

Probably the enzymes which have drawn the most attention in investigations of active site directed modification using "suicide substrates" are those proteins which utilize pyridoxal phosphate in their catalysis. A number of reasons account for the prominence of pyridoxal enzymes in studies with this type of inactivator.

1. Pyridoxal enzymes are involved in transformations of amino acids. A large variety of acetylenic, olefinic, and halide containing amino acids have previously been synthesized chemically and/or have been isolated from natural sources. Synthetic routes to new amino acid derivatives with potentially interesting properties can usually be found.

2. Pyridoxal enzymes catalyze reactions in which the abstraction of a proton is stabilized by conjugation of the resulting carbanion with the pyridinium ring system of the coenzyme. This enables the investigator to place the reactive functional group (acetylene, olefin, or halide) adjacent to the site of proton abstraction, resulting in enzyme catlyzed rearrangement of the substrate analogue to the reactive species which induces alkylation.

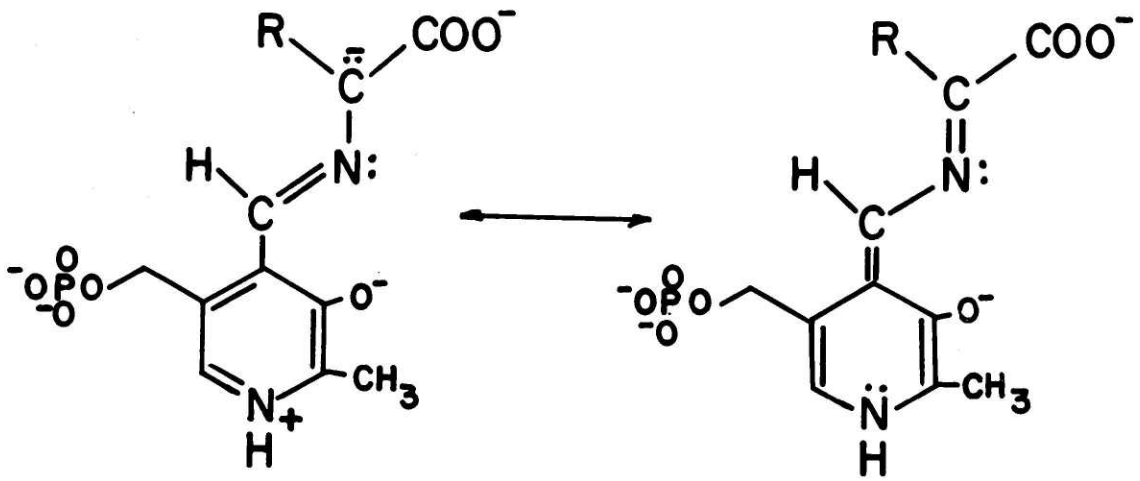
The pyridoxal phosphate dependent enzymes are divided into two general groups. The enzymes catalyzing racemization of amino acids, transamination between amino acids and keto acids, α-decarboxylation, and β-elimination reactions are members of the first group; the catalysis of these reactions requires enzymatic stabilization of carbanions formed only at the α-carbon of the amino acid substrate. The second group of pyridoxal enzymes catalyzes reactions which require stabilization of carbanions formed at both the

α-carbon and at the β-carbon. Enzymes which carry out β-decarboxylations and γ-replacement and γ-elimination reactions belong to this group. An outline of the steps involved in the catalysis of these transformations is provided in any general biochemistry text;[62] the mechanism by which the enzyme uses the pyridoxal coenzyme to provide the necessary stabilization is illustrated in Scheme I.

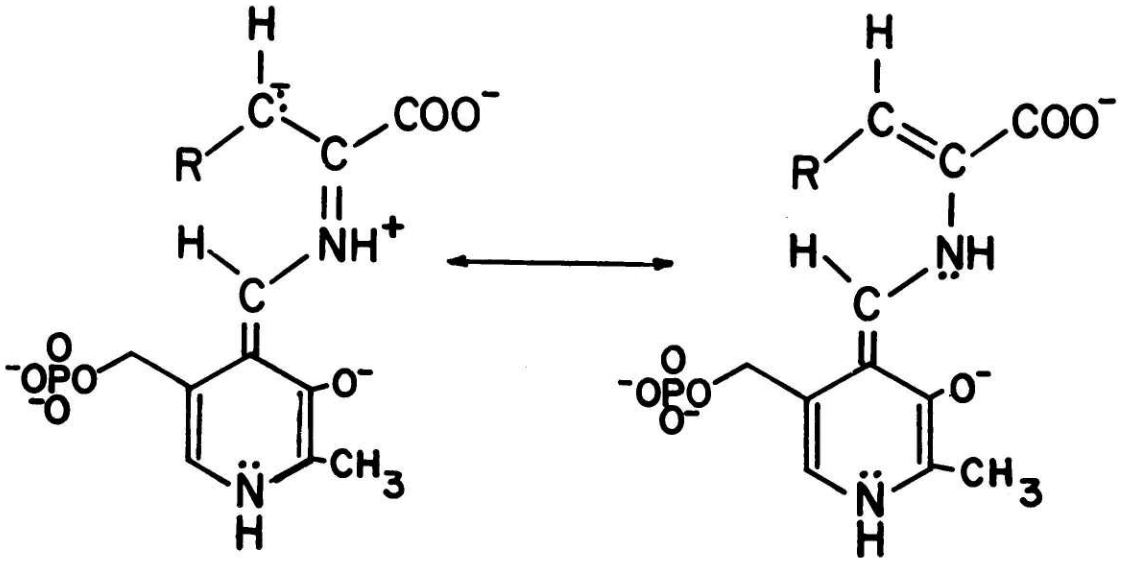
It would be expected that enzymes of the first group would be susceptible to inactivation by amino acids which contain β-halides or β-γ unsaturations. In addition to these potential inactivators, enzymes of the second group might be susceptible to substrate analogues which contain γ-δ unsaturations. It has also been found that (at least) one transaminase, in an activity (superficially) unrelated to its physiological reaction, can catalyze removal of the β-hydrogen of its substrate.[63] The occurrence of this activity renders this transaminase also potentially susceptible to substrate analogues containing γ-δ unsaturations.

A number of reviews have covered the suicide substrates that have been used to inactivate pyridoxal dependent enzymes.[3,4] Therefore, their mention in this Introduction is limited to the listing in Table I. Mechanistic aspects of several of these inactivations will be discussed in a later section.

SCHEME I



STABILIZED ALPHA CARBANION



STABILIZED BETA CARBANION

TABLE I

Pyridoxal enzymes found to be inactivated by "suicide substrates"

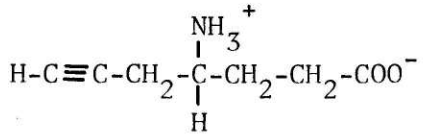
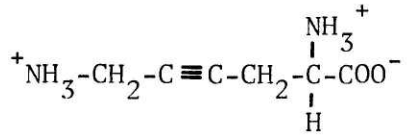
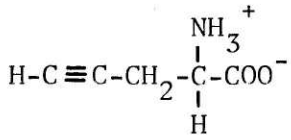
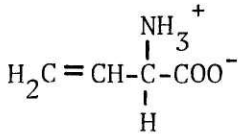
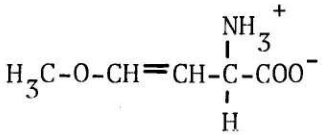
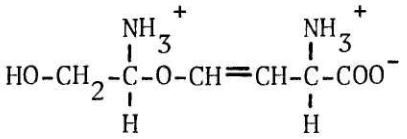
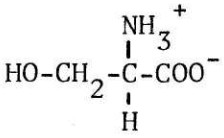
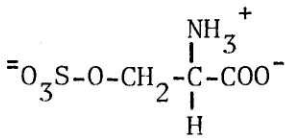
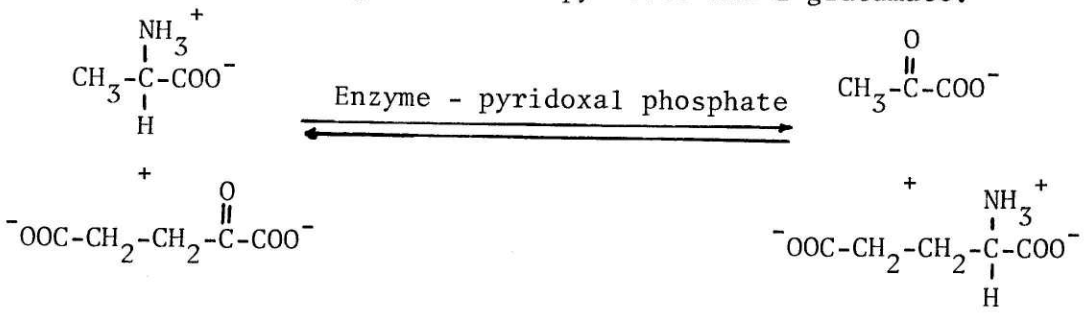
substrate analogue	enzyme	reference
	γ-aminobutyrate transaminase	64
	lysine ε-transaminase	65
	cystathioninie γ-synthase γ-cystathionase L-alanine aminotransferase L-aspartate aminotransferase	49 48 49 50
	L-aspartate aminotransferase D-amino acid transaminase	11 57
	L-aspartate aminotransferase	66
	β-cystathionase	67
	threonine deaminase	68
	L-aspartate aminotransferase	69

TABLE I (continued)

substrate analogue	enzyme	reference
$^{=}O_3S\text{-}O\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$	γ-aminobutyrate transaminase	74
$Cl\text{-}CH_2\text{-}\underset{H}{\overset{NH_3^+}{C}}\text{-}COO^-$	D-amino acid transaminase	70
$X\text{-}CH_2\text{-}\underset{H}{\overset{NH_3^+}{C}}\text{-}COO^-$ X = Cl, F	L-alanine amintransferase L-aspartate aminotransferase alanine racemase	73 71, 72 75
$F_3C\text{-}\underset{H}{\overset{NH_3^+}{C}}\text{-}COO^-$	β-cystathionase γ-cystathionase tryptophanase tryptophan synthase threonine dehydrase	73 73 73 73 73
$Cl_2CH\text{-}\underset{H}{\overset{NH_3^+}{C}}\text{-}COO^-$	γ-cystathionase tryptophanase tryptophan synthase threonine dehydrase	73 73 73 73

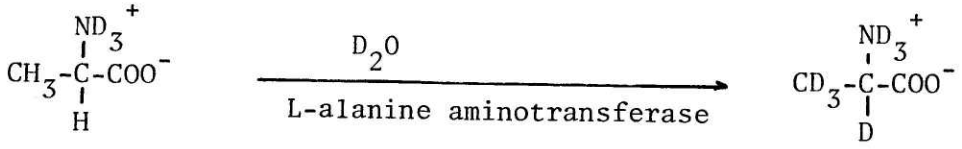
A. L-ALANINE AMINOTRANSFERASE: REACTIONS WITH PROPARGYLGLYCINE AND VINYLGLYCINE

L-alanine aminotransferase is a cytoplasmic, pyridoxal phosphate dependent, transaminase most commonly isolated from pig heart muscle.[76] It has high specificity in the reaction it catalyzes: the conversion of L-alanine and α-ketoglutarate to pyruvate and L-glutamate.

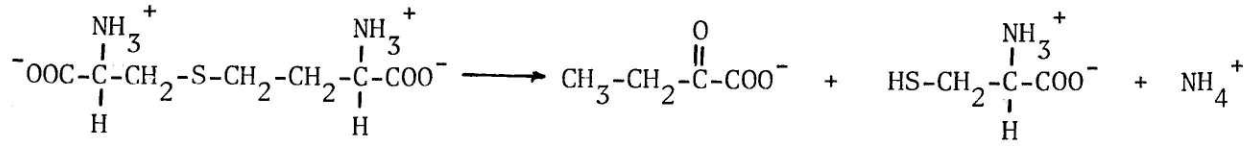


The enzyme has been shown to catalyze the transamination of other L-amino acid and α-keto acid substrates, but the reaction procedes at 1% or less the rate of the physiological substrates.[76]

Oshima and Tamiya were the first to observe the exchange of the β-hydrogens of L-alanine catalyzed by L-alanine aminotransferase.[63] Although the



validity of their experiments was at first doubted,[77] the exchange has recently been confirmed by several laboratories.[78,79] This non-essential property of the enzyme was the rationale for its potential susceptibility to L-propargylglycine, a γ-δ unsaturated amino acid which had previously been found to cause the inactivation of γ-cystathionase,[48] a (rat) liver pyridoxal dependent enzyme



(catalyzing γ-elimination) in which abstraction of the substrate β-hydrogens is a necessary feature of its catalysis.

Vinylglycine had been reported to lead to inactivation of another mammalian transaminase, L-aspartate aminotransferase,[11] which is also most commonly isolated from pig heart muscle.[80] Therefore, vinylglycine was also examined as a substrate and potential inactivator of L-alanine amino-transferase.

EXPERIMENTAL SECTION

Materials

Enzymes: L-alanine aminotransferase was purified to homogeneity (by gel electrophoresis) by the method of Saier and Jenkins[76] from pig hearts purchased from Pel-Freez biologicals. L-lactic dehydrogenase, pyruvate kinase, creatine kinase, furmarase , and phosphorylase A were purchased from Sigma.

Reagents: Propargylglycine was synthesized from diethylacetamidomalonate and propargylbromide as described by Jansen et al.[45] DL-propargyl[2-^{14}C]-glycine (500 cpm/nmole) was synthesized from diethylacetamidomalonate purchased from Amersham. DL-vinylglycine was synthesized by the method of R. Rando;[11] a detailed procedure was provided by Professor Rando.[55] L-vinylglycine, $[\alpha]_D$ +110°, was prepared by treatment of N-chloroacetyl-DL-vinylglycine with hog kidney acylase I in a manner analogous to that described for the resolution of L-alanine.[81] NADH, L-alanine, and α-keto-glutarate were purchased from Sigma. All other reagents were commercially available reagent grade materials.

Instrumentation: Spectrophotometric kinetics were followed by using a Gilford model 222 spectrophotometer. Electronic absorption spectra were recorded using a Beckman model 25 double beam spectrophotometer.

Methods

Enzyme activity: L-alanine aminotransferase was routinely assayed in 1.0 ml of 0.1 M Tris HCl pH 8 at 37°C, containing 0.2 M L-alanine, 0.01 M α-ketoglutarate, 0.2 mM NADH and 50 μg L-lactic dehydrogenase. The rate of

pyruvate formation was measured by the rate of decrease in the absorbance due to NADH at 340 nm (ε 6220).

Spectral changes upon inactivation with propargylglycine. Because of the pH dependence of the visible spectrum of L-alanine aminotransferase,[76] the spectral changes which accompany inactivation with propargylglycine were observed in 0.1 M potassium phosphate pH 6.5, at which pH the native enzyme has a clearly defined absorbance maximum at 425 nm. To the enzyme solution was added 1 mM α-ketoglutarate and 12.5 mM L-propargylglycine. The spectrum of the solution was recorded at intervals over a two hour incubation, at which time less than 3% of the initial activity remained. The inactivated enzyme was dialyzed against 0.1 M Tris HCl pH 8, containing 10^{-4} M pyridoxal phosphate, followed by dialysis against 0.1 M potassium phosphate pH 6.5. After dialysis, the spectrum of the solution was recorded; 1 mM α-ketoglutarate was added to the solution and the spectrum again recorded.

Stoichiometry of alkylation of L-alanine aminotransferase by L-propargylglycine. The enzyme was incubated with DL-propargyl[2-^{14}C]-glycine, followed by passage of the solution through a column of Sephadex G-25 as described in the legend to Figure 4.

Reaction of L-alanine aminotransferase with L-vinylglycine. L-vinylglycine was examined as a substrate and inhibitor of enzyme catalyzed transamination of L-alanine. Experimental details are given in the legend to Figure 5.

RESULTS

Kinetics of inactivation of L-alanine aminotransferase with L-propargylglycine.

Upon incubation of L-alanine aminotransferase with L-propargylglycine, rapid loss of activity is observed. The inactivation appears to be first order early in the reaction but deviations from the simple model are evident as the reaction progresses. Essentially complete (99%) inactivation requires approximately 2 hours of incubation. Exhaustive dialysis does not restore activity, nor does addition of pyridoxal phosphate to the incubation or dialysis prevent inactivation or restore activity. A K_m for L-propargylglycine of 12 mM can be derived from the data of Figure 1A; the maximal initial rate of inactivation is calculated to be 0.6 min^{-1}. It is the pyridoxal form of the enzyme which is subject to inactivation; addition of L-alanine to the incubation rapidly converts the enzyme to the pyridoxamine form, which is unsusceptible to inactivation by propargylglycine.

Large scale inactivation of L-alanine aminotransferase.

In contrast to the findings with catalytic quantities of enzyme, when a spectroscopic concentration of L-alanine aminotransferase (1 mg in 1 ml) was incubated with L-propargylglycine, only 50% of the activity was rapidly lost (assayed with L-alanine and α-ketoglutarate) -- the remainder of the activity was stable for several further hours of incubation. Addition of α-ketoglutarate to the mixture resulted in the remainder of the activity being rapidly lost. The most probable explanation for this occurrence is that the enzyme partitions approximately equally between covalent

Figure 1. Kinetics of inactivation of L-alanine aminotransferase with L-propargylglycine.

In 0.20 ml of 0.1 M Tris HCl, pH 8 at 37°C, the solution contained 10 mM α-ketoglutarate and the desired concentration of L-propargylglycine. The reaction was initiated by addition of L-alanine aminotransferase. At intervals, 10 µl was removed and injected into 1 ml of 0.1 M Tris HCl, pH 8 at 37°C containing 0.2 M L-alanine, 0.01 M α-ketoglutarate, 0.2 mM NADH and 50 µg L-lactic dehydrogenase. The transaminase activity was measured by the rate of decrease in absorbance at 340 nm.

A: Time dependent inactivation of L-alanine aminotransferase at the following concentrations of L-propargylglycine.

(a): 2 mM L-propargylglycine

(b): 3 mM L-propargylglycine

(c): 5 mM L-propargylglycine

(d): 10 mM L-propargylglycine

B: Plot of reciprocal of the initial rate constant on inactivation versus reciprocal of propargylglycine concentration.

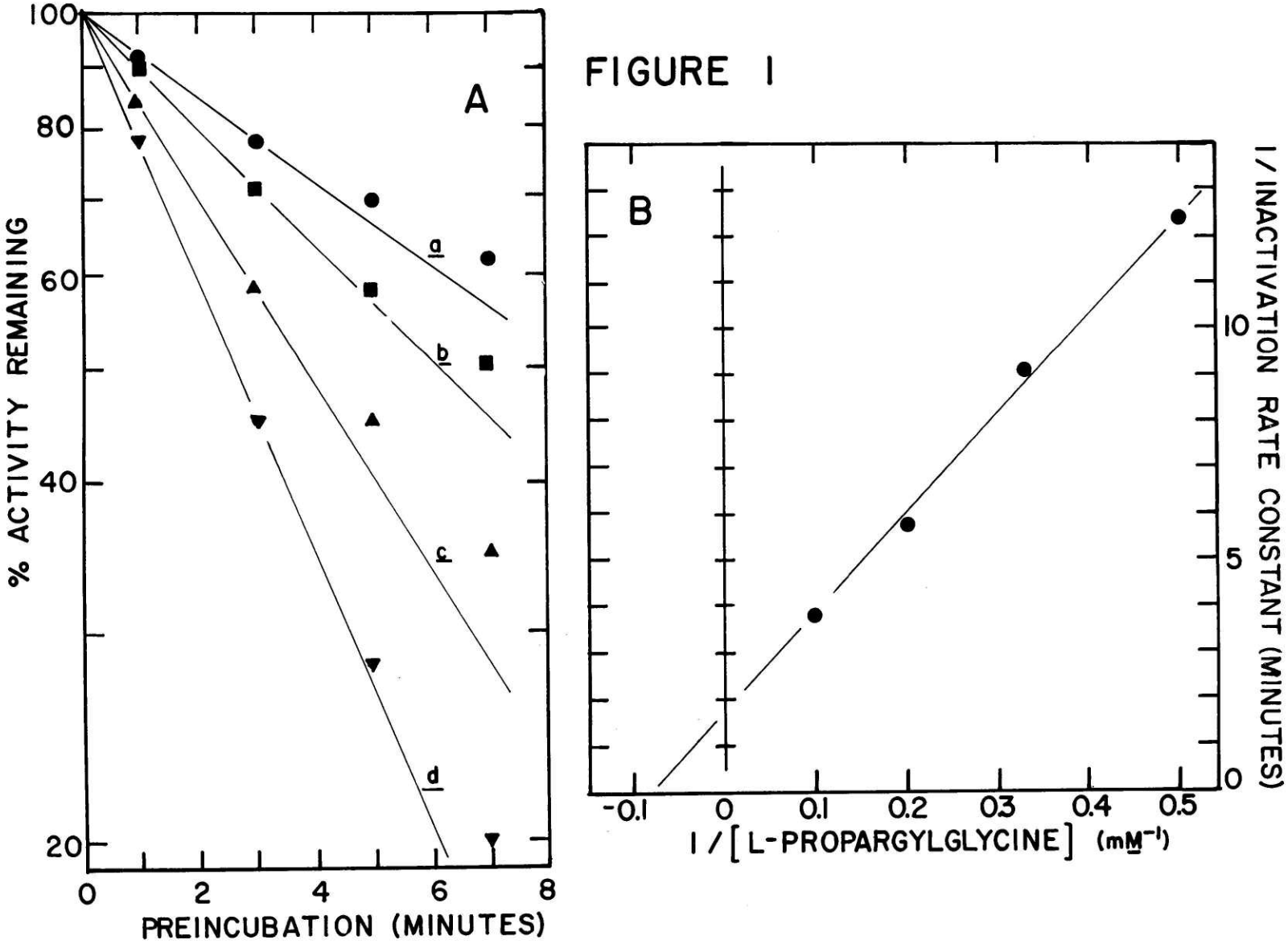

A
% ACTIVITY REMAINING
100
80
60
40
20
0
2
4
6
8
PREINCUBATION (MINUTES)
a
b
c
d
B
1/INACTIVATION RATE CONSTANT (MINUTES)
10
5
0
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[L-PROPARGYLGLYCINE] (mM⁻¹)

FIGURE 1

modification and release of the oxidized product. The enzyme is reduced to the pyridoxamine form by the latter reaction and is incapable of reacting with another molecule of the suicide substrate. Addition of α-ketoglutarate results in reoxidation of the enzyme, which can then react with another propargylglycine molecule. The requirement for added α-keto acid was not observed in catalytic assays; it is probable that sufficient keto acid is present as a trace contaminant of other reagents to return the enzyme to the pyridoxal form.

The electronic absorption spectrum of L-alanine aminotransferase was determined before and after inactivation with L-propargylglycine. As demonstrated in Figure 2, inactivation results in the loss of the long wavelength pyridoxal absorbance and the formation of a new absorbance maximum at 325 nm. Even though there is excess α-ketoglutarate in the incubation, the enzyme remains in the pyridoxamine form after inactivation. After dialysis, the enzyme retains the 325 nm absorbance; addition of α-ketoglutarate to the dialyzed enzyme does not restore the pyridoxal spectrum. Nor does pyridoxal phosphate become bound to the inactivated enzyme when it is added to the dialysis buffer.

L-alanine aminotransferase has thus been shown to be irreversibly converted to the pyridoxamine form upon inactivation by propargylglycine. Inactivation occurs once in every two reactions of enzyme with propargylglycine. Further work is necessary to determine the reasons for the deviations of the kinetics from the simple model. Possible explanations are inhomogeneity in the enzyme preparation (although it was substantially pure by gel electrophoresis) or the existence of a more complicated reaction pathway in

Figure 2. Changes in the spectrum of L-alanine aminotransferase upon reaction with L-propargylglycine.

(a): Spectrum of L-alanine aminotransferase in 0.4 ml of 0.1 M potassium phosphate, pH 6.5 containing 0.001 M α-ketoglutarate. The reference cell contained 0.1 M potassium phosphate with 0.001 M α-ketoglutrate.

(b): Two hours after addition of 10 µl 0.5 M L-propargylglycine to solution in (a).

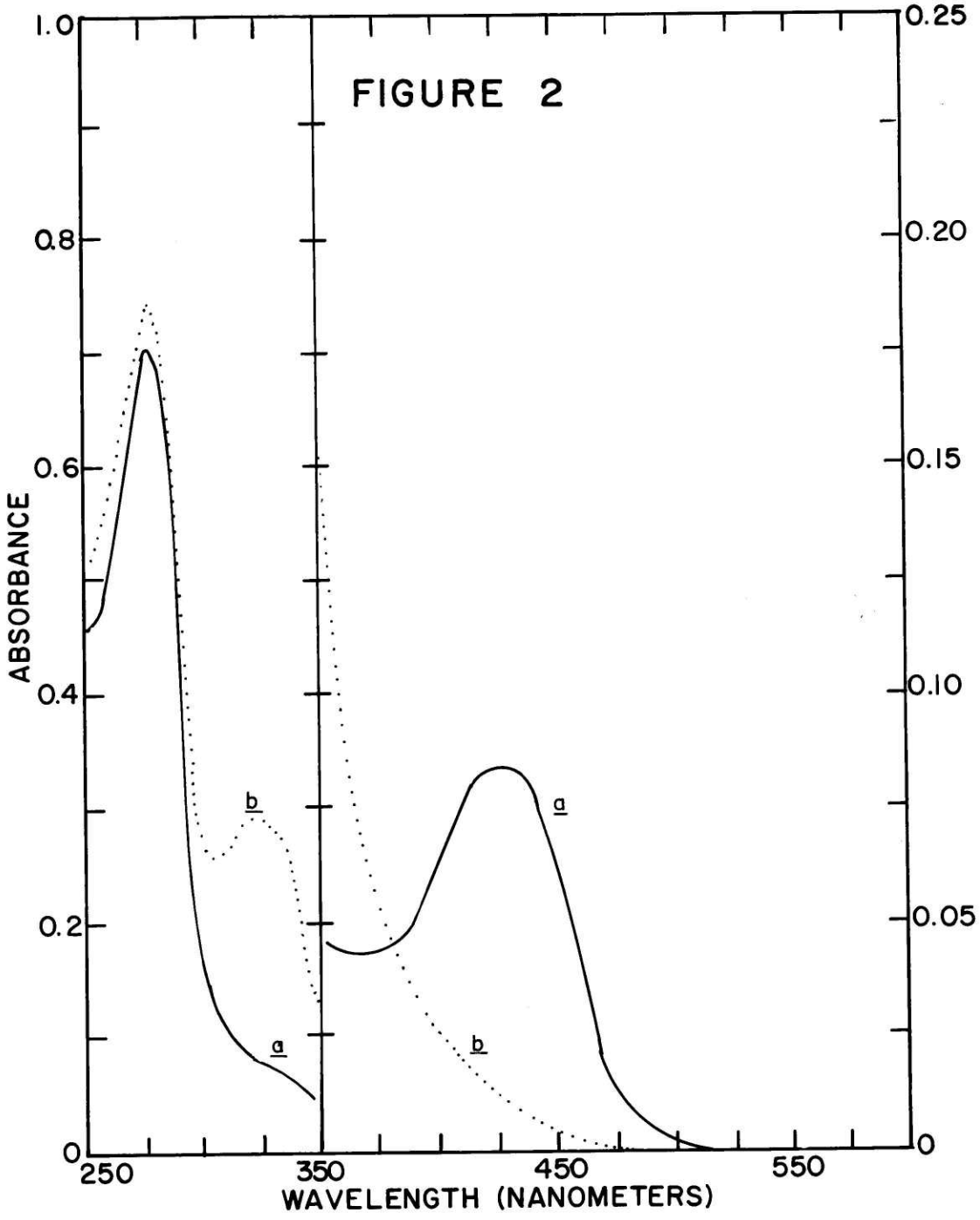


FIGURE 2
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
0.25
0.20
0.15
0.10
0.05
0
250
350
450
550
WAVELENGTH (NANOMETERS)
a
b
a
b

which a slow, but reversible, process is superimposed on the irreversible alkylation. This could be caused by the slow release of the product in those instances when irreversible alkylation does not take place.

Molecular weight and stoichiometry of alkylation of L-alanine aminotransferase.

The information in the literature on the physical characterization of L-alanine aminotransferase is inconsistent. The total molecular weight was determined to be 110,000 by ultracentrifugation,[76] but the same publication reported the pyridoxal phosphate content of the enzyme to be one equivalent per 75,000 - 85,000 daltons.[76] Therefore, data derived from studies on the stoichiometry of alkylation with propargylglycine would be of questionable value without more certain knowledge of the properties of the enzyme.

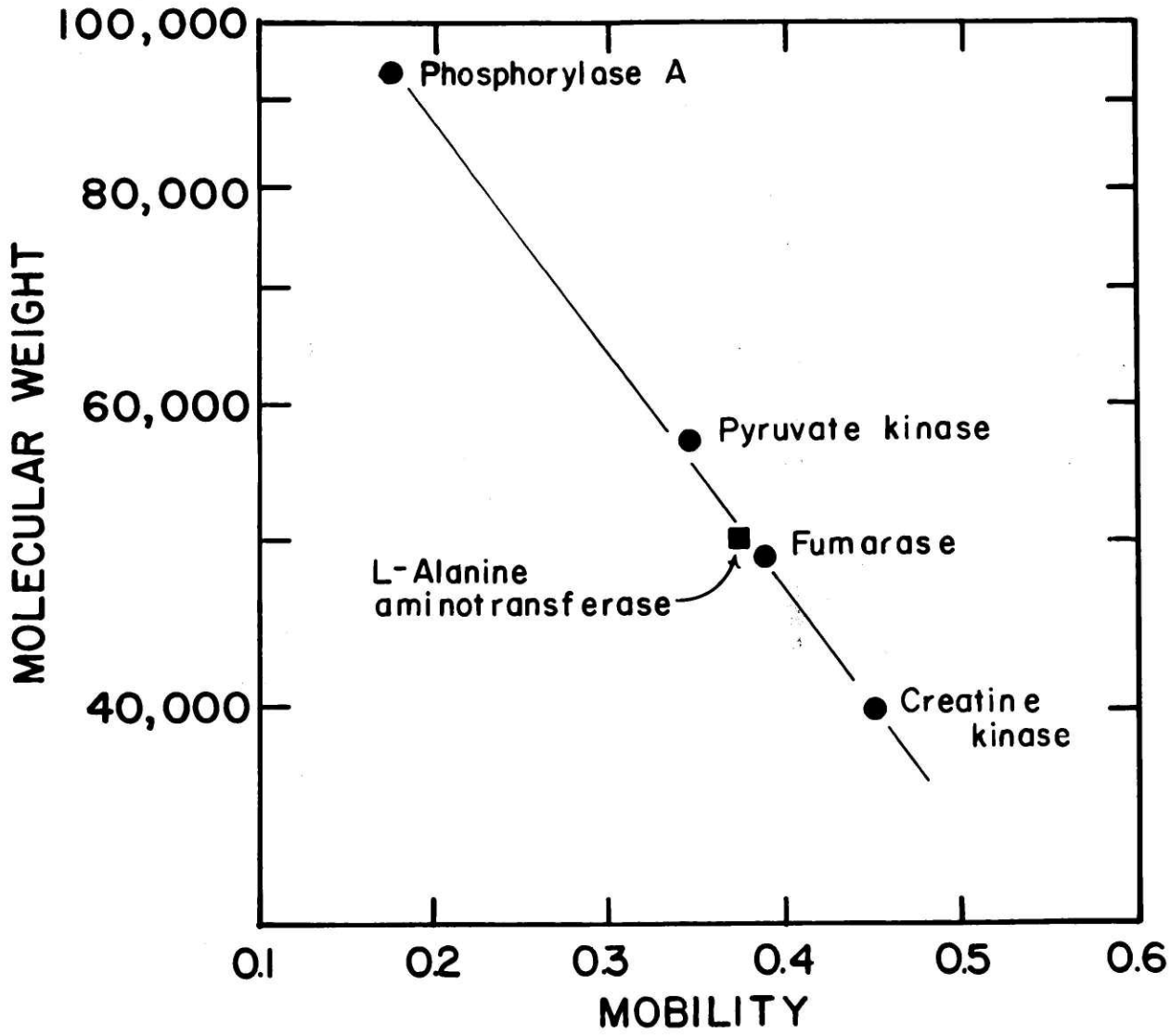
An examination of the subunit composition by means of electrophoresis of protein denatured in sodium dodecylsulfate (the method of Weber and Osborn[82]) revealed one band of molecular weight 50,000 (Figure 3). Using the phenylhydrazone assay of Wada and Snell,[83] the pyridoxal-phosphate content of the preparation used in this work was found to be one equivalent per 95,000 - 100,000 daltons.

Since inactivation of the enzyme had been found to result in total loss of pyridoxal absorbance, it was of interest to determine whether one or more equivalents of propargylglycine per enzyme are incorporated. As shown in Figure 4, it was found that one equivalent of radioactivity is incorporated per 100,000 daltons. Therefore, modification is stoichiometric

Figure 3. Molecular weight of L-alanine aminotransferase as determined by gel electophoresis of protein denatured in sodium dodecyl sulfate.

Figure 4. Elution of Sephadex G-25 column of L-alanine aminotransferase inactivated with propargyl[2-^{14}C]glycine.
In 0.5 ml of 0.1 M Tris HCl, pH 8, at 37°C, 5 mg of L-alanine aminotransferase was incubated with 4 mM α-ketoglutarate and 5 mM DL-propargyl[2-^{14}C]glycine (500 cpm/nmole) for two hours. The solution was layered on a 1.2 x 50 cm column of Sephadex G-25. 1.5 ml fractions were collected. Tubes containing protein were found by their absorbance at 280 nm; a qualitative determination of the protein concentration was then done by the method of Lowry.[84] An aliquot of each tube was injected into scintillation fluid to determine the radioactivity associated with the protein.

FIGURE 3


100,000
80,000
60,000
40,000
MOLECULAR WEIGHT
Phosphorylase A
Pyruvate kinase
Fumarase
L-Alanine
aminotransferase
Creatine
kinase
0.1
0.2
0.3
0.4
0.5
0.6
MOBILITY

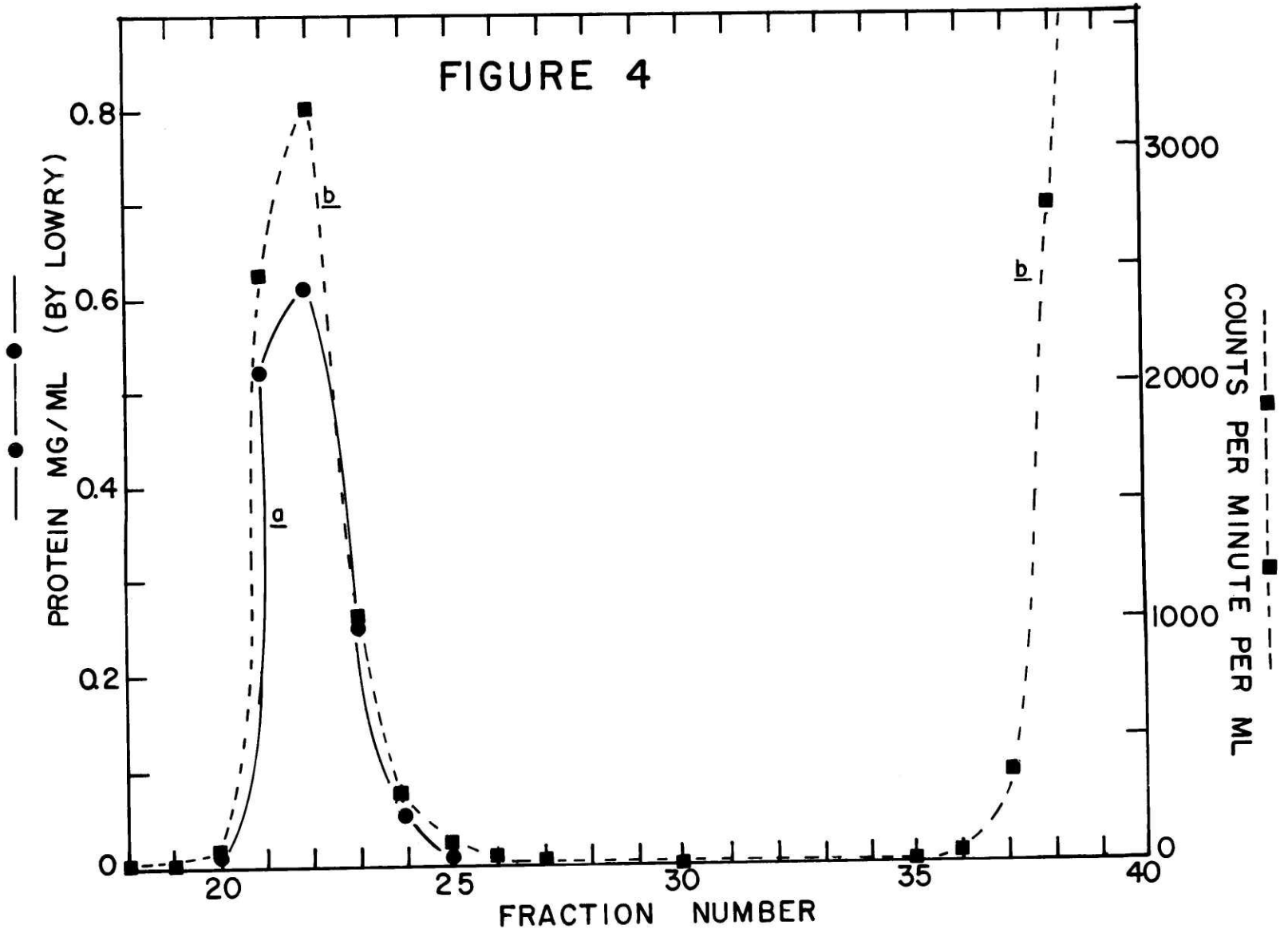


FIGURE 4
PROTEIN MG/ML (BY LOWRY)
COUNTS PER MINUTE PER ML
FRACTION NUMBER
0
0.2
0.4
0.6
0.8
1000
2000
3000
20
25
30
35
40
a
b
b
b

with the coenzyme and there appears to exist one active site per two subunits of the same molecular weight.

It must be pointed out that the evidence presented on the pyridoxal content and stoichiometry of alkylation is dependent on determinations of the protein concentration by the method of Lowry et al.[84] A dry weight analysis was not performed to determine the possible error in these assays. This fact, together with possible inhomogeneity in the enzyme preparation, casts some doubt on the validity of these experiments; they should be repeated with a more carefully characterized enzyme preparation.

Reaction of L-alanine aminotransferase with vinylglycine.

In the first use of this olefinic amino acid with an enzyme, Rando determined that vinylglycine rapidly causes the inactivation of L-aspartate aminotransferase from pig heart.[11] Therefore, its effect on L-alanine aminotransferase was studied.

No inactivation was observed over a 90 minute incubation with 30 mM DL-vinylglycine. The addition of α-ketoglutarate to incubations did not lead to inactivation. This failure could be due to one of two causes: the complete inertness of the amino acid towards transamination or the lack of susceptibility of the enzyme towards alkylation by the conjugated olefin at the active site. Therefore, vinylglycine was examined as a substrate for the transaminase.

In the absence of a specific assay for the vinylglycine transamination product, 2-keto-3-butenoate, the rate of transamination was determined by the concomitant conversion of α-keto[^{14}C] glutarate to L-[^{14}C]glutamate.

The radioactive amino acid can be easily quantitated by its selective adsorption onto DOWEX 50 H^+, from which it can be removed with aqueous ammonia. As shown in Figure 5A, linear production of L-glutamate over 10 minutes is observed, demonstrating that L-alanine aminotransferase utilizes L-vinylglycine as a transamination substrate at 0.3% the rate of L-alanine.

The data of Figure 5A also imply that the K_m of vinylglycine is much lower than that observed for L-alanine and L-α-aminobutyrate (approximately 12 mM for both amino acids). Therefore, vinylglycine was examined as a competitive inhibitor of L-alanine transamination. As shown in Figure 5B, the K_i of L-vinylglycine as an inhibitor of L-alanine is 0.4 mM. The reasons for this very tight binding of vinylglycine to L-alanine aminotransferase have not been determined to date.

Figure 5. A: Reaction of L-alanine aminotransferase with L-vinylglycine. In 1.0 ml of 0.1 M Tris HCl, pH 8, at 37°C, the solution contained 2 mM α-keto[^{14}C]glutarate (500 cpm/nmole) and the desired concentration of L-vinylglycine. The reaction was started by addition of L-alanine aminotransferase (approximately 10 Units). At intervals, 0.1 ml of the incubation was removed and injected onto a 1 ml DOWEX 50 H^+ (400 mesh) column. The column was washed with 10 ml water; then the glutamate was eluted from the resin with 3.0 ml of 4N ammonia.

(a): the solution contained 2 mM L-vinylglycine.

(b): the solution contained 5 mM L-vinylglycine

(c): the solution contained 10 mM L-vinylglycine.

B: Competitive inhibition of L-alanine transamination by L-vinylglycine (Dixon plot).

In 1.0 ml of 0.1 M Tris HCl, pH 8, at 37°C, the solution contained 5 mM α-ketoglutarate, 0.2 mM NADH and 50 μg L-lactic dehydrogenase, and the indicated concentrations of L-alanine and L-vinylglycine. The reaction was initiated by addition of enzyme and the rate of pyruvate formation monitored by the rate of decrease in the absorbance at 340 nm.

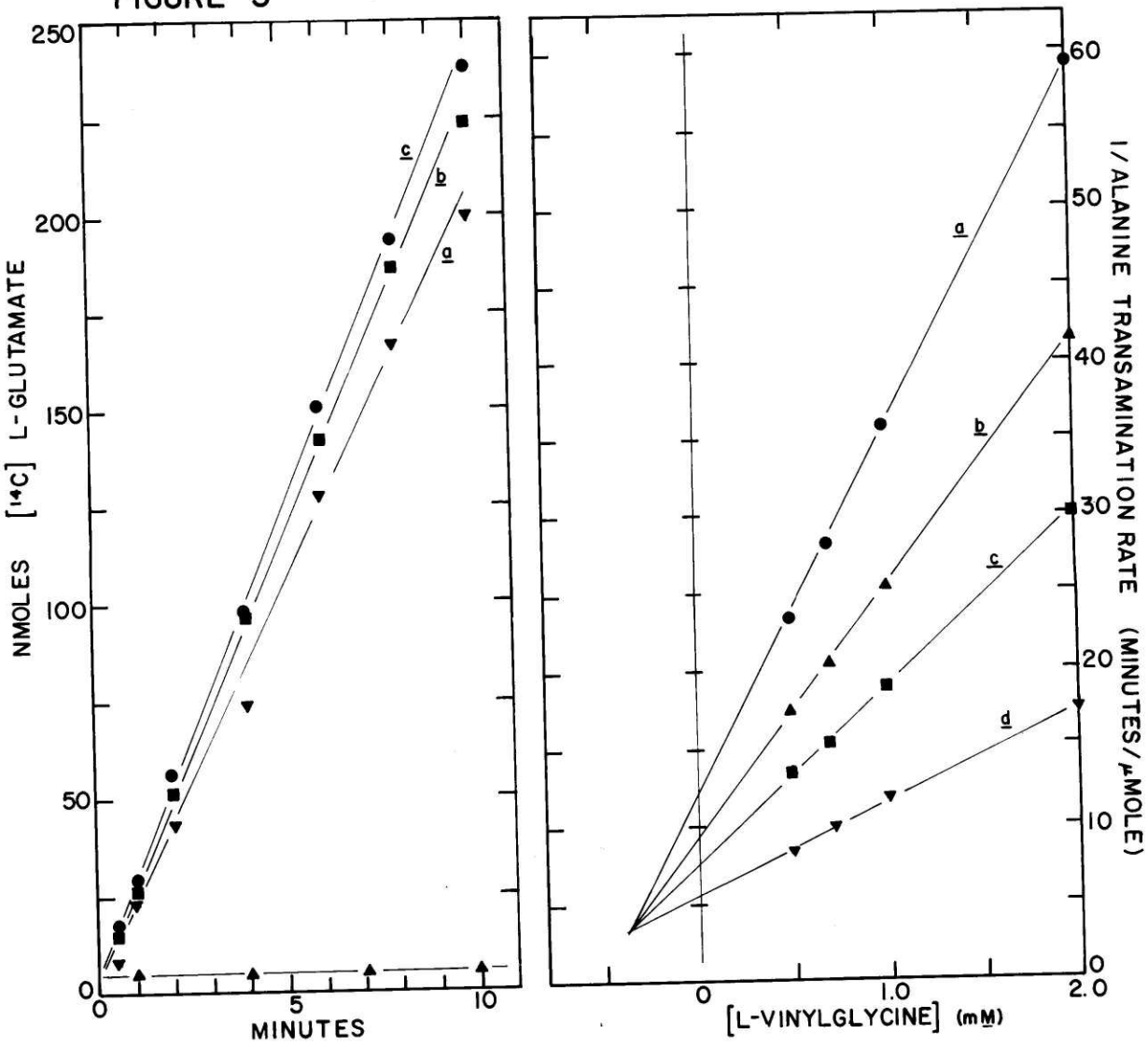
(a): 5 mM L-alanine

(b): 7 mM L-alanine

(c): 10 mM L-alanine

(d): 20 mM L-alanine

FIGURE 5

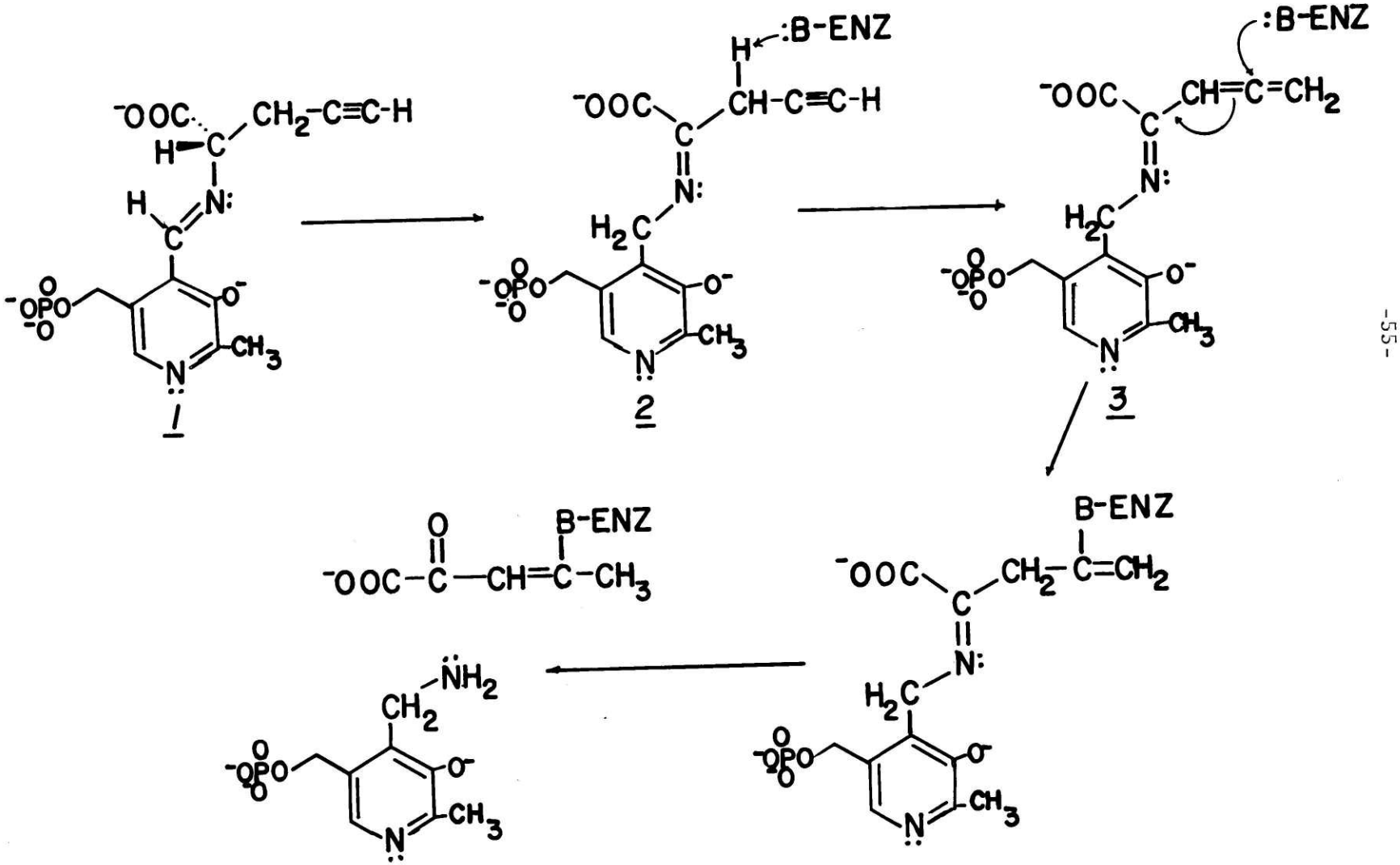

NMOLES [14C] L-GLUTAMATE
250
200
150
100
50
0
0
5
10
MINUTES
c
b
a
1/ALANINE TRANSAMINATION RATE (MINUTES/μMOLE)
60
50
40
30
20
10
0
0
1.0
2.0
[L-VINYLGLYCINE] (mM)
a
b
c
d

DISCUSSION

The inactivation of pyridoxal enzymes caused by propargylglycine has been proposed to occur through the enzyme catalyzed rearrangement (Scheme II) of the phosphopyridoxal linked acetylenic imine (2) to the conjugated allene (3), thus producing at the active site a species which is very susceptible to nucleophilic attack at carbon 4.[48] Propargylglycine was originally considered to be a highly selective enzyme inactivator as "only those pyridoxal dependent enzymes which abstract substrate β-hydrogens should catalyze their destruction by propargylglycine."[48] Therefore, this suicide substrate was first reacted with, and found to inactivate, γ-cystathionase, an enzyme which catalyzes a γ-elimination reaction.[48]

The hypothesis that propargylglycine would be a selective inactivator of the relatively few pyridoxal enzymes which labilize β-hydrogens as a necessary feature of their catalysis has been proven not to be correct, as it has been shown that (at least) one pyridoxal phosphate dependent transaminase, L-alanine aminotransferase, rapidly catalyzes the exchange of β-hydrogens with solvent D_2O. The transaminase catalyzed exchange of the methyl hydrogens of L-alanine was first (in 1959) observed by Oshima and Tamiya[63], who recovered tetradeutero L-alanine (identified by its infrared spectrum) from incubations of enzyme, L-alanine, and a catalytic amount of α-ketoglutarate. To account for their observations, they proposed a mechanism in which the β-hydrogen is removed <u>before</u> the α-hydrogen;[85] this was unfortunate because the proposal brought immediate ridicule[86] and led to acceptance that "it seems more likely that the observed exchange is due to the enolization

SCHEME II



of the pyruvate formed in the reversible transamination reaction."[77]

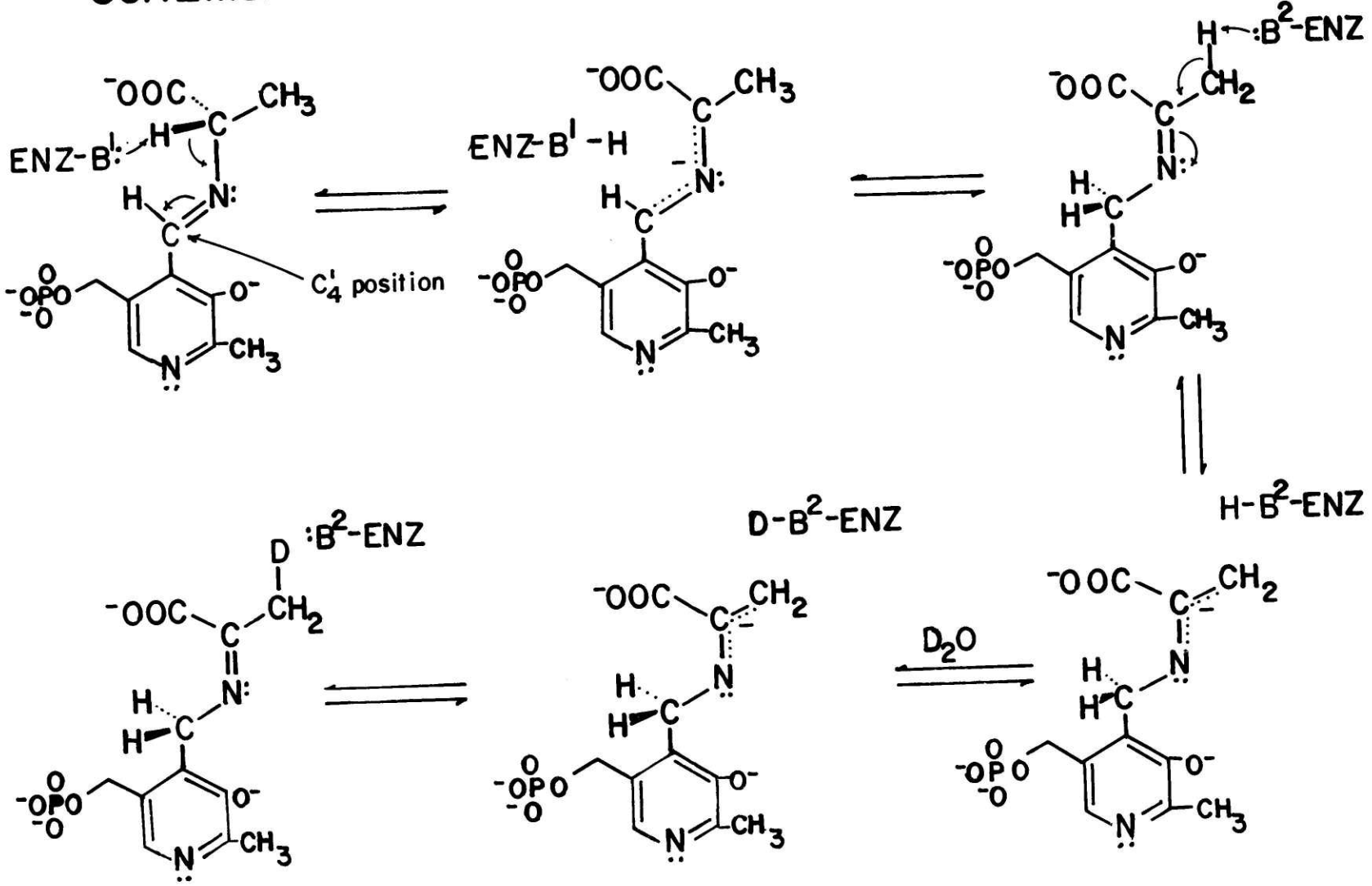
Oshima and Tamiya did not examine carefully the kinetics of the exchange and they did not report the appropriate control experiments to rule out the non-enzymatic reaction as the cause of their observations. However, in retrospect it is obvious that the rate of exchange implied by their data is far greater than can be accounted for by pyruvate enolization ($t_{1/2}$= 100 min at pH 8).[88] It was many years before the problem was properly reexamined.

In 1974, Babu and Johnston reported that the nuclear magnetic resonance signals of both the α and β hydrogens of L-alanine rapidly disappear upon incubation of L-alanine , L-alanine aminotransferase, and a catalytic quantity of pyruvate.[78] In the absence of added keto acid, the enzyme is wholly converted to the pyridoxamine form (only a stoichiometric amount of keto acid is produced) and further reaction with L-alanine cannot occur. The added pyruvate ensures that the equilibrium reaction will continue. Following the work of Babu and Johnston, three other groups[87 88] have independently examined the problem. The most complete work is that of Cooper,[79] who used pulsed fourier transform NMR to study the exchange reaction. The following is a summary of the evidence on the mechanism of the exchange reaction.

1. The exchange rate of the α-hydrogen is 30[79]- 37% the transamination rate: this implies a large degree of sequestration of the α-proton, probably at the C_4' position of the pyridoxal coenzyme. (Scheme III)

2. The rates of disappearance of the resonances of the α and β hydrogens are nearly identical;[79] therefore, the rate of exchange of each

SCHEME III



β-hydrogen is approximately three times that of the α hydrogen.

3. Intermediate species can be observed in the incubation which have ^{1}H at carbon 2 while ^{2}H at carbon 3 of L-alanine.[79] Since the β-hydrogen cannot be removed before the α , this evidence is corroborative of the sequestration of the α hydrogen at C_4' of the coenzyme.

4. It is possible that there could exist transfer of the β-hydrogen to the α-carbon. Attempts to demonstrate this phenomenon with 2-[^{2}H]-3-[^{1}H]-alanine were unsuccessful; no hydrogen was visibly incorporated into the α position.[79]

5. The exchange rates are independent of pH between 5 and 8.[87]

6. There is no detectable exchange of the β-hydrogens of L-glutamate under the same conditions.[79]

Whether there are one or more bases at the active site is a matter open to speculation. The lysine which is linked to the coenzyme in the resting enzyme has the required flexibility, but the polyprotic nature of the amino group would make likely transfer of hydrogens from the β to the α position. Also, since lysine has been found to be at the active site of all pyridoxal enzymes examined;[62] rapid β-hydrogen exchange might be expected to be a more general feature of pyridoxal catalysis. L-alanine aminotransferase is the only transaminase which has been reported to carry out such an exchange. Finally, the rate of exchange of a ε-amino group of a lysine has been estimated at 10 sec^{-1};[89] unless another base is present at the active site to facilitate exchange to the media, lysine is kinetically incompetent to ex-

the β-hydrogens at a rate approximately equal to the transamination rate ($1100\ sec^{-1}$).[76]

If there is a second base present at the active site, the imidazole moiety of a histidine residue is a possibility. The rate of exchange of histidine with solvent D_2O could be fast enough (10^3-$10^4\ sec^{-1}$) to account for the observed rate, but a significant perturbation of the normal pK_a of histidine (6.0) would be required to account for the pH independence of the rate of exchange. Perhaps the most attractive possibility is a carboxylate base (from glutamate or aspartate), since it would exchange very fast ($10^7\ sec^{-1}$)[89] and would have no pH sensitivity over the observed range.

Since the exchange reaction has been shown to proceed so rapidly, the only probable obstacle in the inactivation of this enzyme with propargylglycine is the severe substrate specificity of the enzyme. L-α-aminobutyrate is transaminated at 1% and L-norvaline at 0.2% the rate of L-alanine.[76] Propargylglycine causes rapid inactivation; because the enzyme catalyzes one catalytic transamination before suffering loss of activity, the rate of reaction of the suicide substrate and the enzyme can be estimated at twice the observed inactivation rate. Therefore, propargylglycine has been shown to react with the enzyme ($1.2\ min^{-1}$) at less that 0.01% the rate of L-alanine ($1100\ sec^{-1}$) but this does not prevent rapid inactivation of the enzyme.

The residue involved in the inactivation has not yet been identified; it will be of interest to determine whether the residue alkylated is the lysine involved in pyridoxal binding or a second base at the active site.

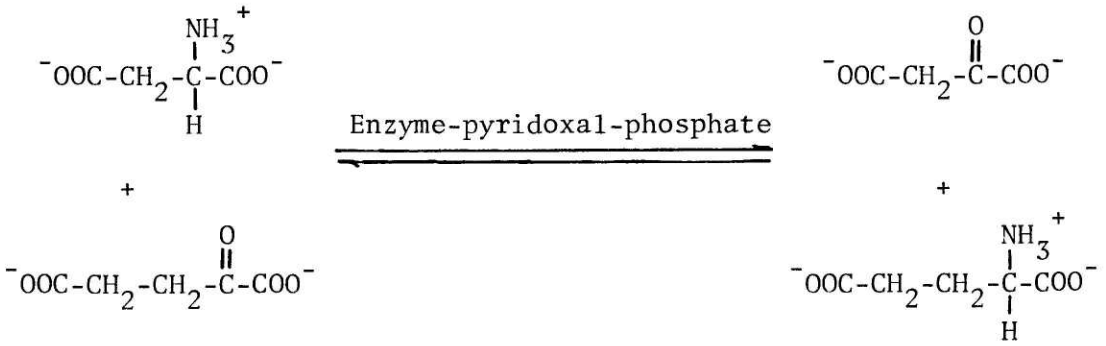
The fact that the enzyme remains in the pyridoxamine form upon inactivation implies that the enzyme has lost the ability to catalyze the transaldimination reaction after suffering alkylation. In contrast, γ-cystathionase has been found to be in the pyridoxal form after inactivation by propargylglycine[90]; therefore, that enzyme retains the capability to catalyze the transaldimination and release the amino acid. Washtein and Abeles were unable to isolate the alkylated amino acid from inactivated γ-cystathionase (the linkage is too rapidly hydolyzed under the isolation procedures); studies on the kinetic lability of the linkage led them to propose a cysteine or tyrosine residue as the susceptible nucleophile.[90]

The inactivation of L-alanine aminotransferase with L-propargylglycine occurs with incorporation of one equivalent of alkylating agent per bound pyridoxal phosphate coenzyme. The enzyme has been found to consist of two subunits of molecular weight 50,000 with only one pyridoxal for the two subunits. No other pyridoxal dependent enzyme has been shown to have a similar structure.

The other major transaminase of heart muscle, L-aspartate aminotransferase,[80] has also been examined as to its exchange of the β-hydrogens. Babu and Johnston reported that this enzyme "catalyzed similar exchanges of α and β-hydrogens of L-alanine but at a much slower rate" than L-alanine aminotransferase.[78] Cooper observed a similar loss of NMR signals but could not rule out the nonenzymatic reaction as the cause.[79] Cooper also reported no enzyme catalyzed exchange of the β-hydrogens of L-glutamate. In contrast, Walter et al[88] stated that radioactivity from 3-[^{3}H]-L-glutamate is released into the medium. However, this rate is slow (1.4% of the transamination

rate) and it is uncertain that nonenzymatic exchange was properly ruled out.

L-aspartate aminotransferase is highly specific in the reaction it catalyzes: the interconversion of the four and five carbon α-amino and α-keto dicarboxylic acids.[91]



L-alanine is a poor substrate (0.01%) and exhibits a very high K_m (>2M). Interestingly, addition of high concentrations of monocarboxylic anions greatly enhances the rate of alanine transamination; 3 N potassium formate causes a 200 fold increase in the rate.[71] As the size of the carboxylic anion is increased, its ability to enhance the reaction decreases; however, acetate, propionate, and butyrate all cause significant increases in the rate of enzyme catalyzed transamination of L-alanine.[92]

The mechanism of the enhancement of L-alanine transamination has been proposed to occur through the binding of the carboxylate anion at the binding site of the γ-carboxyl of L-glutamate. Space filling models have purportedly shown the similarity between the shape of glutamate and the combination of alanine and formate.[92] This simplistic rationale cannot be wholly correct, as the combination of alanine and the other anions is much larger than can be accomodated in a site suited for glutamate.

L-aspartate aminotransferase was found by Tanase and Morino to be

only slowly inactivated by 0.1 M L-propargylglycine ($t_{1/2}$ = 5 hrs).[50] The rate of inactivation was greatly increased by addition of 3 N potassium formate ($t_{1/2}$ = 5 min). Addition of keto acid is necessary for complete inactivation, implying a partition between catalytic turnover and inactivation. Tanase and Morino did not report a partition ratio, but an examination of their spectral data reveals no more than 5 turnovers before inactivation.[93] As with L-alanine aminotransferase, the enzyme remains in the pyridoxamine form after suffering inactivation. The identity of the alkylated residue has not yet been reported.

Inactivation of a pyridoxal enzyme by propargylglycine has been claimed to be "diagnostic of the catalytic capacity of that enzyme to form β-carbanionic species."[49] However, the methylene protons of the pyridoxal bound acetylenic imine (2 of Scheme II) are rendered much more acidic than the alanine protons; and examination of the reactions of this type of molecule will be presented in Chapter III. This enhanced reactivity of the β-hydrogens makes it possible that enzymes will be found to be susceptible to propargylglycine which, like L-aspartate aminotransferase, do not in any other way reveal their latent capacity to catalyze removal of the β-protons. Therefore, in the design of inactivator specific for a target enzyme, all latent activities, as well as those known to be necessary in catalysis, must be taken into account.

B. D-AMINO ACID TRANSAMINASE: REACTIONS WITH VINYLGLYCINE AND PROPARGYLGLYCINE.

One of the distinct properties of bacterial metabolism is the use of D-alanine and D-glutamate in the construction of bacterial cell walls. Thus, it is not surprising that bacteria contain enzymes specific for D-amino acids as well as the L-amino acids which constitute their proteins. Enzymes which synthesize D-amino acids by epimerization are known, such as the pyridoxal phosphate dependent enzyme alanine racemase,[94] as well as transaminases which utilize D-amino acids and the corresponding keto acids.

The D-amino acid transaminases isolated from species of Bacillus have been found[95,96] to be relatively non-specific in their action of D-amino acids and keto acids; therefore, it would be expected that vinylglycine and propargylglycine would serve as good substrates, as well as potential inactivators. The following section of this paper reports on the reactions of these amino acids with the D-amino acid transaminases.

The preparation of the D-amino acid transaminases from Bacillus subtilis and Bacillus sphaericus was performed in the laboratory of Professor James Manning at Rockefeller University. The experiments on the B. subtilis enzyme were done by the collaborators in New York and are included for comparison with the results of studies with the B. sphaericus enzyme done as a part of this work.

EXPERIMENTAL SECTION

Enzymes and Reagents: D-amino acid transaminase from Bacillus subtilis 168 and Bacillus sphaericus ATCC 14577 were prepared in the laboratory of Professor Manning using modifications[70] of published procedures.[95,96] L-aspartate aminotransferase, lactic dehydrogenase, NADH, α-amino acids and α-keto acids were purchased from Sigma. DL-vinylglycine was prepared by the method of Rando.[11] All other chemicals were commercial available reagent grade materials.

Methods: D-amino acid transaminase activity was observed by its transamination of D-alanine and α-ketoglutarate at pH 8.5, in the presence of NADH and lactic dehydrogenase. The rate of pyruvate formation was measured by the rate of decrease in the absorbance of NADH at 340 nm (ε 6220). Experimental details of the work on the inactivation of the D-amino acid transaminases with vinylglycine is provided in the legends of Figures 6-10.

RESULTS

Inactivation of Bacillus subtilis and Bacillus sphaericus D-amino acid transaminases by vinylglycine.

Upon incubation of the D-amino acid transaminases from B. subtilis and B. sphaericus with vinylglycine, no loss of activity can be observed. Addition of α-keto acid to an incubation of the enzyme and vinylglycine results in the rapid loss of transaminase activity.

The kinetics of inactivation of the B. subtilis and B. sphaericus enzymes are presented in Figures 6A and 7A. Both inactivation processes are first order in the early part of the reaction, but both show deviations from simple kinetics as the reaction procedes towards completion. The K_m's of DL-vinylglycine derived from Figures 6B and 7B are 2 mM for both enzymes but the maximal rate of inactivation observed for the B. sphaericus enzyme (1.9 min^{-1}) is five fold higher than that observed for the B. subtilis enzyme (0.36 min^{-1}). The K_m of DL-vinylglycine is approximately the same concentration as the K_m of D-alanine with these enzymes,[96] consistent with vinylglycine being recognized by the enzyme as a good substrate.

Direct analysis of vinylglycine transamination.

Because no inactivation is observed upon incubation of vinylglycine and the D-amino acid transaminases in the absence of added α-keto acid it was considered probable that the alkylation process occurs only rarely in the reaction of vinylglycine and enzyme; i.e., for the most part the reaction results in catalytic transamination as with any other amino acid

Figure 6. A: Time dependent inactivation of Bacillus sphaericus D-amino acid transaminase by DL-vinylglycine.

In 1.0 ml of 0.05 M potassium pyrophosphate, pH 8.5, the solution contained at 25°C:

10 mM α-ketoglutarate,

desired concentration of DL-vinylglycine,

200 μM NADH, and 50 μg L-lactic dehydrogenase.

The reaction was initiated by addition of 1.3 μg D-amino acid transaminase. After the desired interval of incubation, the remaining enzyme activity was measured by addition of 25 μl of 1 M D-alanine to the solution. The rate of alanine transamination was obtained from the rate of decrease in the absorbance of NADH at 340 nm.

(a): 0.5 mM DL-vinylglycine

(b): 1.0 mM DL-vinylglycine

(c): 2.0 mM DL-vinylglycine

(d): 5.0 mM DL-vinylglycine

B: A plot of the reciprocal of the rate constant for inactivation from A versus the reciprocal of DL-vinylglycine concentration.

FIGURE 6

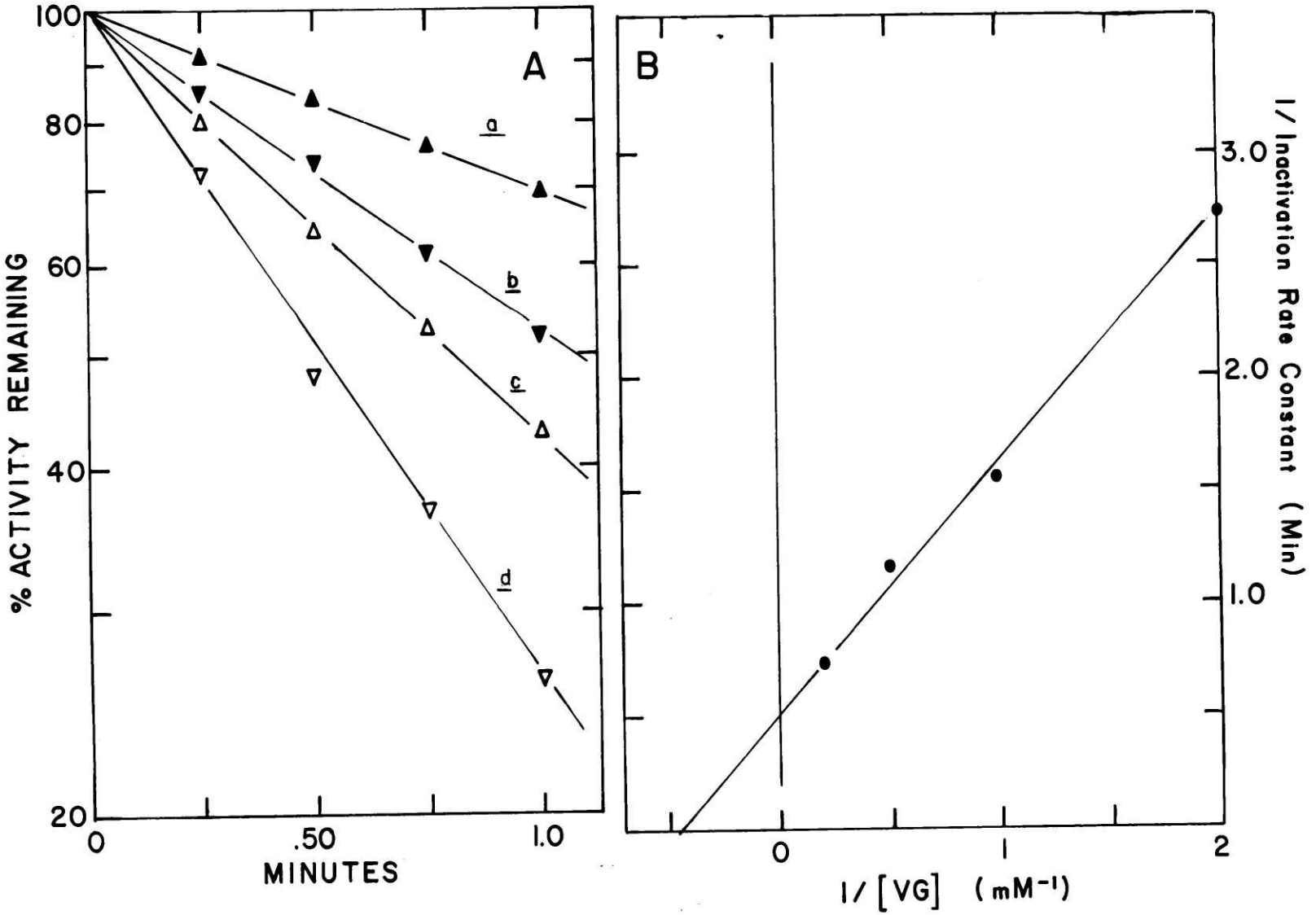

A
B
a
b
c
d
100
80
60
40
20
% ACTIVITY REMAINING
0
.50
1.0
MINUTES
3.0
2.0
1.0
1/ Inactivation Rate Constant (Min)
0
1
2
1/ [VG] (mM⁻¹)

Figure 7. A: Time dependent inactivation of Bacillus subtilis D-amino acid transaminase by DL-vinylglycine.

In 3.0 ml of 0.33 M potassium phosphate, pH 8.5, at 25°C, the solution contained 1.0 mM DL-vinylglycine.

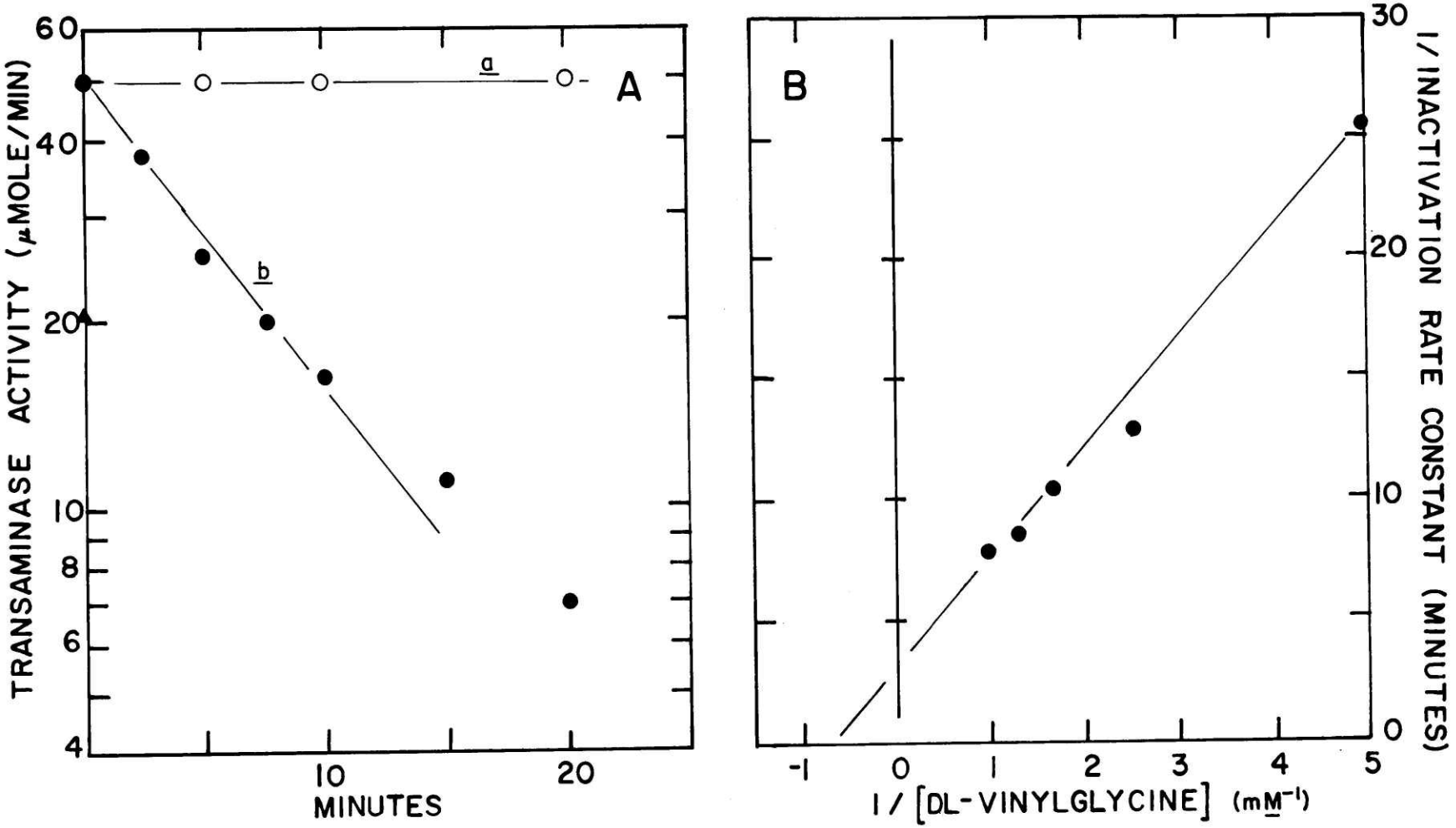
The reaction was initiated by addition of 60 µg D-amino acid transaminase. After the desired interval of incubation, D-alanine was added to the incubation (final concentration 25 mM) and the transaminase activity determined with the NADH / L-lactic dehydrogenase coupled assay of pyruvate.

(a): no added α-keto acid

(b): with 25 mM α-ketoglutarate.

B: Plot of the reciprocal of the rate of inactivation of Bacillus subtilis D-amino acid transaminase versus the reciprocal of DL-vinylglycine concentration. D-amino acid transaminase was incubated at 25°C with 25 mM α-ketoglutarate and the indicated concentrations of DL-vinylglycine. After 10 minutes, the inactivation was stopped by addition of 25 mM D-alanine and the transaminase activity determined with the NADH / lactic dehydrogenase coupled assay of pyruvate.

FIGURE 7


A
a
b
TRANSAMINASE ACTIVITY (μMOLE/MIN)
60
40
20
10
8
6
4
10
20
MINUTES
B
30
20
10
0
-1
0
1
2
3
4
5
1/ INACTIVATION RATE CONSTANT (MINUTES)
1/ [DL-VINYLGLYCINE] (mM⁻¹)

substrate. Therefore, a relatively large number of turnovers before inactivation was expected, and the partition should be directly measurable by the number of α-keto acid molecules produced by a given quantity of enzyme. Since there is no convienint assay for the vinylglycine transamination product, 2-keto-3-butenoate (radioactively labeled vinylglycine was not synthesized), catalytic transamination was observed by the concomitant conversion of α-keto[^{14}C]glutarate into D-[^{14}C]glutamate.

The procedures used for the analysis of the number of turnovers before inactivation of the two enzymes were somewhat different, but based on a similar principle. The experiments on the B. sphaericus enzyme were perfomed by selectively adsorbing the glutamate formed onto a DOWEX 50 H^+ resin. After an elution with water to remove α-ketoglutarate, the amino acid was eluted from the resin with 4N ammonia. The Rockefeller group performed similar experiments on the B. subtilis enzyme; they used an amino acid analyzer of the design of Spackman et al[97] to separate the α-ketoglutarate and glutamate.

The data of Figure 8 illustrate the production of [^{14}C] glutamate as a function of time at three concentrations of B. sphaericus D-amino acid transaminase. The amount of [^{14}C] glutamate at 20 minutes was 58 nmoles for 6.7 μgrams of enzyme, 100 nmoles for 13.3 μgrams, and 128 nmoles for 20 μgrams. As the enzyme concentration is increased, it is likely that a fraction of the enzyme is alkylated by a molecule of 2-keto-3-butenoate produced by transamination by another enzyme molecule, resulting in a progressive decrease in the number of catalytic transaminations per equivalent of enzyme. The data reveal that approximately 450 turnovers

Figure 8. Production of D-[^{14}C]glutamate from α-keto[^{14}C]glutarate and DL-vinylglycine catalyzed by Bacillus sphaericus D-amino acid transaminase.

In 1.0 ml of 0.05 M potassium pyrophosphate, pH 8.5, the solution contained at 25°C:

2 mM DL-vinylglycine

1 mM α-keto[^{14}C]glutarate (1000 cpm/nmole).

The reaction was initiated by addition of D-amino acid transaminase. At intervals, 100 µl was removed from the incubation and injected onto a 1 ml DOWEX 50 H^+ (400 mesh) column. Each column was washed with 10 ml water, then eluted with 3.0 ml 4 N ammonia to determine the [^{14}C] glutamate attached to the resin.

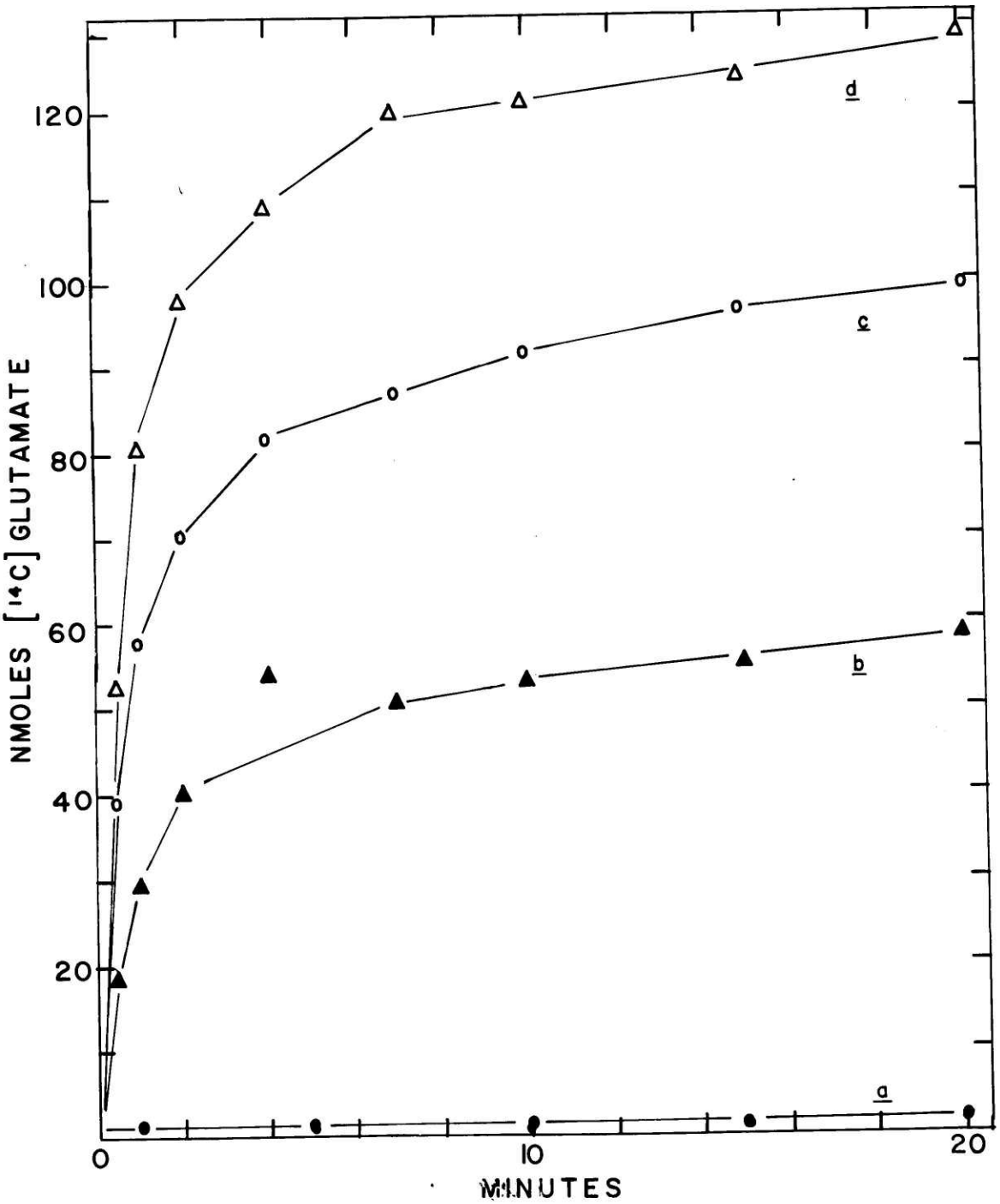
(a): 0 µg D-amino acid transaminase (control).

(b): 6.7 µg transaminase

(c): 13.3 µg transaminase

(d): 20 µg transaminase

FIGURE 8



are catalyzed by the enzyme before suffering loss of activity.

The data of Figure 8 futher indicate that there is a slow but real increase in the amount of [^{14}C] glutamate formed at 10, 15, and 20 minutes. The residual rate corresponds to about 1% of the estimated initial rate, i.e., the enzyme is 99% inactivated. Large enough quantities of enzyme were not available to conduct spectroscopic experiments which would demonstrate the catalytic competence of all of the enzyme molecules following alkylation.

The number of turnovers before inactivation of the B. subtilis enzyme upon incubation with vinylglycine was also determined. Figure 9 shows the relationship between [^{14}C] glutamate production and remaining enzyme activity. With time, both the rate of glutamate production and remaining enzyme activity fall off in parallel. After 10 minutes, at 50% inactivation, about 20 nmoles of [^{14}C]glutamate had been generated resulting in inactivation of 0.025 nmole of transaminase--about 800 turnovers before inactivation.

Effect of thiol nucleophiles on inactivation of D-amino acid transaminase by vinylglycine.

It is possible that the inactivation of the enzymes could occur by attack on enzyme nucleophiles not at the active site by the 2-keto-3-butenoate released into solution. To test this possibility, incubations of B. sphaericus enzyme, DL-vinylglycine, and α-ketoglutarate were performed in 5 mM dithiothreitol, which has been found[42] to protect L-hydroxy acid oxidase from inactivation by DL-vinylglycolate (2-hydroxy-3-butenoate),

Figure 9. Relationship between production of D-[^{14}C]glutamate from DL-vinylglycine and α-keto[^{14}C]glutarate and the inactivation of D-amino acid transaminase from Bacillus subtilis.

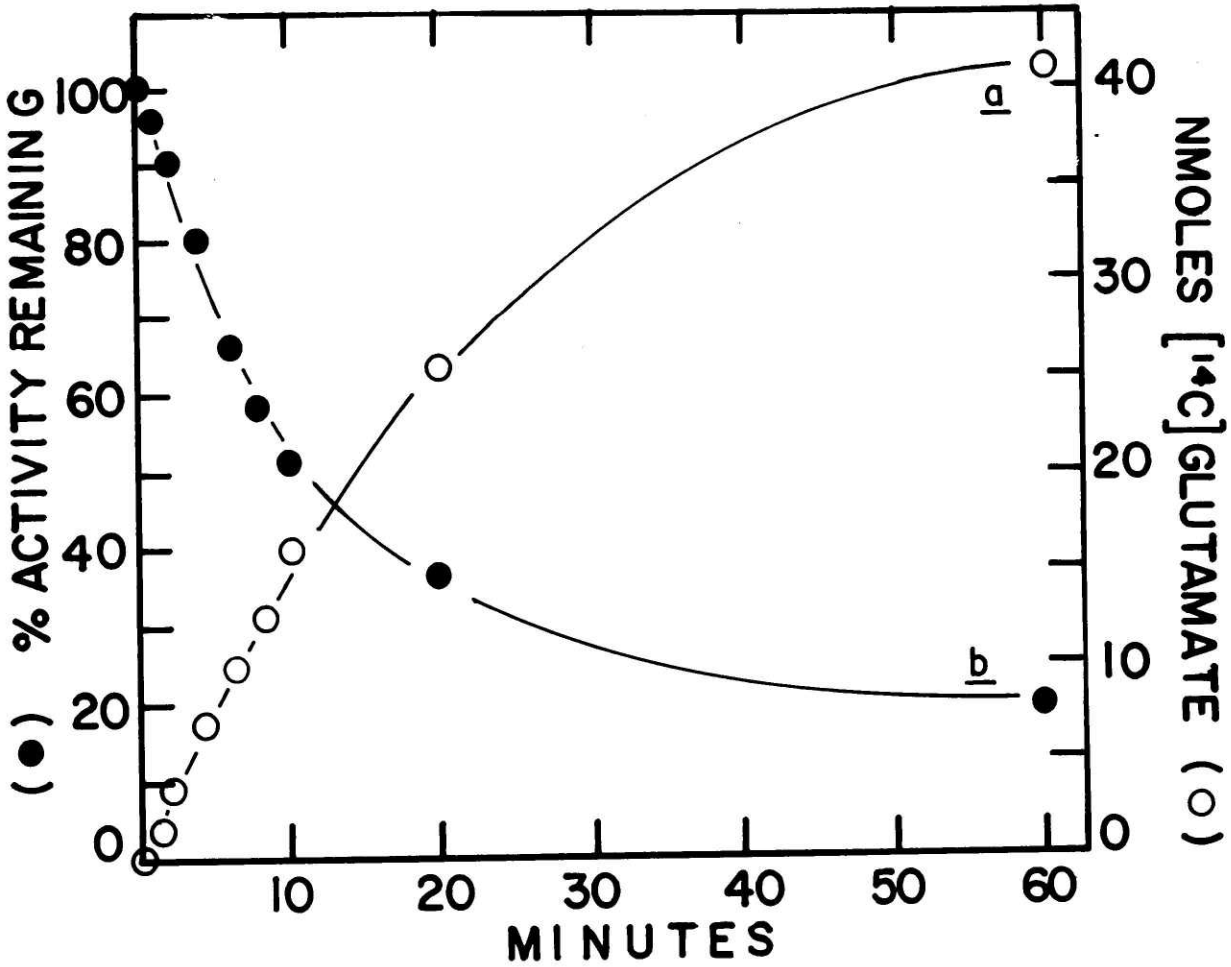
In 2.0 ml of 0.33 M potassium phosphate pH 8.5, the solution contained at 22°C:

1.0 mM DL-vinylglycine,

2.0 mM α-keto[^{14}C]glutarate (6000 cpm/nmole).

The reaction was initiated by addition of 30 µg D-amino acid transaminase. At each interval, two 0.1 ml aliquots were removed. One aliquot was added to citrate buffer, pH 2.2, and a portion of this sample applied to the amino acid analyzer. At this pH, the D-amino acid transaminase is rapidly inactivated; this aliquot measures the production of glutamate in the reaction mixture from vinylglycine and α-ketoglutarate. To the second 0.1 ml aliquot, 3 µl of D-alanine (0.75 M) was added and incubated at 22°C for 5 minutes. The increase in [^{14}C] glutamate was determined as indicated above. The difference in the amounts of [^{14}C] glutamate in the two aliquots represents the activity remaining in the reaction mixture.

FIGURE 9


(●) % ACTIVITY REMAINING
NMOLES [14C]GLUTAMATE (○)
MINUTES
0
20
40
60
80
100
10
20
30
40
50
60
a
b

the enzymatic oxidation of which results in the formation of the same electrophile.

Although this concentration of dithiothreitol had no effect on enzyme stability or the rate of transmamination of D-alanine and α-ketoglutarate, the rate of inactivation was increased. As shown in Figure 10, 25% fewer tunovers were observed from incubations containing dithiothreitol than those of controls. Overnight dialysis versus 1 mM dithiothreitol did not produce an enzyme more susceptible towards vinylglycine. The role of thiol in enhancing the inactivation of D-amino acid transaminase with vinylglycine has not yet been determined.

Reaction of propargylglycine with the D-amino acid transaminases.

In preliminary experiments, propargylglycine has been found to slowly inactivate the B. subtilis enzyme, but only after the enzyme catalyzes at least several thousand transaminations of propargylglycine. The B. sphaericus enzyme was found to have no susceptibility to propargylglycine in a 30 minute incubation. Whether these transaminases have the capacity to exchange the β-hydrogens of D-alanine has not yet been determined.

Comparison of the reaction of D-amino acid transaminase and L-aspartate aminotransferase with vinylglycine.

As stated previously in this paper, L-aspartate aminotransferase from pig heart is rapidly inactivated upon incubation with vinylglycine.[11] But in marked contrast to the bacterial D-amino acid transaminases, no stimula-

Figure 10: Effect of dithiothretol on the number of catalytic transaminations of vinylglycine by Bacillus sphaericus D-amino acid transaminase before suffering inactivation.

The assay of the production of D-[^{14}C]-glutamate was by the same method as that described in the legend to Figure 8.

In 1.0 ml of 0.05 M potassium pyrophosphate, pH 8.5, the solution contained at 25°C:

2 mM DL-vinylglycine

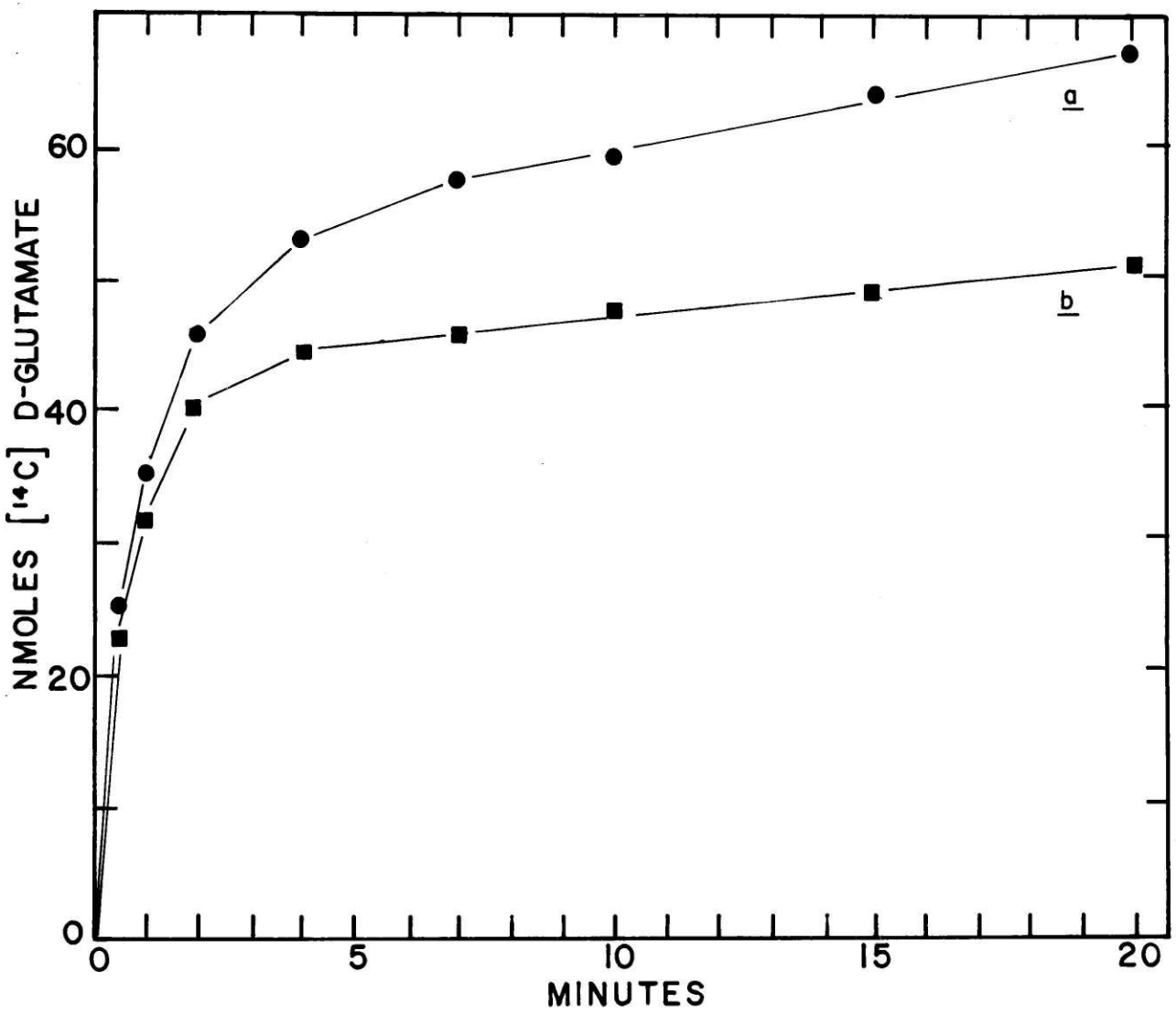
1 mM α-keto[^{14}C]glutarate (1000 cpm/nmole).

The reaction was initiated with 13 µg D-amino acid transaminase.

(a): the solution contained 0 mM dithiothreitol.

(b): the solution contained 5 mM dithiothreitol.

FIGURE 10



tion by α-keto acids on the rate of inactivation is observed. Also no significant formation of L-[^{14}C]glutamate from α-keto[^{14}C]glutarate is detected upon reaction of L-aspartate aminotransferase and vinylglycine in the presence of the radioactive keto acid. Therefore, this inactivation must procede very efficiently, i.e., with no significant partition between inactivation and transamination.

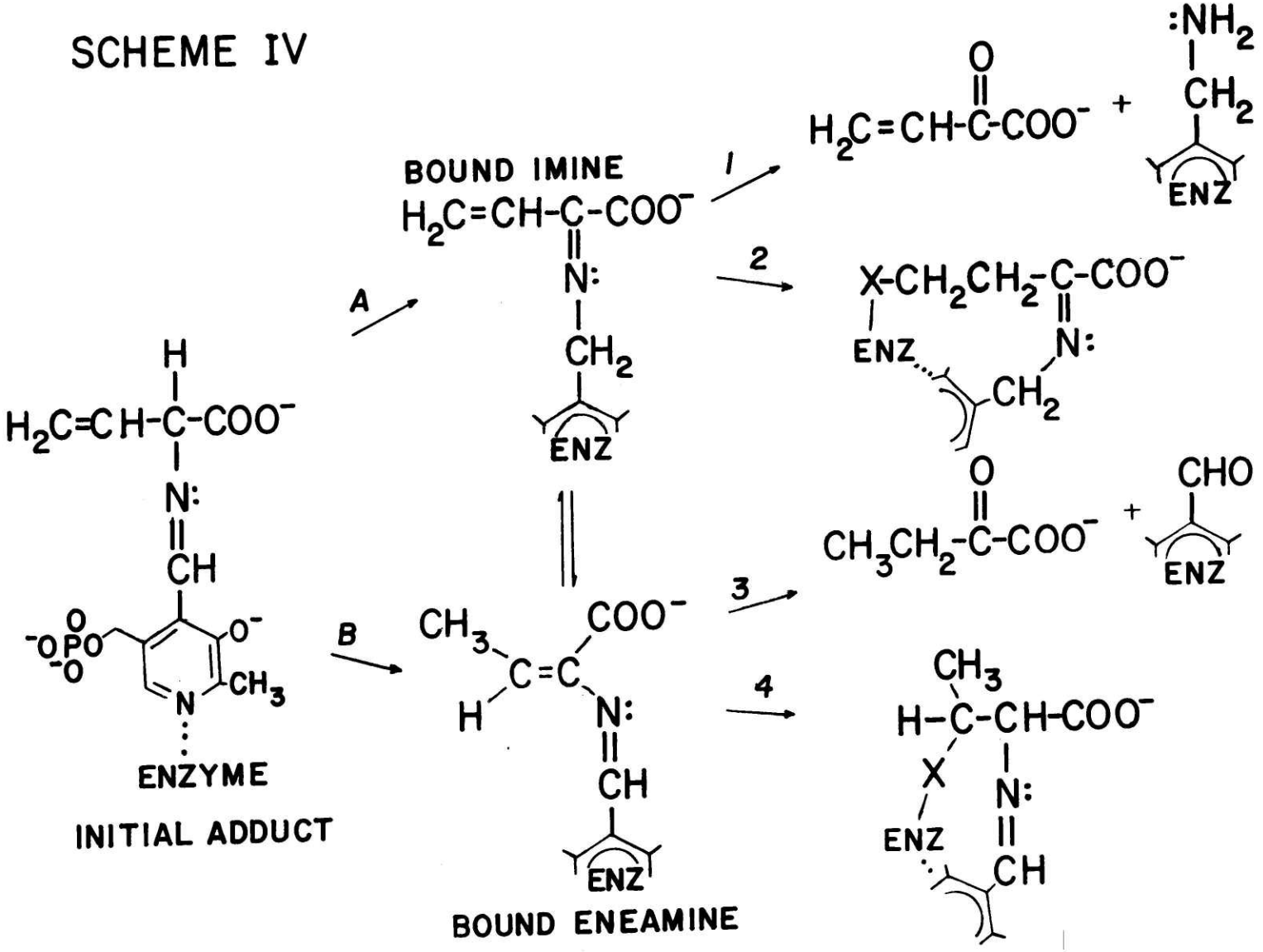
DISCUSSION

The possible pathways for the reaction of vinylglycine with the D-amino acid transaminases are delineated in Scheme IV. Two of these mechanisms lead to enzyme inactivation by covalent alkylation (pathways 2 and 4) while the other two (pathways 1 and 3) lead to catalytic turnover and α-keto acid production: 2-keto-3-butenoate by pathway 1 and 2-ketobutyrate by pathway 4. It is also clear from Scheme IV that these four routes are grouped pairwise from two different intermediates, which are formed by prototropic shifts from the initial aldimine adduct between vinylglycine and the pyridoxal coenzyme.

If the initial adduct is converted by route A to the "normal" transaminase ketimine complex, then hydrolysis of the imine (pathway 1) would yield 2-keto-3-butenoate, with the enzyme being converted to the pyridoxamine form. Should inactivation take place from this complex, it would be the result of attack by an enzyme nucleophile at carbon 4 of the vinylglycine molecule (pathway 2).

On the other hand, if the initial aldimine adduct, after enzymatic abstraction of the α-hydrogen as a proton, can undergo a 1,3 prototropic shift, the product (route B) will be an enzyme bound eneamine. Hydrolysis of the eneamine (pathway 3) would yield 2-ketobutyrate and the pyridoxal-phosphate form of the transaminase. If the product eneamine complex is captured instead by an enzyme nucleophile, inactivation would again ensue. But pathway 4 is distinct from pathway 2 in that pathway 4 would predict covalent bond formation at carbon 3 of the inactivator. The situation is

SCHEME IV


BOUND IMINE
ENZYME
INITIAL ADDUCT
BOUND ENEAMINE
A
B
1
2
3
4
ENZ

further complicated by the possibility that the bound eneamine and bound imine might well be in equilibrium, as is shown in Scheme IV.

Mechanism B3 has been proposed to explain the production of 2-ketobutyrate from L-vinylglycine by the following pyridoxal-phosphate dependent enzymes: sheep liver threonine deaminase,[59] rat liver γ-cystathionase,[61] Escherichia coli tryptophan synthetase,[58] and Salmonella typhimurium cystathionine-γ-synthase.[60] However, no catalytic production of 2-ketobutyrate is observed on incubation of vinylglycine and the D-amino acid transaminases, which rules out possibility B3 as a significant path to product formation.

D-Vinylglycine is a transaminase substrate when α-ketoglutarate is present as measured by the conversion of radioactive α-ketoglutarate to radioactive D-glutamate. This result suggest pathway A1 is responsible for vinylglycine turnover and implies that the product is 2-keto-3-butenoate. If path A1 is responsible for catalytic turnover, then it seems more probable that inactivation occurs by way of pathway A2 rather than B4. However, despite the fact that there is no evidence that the D-specific transaminase can isomerize vinylglycine to an eneamine product, it is possible that branch B could occur occasionally, and could be responsible for inactivation. This issue must be left in doubt until the identification of the enzyme inactivator linkage is elucidated, indicating which carbon is attached to the enzyme residue.

The production of 2-keto-3-butenoate during catalytic turnover leaves the enzyme molecules in the pyridoxamine-phosphate form, unable to undergo further reaction with another molecule of vinylglycine, or any other D-amino

acid, in the absence of added keto acid. These enzyme molecules are thus protected from inactivation that would proceed during many catalytic cycles, explaining the insensitivity of the D-transaminase in the absence of keto acid. The efficiency of vinylglycine in causing inactivation of the D-amino acid transaminases is low for both enzymes, 0.22% for any given catalytic cycle for the B. sphaericus enzyme and 0.15% for the B. subtilis enzyme.

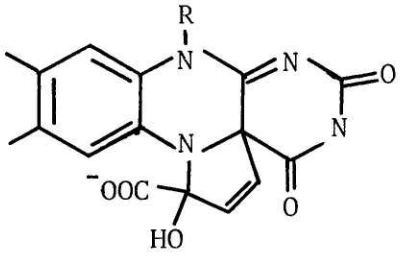
These low partitioning ratios between inactivation and normal catalytic transamination contrast with the susceptibility of pig heart L-aspartate aminotransferase to L-vinylglycine.[11] This enzyme must be nearly 100% inactivated in any cycle in which it acts upon vinylglycine. The basis for the different partitioning ratios must be due to the detailed geometry of the active site and the position of the susceptible nucleophile relative to the electrophile during the catalysis. Such factors could lead to a kinetic difference between the rates of hydrolysis of the bound imine versus the rate of nucleophilic attack. Similarly unclear at the molecular level is why L-alanine aminotransferase binds L-vinylglycine more tightly than its physiological substrates, has been shown to have an appropriately placed base at the active site, yet undergoes no inactivation while carrying out the transamination reaction.

CHAPTER III

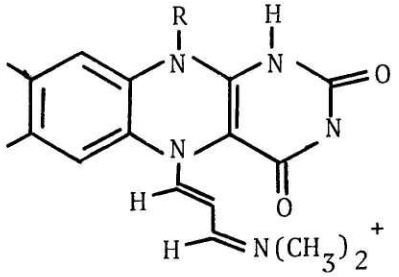
ACTIVE SITE DIRECTED INACTIVATION OF FLAVOPROTEIN AMINO ACID OXIDASES WITH ACETYLENIC AND OLEFINIC SUBSTRATE ANALOGUES

Unlike pyridoxal enzymes, for which many active site directed enzyme inactivators have been developed, the flavoprotein oxidases of amino acids, D-amino acid oxidase (usually isolated from hog kidney) and L-amino acid oxidase (from rattlesnake venom) have proven much less susceptible to inactivation.

Flavoprotein oxidases of α-hydroxy acids and alkyl amines have been found to be inactivated upon incubation with acetylenic substrate analogues, the unsaturation being placed adjacent to the carbon undergoing oxidation.[42 98 99] The invariant result is loss of activity due to modification of the coenzyme; the structure of the covalent flavin adducts produced in two such cases have been determined.[99 100]



from inactivation of
lactic 2-monooxygenase
with 2-hydroxy-3-butynoate

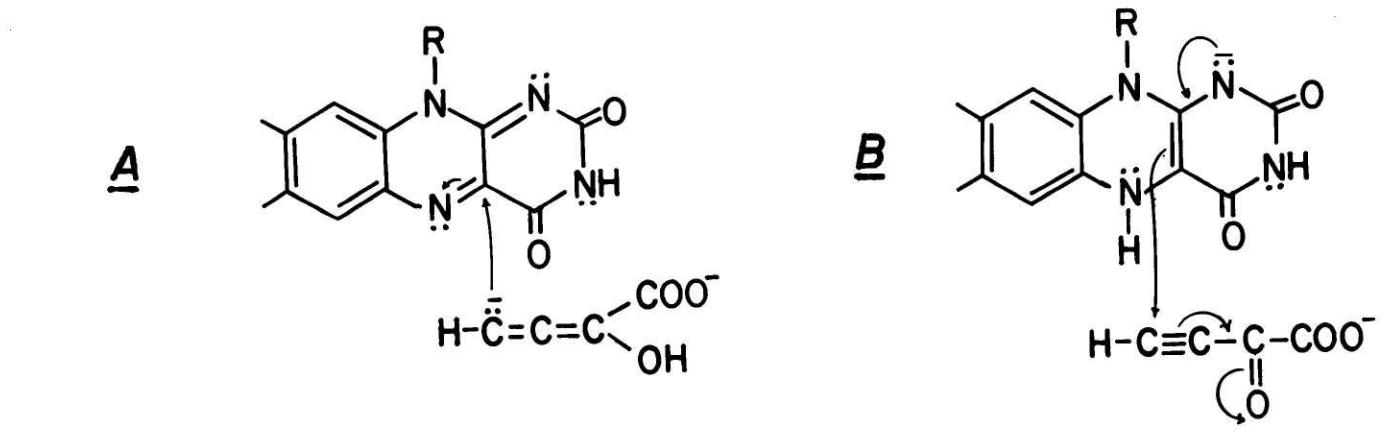


from inactivation of
monoamine oxidase with
N,N dimethylpropargylamine

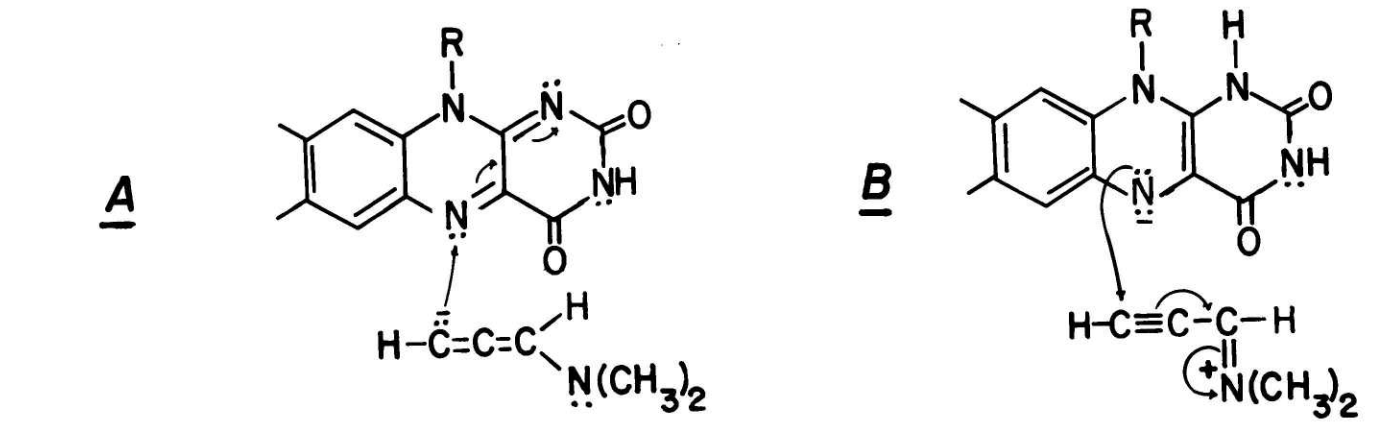
As shown in Scheme I, the modification of the flavin arises by one of two mechanisms: A, removal of the α-hydrogen as a proton followed by attack of the allenic anion thus formed on the oxidized coenzyme; or B, oxidation of the substrate followed by attack of the reduced coenzyme on the electrophilic product before it can be released. Since the two pathways lead to the same modified coenzyme structures, a conclusive argument as to the mechanism cannot be made. However, incubation of the enzymes

SCHEME I

L-LACTATE-2-MONOOXYGENASE

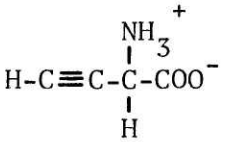


MONOAMINE OXIDASE



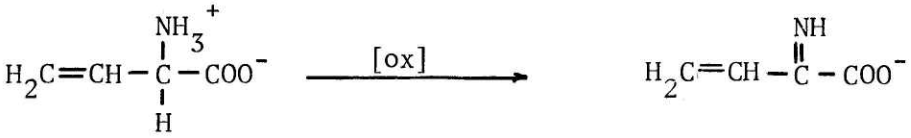
with the corresponding olefinic substrate analogues has never been found to result in coenzyme modification. Since the oxidation product, a conjugated olefin, should also be susceptible to attack by the reduced coenzyme (analogous to pathway B), the failure to observe such inactivation has been argued[42,100] to favor the allenic anion mechanism (pathway A) for the acetylene induced inactivation.

In view of the preceding discussion, the logical compound to similarly inactivate the amino acid oxidases would be 2-amino-3-butynoate. It would



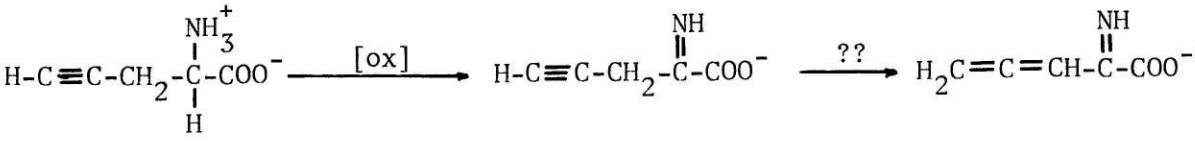
be interesting to compare the interaction of vinylglycine and "ethynylglycine" with the pyridoxal and flavin dependent enzymes studied in this work. However, the synthesis of this amino acid has not to date been accomplished in spite of the efforts of a number of research laboratories.[101]

Vinylglycine and propargylglycine, the two amino acids used earlier in studies with pyridoxal enzymes, were examined as substrates and possible inactivating agents of the flavoprotein amino acid oxidases. Vinylglycine was considered a likely inactivator; oxidation of this amino acid would result in the formation of 2-imino-3-butenoate at the active site,



a conjugated olefin similar to that previously shown to be responsible for inactivation of several pyridoxal enzymes.

On the other hand, propargylglycine oxidation yields 2-imino-4-pentynoate, a species which should not be reactive at the active site. Only upon isomerization of the acetylene to the electophilic conjugated allene should



inactivation be induced. Neither flavoprotein oxidase had been shown to catalyze removal of the β-hydrogen, the necessary requirement for the rearrangement to occur. (Because of the irreversibility of the reaction and the acidity of the β-hydrogens of the keto-acid product, it would be difficult to conclusively prove or disprove this point.) Thus susceptibility of either of the two enzymes to propargylglycine would demonstrate a catalytic potential not before recognized.

Complementary specificity towards inactivation of these two enzymes was observed -- L-amino acid oxidase being alkylated upon its oxidation of vinylglycine and D-amino acid oxidase upon oxidation of propargylglycine. Both inactivations resulted from modification of an amino acid residue rather than the bound flavin coenzyme. The remainder of this chapter describes the experiments on the reactions and inactivation of these flavoproteins with these amino acids.

A. REACTION OF VINYLGLYCINE WITH L-AMINO ACID OXIDASE AND D-AMINO ACID OXIDASE

EXPERIMENTAL SECTION

Materials: L-amino acid oxidase (listed as 5.2 units/mg) was purchased from Sigma chemical and dialyzed against 0.1 M Tris HCl pH 8 before use. The ratio of absorbances at 275 nm and 463 nm was 20 for the commercial enzyme, two times that of the crystalline enzyme.[102] D-amino acid oxidase was purified to 70% homogeneity (through the calcium phosphate/cellulose column step) by the method of Brumby and Massey.[103] DL-vinylglycine was synthesized as described by R. Rando.[11] Other chemicals were reagent grade.

Methods: The enzymatic oxidation of vinylglycine was observed by the consumption of oxygen in a 0.4 ml cell fitted with a Clarke-type electrode (Yellow Springs Instruments Model 53 oxygen monitor). Kinetic assays were performed on a Gilford Model 222 spectrophotometer. Electronic absorption spectra were recorded on a Beckman model 25 spectrophotometer.

The enzymes were preincubated with vinylglycine by stirring a solution of vinylglycine and the enzyme in the appropriate buffer containing catalase (and FAD with D-amino acid oxidase). Aliquots of the solution were removed at intervals to determine the residual enzyme activity. D-amino acid oxidase was assayed by its oxidation of D-alanine; L-amino acid oxidase by its oxidation of L-α-aminobutyrate. In both cases, the rate of reaction was measured by coupled spectrophotometric assay of the products with NADH and L-lactic dehydrogenase.

RESULTS

Reaction of L-amino acid oxidase with vinylglycine.

Under initial velocity conditions, vinylglycine is a good substrate for L-amino acid oxidase. The measured maximal velocity of oxidation was 11 μmoles min^{-1} mg^{-1} (25°C, 0.1 M Tris HCl, pH 8), which was found to be near the maximal velocity of L-leucine (the most commonly assayed substrate) under the same conditions. The K_m of DL-vinylglycine is, however, higher--at 8 mM compared with 1 mM reported for L-leucine.[104]

Initially, the reaction of L-amino acid oxidase with vinylglycine was examined in the hopes of using the enzyme to selectively oxidize L-vinylglycine in a racemic mixture of the amino acid so that the D-isomer could be recovered and used with the pyridoxal transaminases discussed earlier. An incomplete resolution by this procedure had been reported;[54] when efforts to produce optically pure D-vinylglycine in this manner were similarly unsuccessful, the reasons for this failure were investigated.

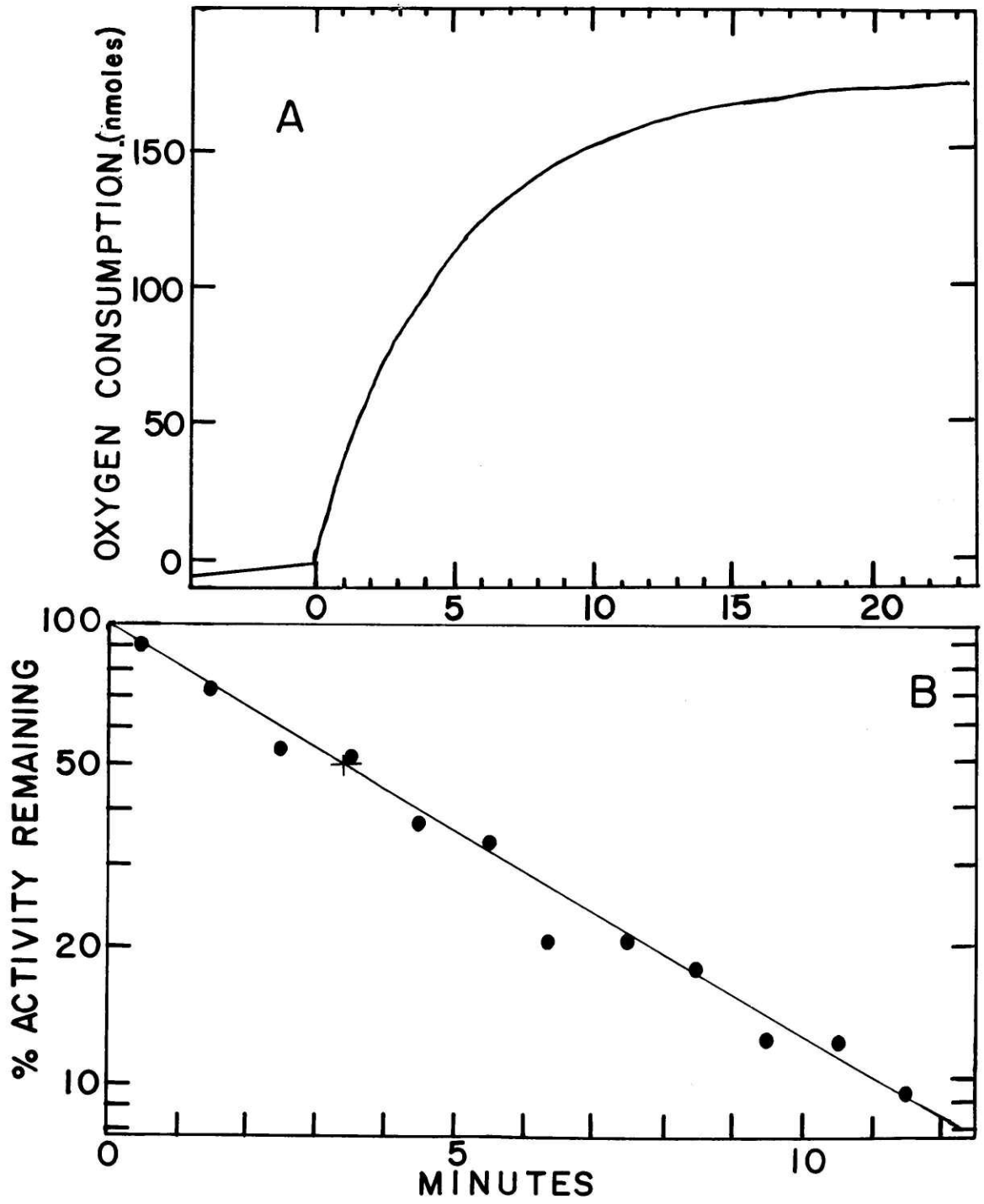
The oxidation of vinylglycine by L-amino acid oxidase results in progressive, time-dependent inactivation of the enzyme (Figure 1A). The inactivation is irreversible; dialysis overnight against a large excess of buffer restores no more than 3% of the original activity. By analysis of the amount of oxygen consumed before inactivation is complete, it can be calculated that each molecule of L-amino acid oxidase oxidized about 2000 vinylglycine molecules before suffering loss of activity.

On chemical grounds, the likely inactivator is not the amino acid substrate but rather the product of the enzymatic oxidation, 2-imino-3-

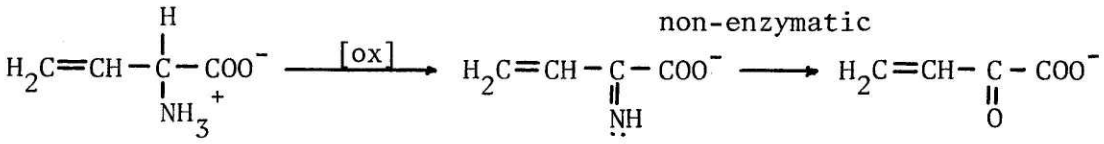
Figure 1. A: Time course of reaction of L-amino acid oxidase with DL-vinylglycine, The oxygen elctrode chamber contained, in 0.4 ml 0.1 M Tris HCl, pH 8.0, 6.25 mM DL-vinylglycine and 2 µg catalase. At 0 minutes, 8 µg oxidase was added.

B: The rate of inactivation of L-amino acid oxidase with DL-vinylglycine derived from data in part A. The $T_{1/2}$ of inactivation was found to be 3.4 minutes.

FIGURE 1

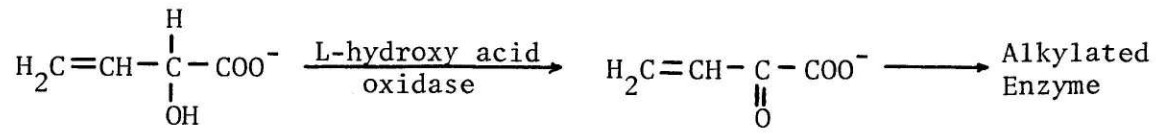

A
OXYGEN CONSUMPTION (nmoles)
150
100
50
0
0
5
10
15
20
B
% ACTIVITY REMAINING
100
50
20
10
0
5
10
MINUTES

butenoate. Because of accumulation of the electrophilic olefinic keto acid, produced via non-enzymatic hydrolysis of the imino acid, it was possible that the observed inactivation was the result of non-specific alkylation of available enzyme nucleophilic groups.



The data of Figure 1B suggest that the inactivation follows first order kinetics. At 6.25 m<u>M</u> DL-vinylglycine, the half time for inactivation at room temperature is 3.4 minutes. This rate behavior is consistent with inactivation proceding from an enzyme-substrate (or enzyme-product) complex rather than by some collisional interaction of enzyme and inactivator.

The non-specific alkylation of enzyme nucleophiles appears to operate in the inactivation of the FMN dependent (rat) kidney L-hydroxy acid oxidase by 2-vinylglycolate.[42] In that case, a time dependent inactivation is observed, but addition of exogenous low molecular weight nucleophiles, such as dithiothreitol, completely protects the hydroxyacid oxidase by removing the electrophile from solution.[42]



When dithiothreitol was added to incubations of L-amino acid oxidase and DL-vinylglycine, similar protection was not observed. Since the same electrophilic inactivator is presumed to accumulate in both cases, this lack of protection suggest that L-amino acid oxidase in inactivated due

to covalent modification at the active site of the enzyme prior to release.

A point of interest is whether inactivation involves an active site amino acid residue or involves reaction with the flavin coenzyme. Addition of FAD to incubations did not alter the kinetics of inactivation, nor did dialysis after inactivation against buffer containing 10 μM FAD lead to any significant recovery of activity. The visible absorbance spectrum of the enzyme was examined before and after incubation with DL-vinylglycine (Figure 2). The spectrum was altered, but the long wavelength absorbance of FAD had not been bleached. If addition to the isoalloxazine ring of the coenzyme had occurred, major electronic perturbations would have been detected.[42 98] The flavin spectrum of vinylglycine inactivated L-amino acid oxidase is quite similar to the bound flavin spectrum of 2-vinylglycolate inactivated L-hydroxyacid oxidase.[42] In the latter case, the released flavin was not radioactive when 2-vinyl[1-^{14}C]glycolate had been used. Therefore, it is likely that vinylglycine inactivates L-amino acid oxidase by an amino acid modification at the active site.

Reaction of D-amino acid oxidase with DL-vinylglycine.

With D-amino acid oxidase, DL-vinylglycine is a good substrate. It has a K_m of 0.96 mM and a maximal velocity 125% that of D-alanine. In sharp contrast to the behavior of L-amino acid oxidase, no inactivation was observed during a one hour incubation. Since the stability in solution of the oxidation product, 2-keto-3-butenoate, has not been determined, its concentration in these incubations is unknown. However, it does accumu-

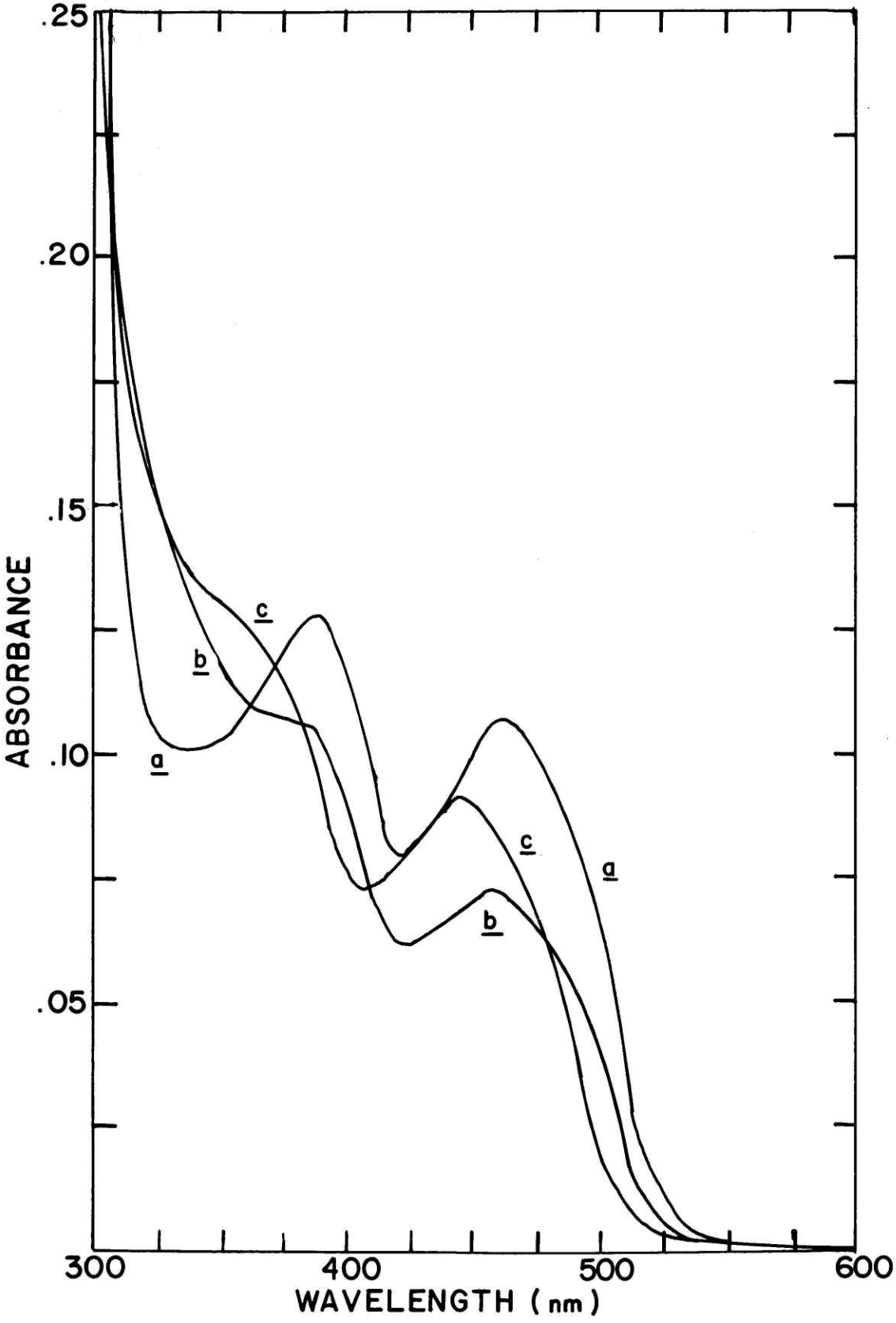
Figure 2. Spectral changes on inactivation of L-amino acid oxidase with DL-vinylglycine.

(a): spectrum of 1 mg of L-amino acid oxidase in 1 ml of 0.1 M Tris HCl, pH 8.

(b): after complete inactivation with 10 mg of DL-vinylglycine followed by dialysis against Tris HCl buffer (spectrum corrected for changes in concentration by normalizing absorbaces at 275 nm).

(c): Inactive enzyme of B heated to 100°C for 5 minutes, protein removed by membrane filtration, and spectrum of the supernatant recorded.

FIGURE 2


.25
.20
.15
.10
.05
ABSORBANCE
a
b
c
c
b
a
300
400
500
600
WAVELENGTH (nm)

late sufficiently to cause inactivation of L-hydroxy acid oxidase[42] whereas D-amino acid oxidase appears to be insensitive.

DISCUSSION

A number of points in the mechanism of the inactivation of L-amino acid oxidase with vinylglycine could be clarified with the use of radioactively labeled vinylglycine. These points concern the stoichiometry of modification and a conclusive demonstration of whether the inactivation involves apoenzyme or coenzyme modification. To date these experiments have not been done; the reasons are due to the low yield (15%)[55] of the synthesis of vinylglycine from vinylglycolate and the large number of catalytic oxidations (2000) before inactivation. These two reasons dictated the use of indirect methods to examine the questions more directly answerable with radioactive material.

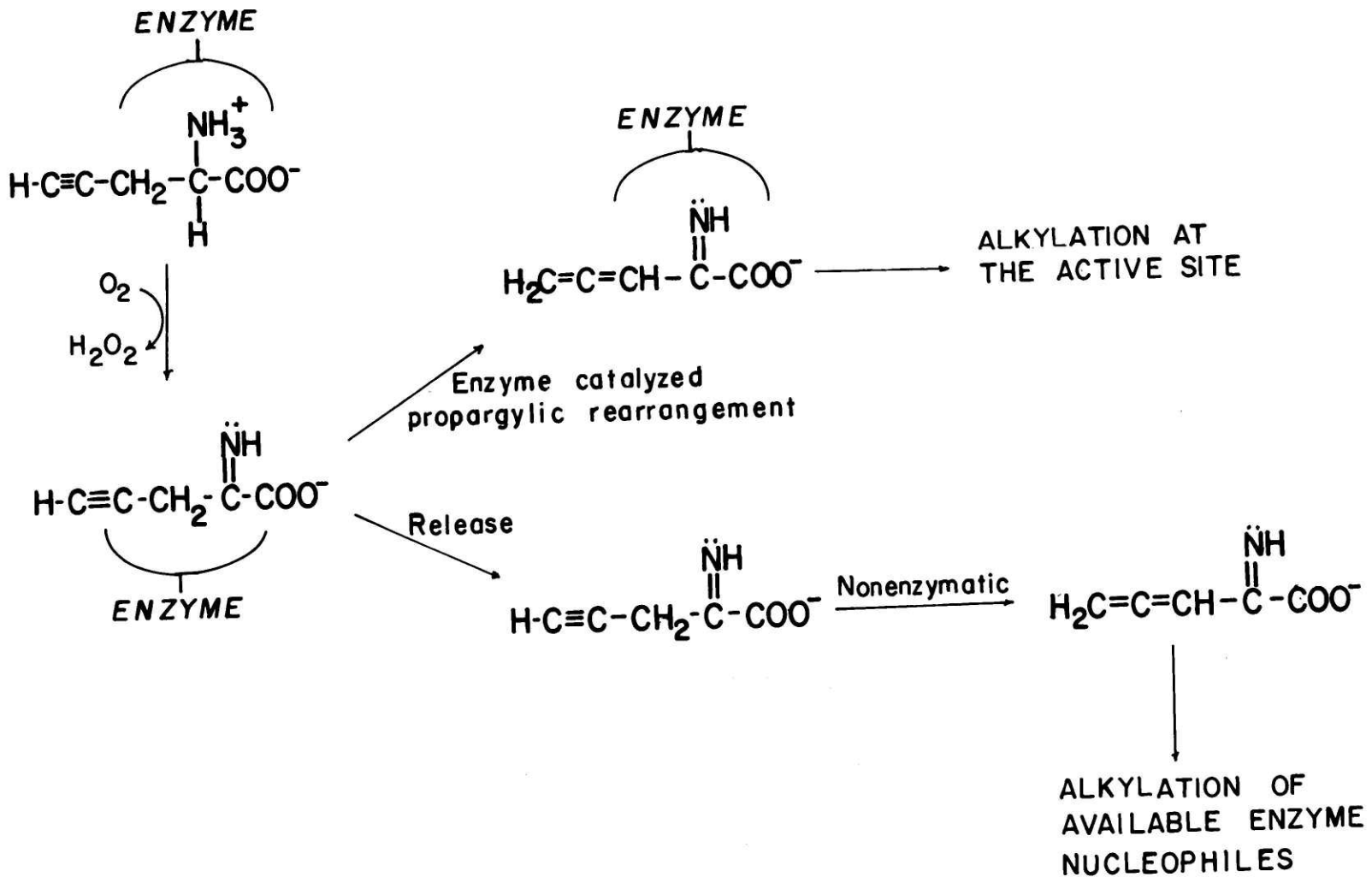
Although vinylglycine is a good substrate for both enzymes, only L-amino acid oxidase is inactivated. There is kinetic evidence with each enzyme[40 104] that product release is a slow step in the catalysis of the oxidation. Therefore the product should be retained long enough for attack should there be an appropriately placed nucleophile. The evidence suggests only L-amino acid oxidase has such a susceptible nucleophile at the active site.

B. REACTION OF D-AMINO ACID OXIDASE AND L-AMINO ACID OXIDASE WITH PROPARGYLGLYCINE

The inactivation of D-amino acid oxidase upon oxidation of D-propargylglycine was first observed by Horiike et al;[51] the enzyme being reported to carry out 600 - 800 catalytic oxidations before suffering loss of activity. That work demonstrated that this inactivation is not a simple phenomenon--the kinetics of inactivation showed substantial deviations from first order behavior and 20% of the original activity was insensitive to inactivation. The work of Horiike et al did not produce any evidence on the mechanism of inactivation nor did they provide more than conjecture as to the reasons for the observed deviations from the simple model. Therefore, this inactivation was of interest to this work, in view of the previous examination of the action of propargylglycine on pyridoxal enzymes.

This study was originally undertaken in the hopes of demonstrating that the observed loss of activity was due, not to an active site directed modification, but to alkylation proceding from solution as in the case (dicussed previously) of the inactivation of L-hydroxyacid oxidase upon oxidation of 2-vinylglycolate.[42] An illustration to contrast the mechanism of propargylglycine induced inactivation by the two processes is provided in Scheme II. In both cases, the probable alkylating agent is the conjugated allene, 2-imino-3,4-pentadienoic acid (3), produced via propargylic rearrangement of the original oxidation product, 2-imino-4-pentynoic acid (2). The difference between the two mechanisms concerns whether this

SCHEME II


ENZYME
NH3+
H-C≡C-CH2-C-COO-
H
O2
H2O2
ENZYME
NH
H-C≡C-CH2-C-COO-
ENZYME
Enzyme catalyzed
propargylic rearrangement
ENZYME
NH
H2C=C=CH-C-COO-
ALKYLATION AT
THE ACTIVE SITE
Release
NH
H-C≡C-CH2-C-COO-
Nonenzymatic
NH
H2C=C=CH-C-COO-
ALKYLATION OF
AVAILABLE ENZYME
NUCLEOPHILES

rearrangement, which leads to modification, is enzyme catalyzed or whether the observed inactivation is a fortuitous result of alkylation of amino acid residues not at the active site by allene produced via the propargylic rearrangement having occured in solution. Inactivation by this second mechanism would not be expected to follow simple kinetics; furthermore, alkylations away from the active site could well be imagined to result in the formation of an enzyme species with substantial residual activity. Therefore, inactivation by this mechanism was considered a likely possibility; it had not been considered in the publication by Horiike et al;[51] but nothing in their work ruled it out as a possibility.

Attempts to demonstrate unambiguously that the alkylating agent can be generated in solution were unsuccessful. However, a number of experimental observations imply that the inactivation mechanism involves a species which is loosely associated with the enzyme. The inconclusive results of the initial experiments did not signal the end of the work on this inactivation, as several other aspects of the reaction were found to be of interest. The discussion will be divided into three parts.

1. It has been found that a number of interesting non-enzymatic reactions follow the initial enzymatic oxidation of propargylglycine to the corresponding imino acid. Three discrete species have been identified in the reaction mixture, two of which are tight competitive inhibitors of D-amino acid oxidase and lead to the formation of a complexes with intense long wavelength ("charge transfer") absorbance upon binding to the enzyme. The probable structures of the compounds produced following enzymatic oxidation

of propargylglycine, and the mechanism of their formation, are the first matter to be discussed.

2. The covalent modification of D-amino acid oxidase upon its oxidation of D-propargylglycine results in the formation of a catalyst with markedly changed catalytic properties. The second part of the discussion will describe the preparation and characterization of the modified D-amino acid oxidase, and demonstrate that the modified enzyme has greatly changed substrate specificity and exhibits a different rate determining step from native enzyme.

3. The final section is a description of the likely mechanism of the covalent modification of D-amino acid oxidase upon its oxidation of D-propargylglycine.

In contrast to the susceptibility of D-amino acid oxidase, no modification of kinetic parameters is observed upon incubation of L-amino acid oxidase and L-propargylglycine. Since L-propargylglycine is a good substrate for the enzyme, this reaction proved very useful for the synthesis of the product species the structure determination of which is discussed in this chapter.

1. THE REACTION PATHWAY FOLLOWING ENZYMATIC OXIDATION OF PROPARGYLGLYCINE

EXPERIMENTAL SECTION

Materials

Enzymes and reagents: L-amino acid oxidase was purchased from Sigma chemical. D-amino acid oxidase was purified to 70% homogeneity (through the calcium phosphate cellulose column step) by the method of Brumby and Massey.[103] DL-2-amino-4-ketovaleric acid was synthesized as reported by Wiss and Fuchs.[105] Propargylglycine was synthesized and resolved by the method of Jansen et al.[45] DL-propargyl[^{14}C]glycine (500 cpm/nmole) was synthesized from diethyl[2-^{14}C]malonate purchased from Amersham. All other amino acids, FAD, NADH, acetopyruvic acid, Hepes, and DL-allylglycine and catalase were purchased from Sigma. DL-vinylglycine was synthesized by the method of Rando.[11] Buffer salts and solvents were commercially available reagent grade materials.

Instrumentation: Electronic absorption spectra were recorded using a Perkin Elmer model 200 double beam spectrophotometer. Spectroscopic kinetics were followed with a Gilford model 222 sigle beam spectrophotometer.

Methods

Ultraviolet spectrum of the product of propargylglycine oxidation: 1 µl of 0.1 M L-propargylglycine (0.1 µmole) was added to a cuvette containing 1.0 ml of buffer 10 µg catalase and 125 µg L-amino acid oxidase. After absorbance changes stabilized (about 2 hours), the spectrum was recorded

against a reference cell containing buffer, catalase, and oxidase. A variety of buffers, based on Hepes, Tris, and pyrophosphate were used; primary amine, thiol, and imidazole nucleophiles were added to the buffer solution in some experiments.

Chromatographic identification of the product of propargylglycine oxidation: In 0.2 ml 0.1 M Hepes pH 8, 2.5 μmole of DL-propargyl[2-^{14}C]-glycine was reacted with 95 μg L-amino acid oxidase and 20 μg catalase. After a 90 minute incubation, the reaction mixture was passed through two 1 ml DOWEX 50 H^+ columns. The solution was evaporated to a small volumne and spotted on 12 x 4 cm Baker flex silica gel 1B plates. The plates were developed with n-butanol-acetic acid-water (12-3-5) or water saturated ethyl ether-formic acid (7-1). The plates were sprayed with a solution of 3% vanillin-5% concentrated sulfuric acid in absolute ethanol, and then heated to 120°C.[106] Authentic acetopyruvic acid yields a brown spot on the plated developed in the n-butanol-acetic acid-water system and a purple spot in the ether-formic acid system. The plates were cut into 0.5 cm sections and eluted with scintillation fluid which was counted using a Beckman LS-100C scintillation counter.

Characterization of the accumulated product following enzymatic oxidation of propargylglycine and 2-amino-4-ketovaleric acid: At room temperature, 50 nmoles L-propargylglycine or 100 nmoles DL-2-amino-4-ketovaleric acid were mixed with 25 μg catalase and 0.2 nmoles L-amino acid oxidase in 1.0 ml 0.1 M Hepes, pH 8.0. Complete oxidation of the amino acids required 3-4 hours at room temperature. Oxidation was effected similarly with 1 nmole of D-amino acid oxidase. Reduction of the oxidation products

(and also of authentic acetopyruvate) was accomplished by addition of 10 µl 1.0 M hydroxylamine HCl or 1.0 M hydrazine HCl (both stock solutions at pH 8) to 1.0 ml of product solution. Reduction with sodium borohydride was done by addition of a speck of solid $NaBH_4$ to a solution of product. Reduction was also attempted by addition of 100 nmoles NADH followed by 250 µg of L-lactic dehydrogenase.

To test for the solubility of the products in diethyl ether, a 3 ml solution containing 150 nmoles of the oxidation products from propargylglycine or 2-amino-4-ketovaleric acid were mixed with 3 ml of ether. After vigorous mixing, the aqueous and organic layers were separated and the ultraviolet spectrum of each recorded. To recover the ether extracted product, 10 ml of 0.1 M Hepes pH 8 containing 0.5 µmole of oxidation product of propargylglycine was extracted 3 times with 5 ml of diethyl ether. The ether was dried with $MgSO_4$, after which 250 µl 0.1 M Hepes pH 8 was added to the solution. The ether was then evaporated with a stream of dry argon.

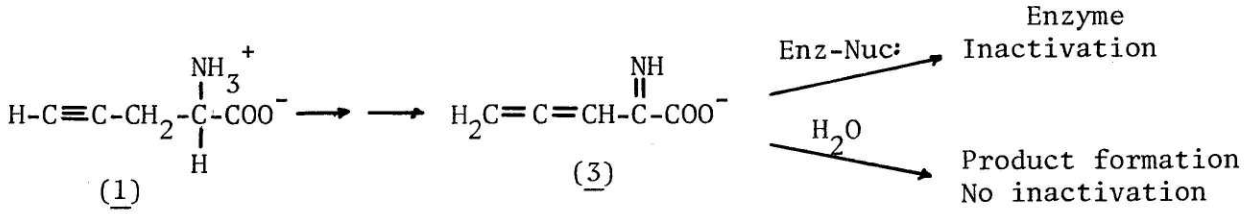
Charge transfer complexes between D-amino acid oxidase and amino acid oxidation products: Spectra of the product complexes of D-amino acid oxidase were observed at 10°C so as to minimize turbidity in the enzyme sample. A 1-5 µl aliquot of the amino acid to be oxidized (D-propargylglycine, DL-allylglycine, DL-2-amino-4-ketovaleric acid, or DL-vinylglycine) was added to a solution of D-amino acid oxidase in 0.4-0.5 ml 0.05 M pyrophosphate buffer pH 8.5. When only a slight molar excess of amino acid is used, reaction is complete in seconds and the spectrum of the complex is recorded immediately. 25 nmoles of L-propargylglycine was

added to 10 nmoles D-amino acid oxidase, followed by 0.15 nmole L-amino acid oxidase, to record the charge transfer complex formed in that manner.

Attempted inactivation of D-amino acid oxidase upon incubation with L-propargylglycine and L-amino acid oxidase. In 0.25 ml of 0.05 M sodium pyrophosphate, the solution contained 40 mM L-propargylglycine, 0.02 mM FAD , and 25 µg catalase. To this solution was added 2.1 nmoles of D-amino acid oxidase and 20 µg L-amino acid oxidase. The solution was gently stirred at room temperature for two hours. At intervals, 10 µl aliquots were removed and diluted into 1 ml of pyrophosphate buffer containing 0.2 M D-alanine, 0.01 mM FAD, 0.2 mM NADH, and 50 µg L-lactic dehydrogenase.

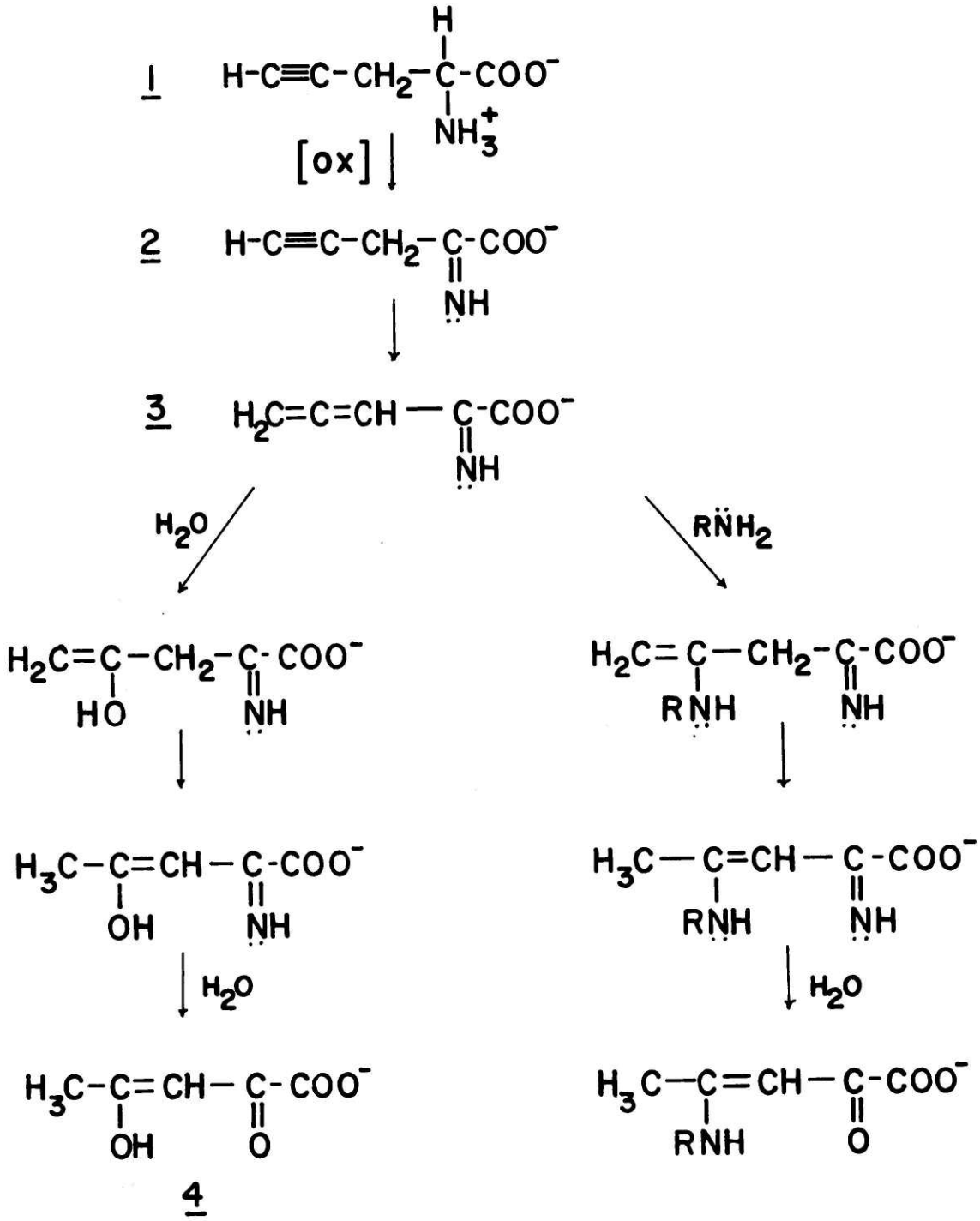
RESULTS

The identification of the product(s) of oxidation of propargylglycine was originally pursued to produce evidence confirming the probable structure of the species responsible for the alkylation of D-amino acid oxidase. Regardless of whether or not the modification is active site directed, the observed inactivation was considered to be the result of attack of an enzyme nucleophile at the electrophilic fourth carbon of 2-imino-3,4-pentadienoate (3). Since the enzyme had been observed to catalyze at least several hundred oxidations before suffering loss of activity, a favored hypothesis for the inactivation involved a partition of the inactivating species between attack by an enzyme nucleophile and attack by water, the result of which would be the accumulation in solution of such a hydrated product.



A reasonable scheme for the pathway following oxidation of propargylglycine is shown in Scheme III. The end product of this speculative pathway is acetopyruvic acid (2,4 diketovaleric acid). Because the expected short lifetime of the conjugated allene would make its direct observation an unlikely possibility, the identification of acetopyruvate in the reaction mixture would be evidence that there had existed in the pathway a species subject to nuceleophilic attack at carbon 4. The pathway proposed in Scheme III also predicts that the addition of nucleophiles to the incubation

SCHEME III


1
H-C≡C-CH2-C-COO-
NH3+
[ox]
2
H-C≡C-CH2-C-COO-
NH
3
H2C=C=CH-C-COO-
NH
H2O
RNH2
H2C=C-CH2-C-COO-
HO
NH
H2C=C-CH2-C-COO-
RNH
NH
H3C-C=CH-C-COO-
OH
NH
H3C-C=CH-C-COO-
RNH
NH
H2O
H2O
H3C-C=CH-C-COO-
OH
O
H3C-C=CH-C-COO-
RNH
O
4

would cause the formation of a trapped product. This provided another experimental approach for the examination of the pathway.

Because of the inactivation of D-amino acid oxidase by propargylglycine, L-amino acid oxidase was routinely used for oxidation of the amino acid in studies on the nature of the products. No difference in the pathway following oxidation of propargylglycine by the two enzymes was ever observed, evidence that neither enzyme does more than carry out the initial oxidation. This point will be addressed in some detail in a later section.

Ultraviolet absorption spectra of the products derived from the oxidation of propargylglycine.

One of the first observations to be made concerning the pathway following enzymatic oxidation of propargylglycine was that the oxidation results in the formation of a product with intense absorbance in the near ultraviolet. By subjecting a known quantity of amino acid (0.1 μmole in 1 ml) to exhaustive oxidation, the extinction coefficient of the ultraviolet absorbing material could be calculated. As would be expected if product formation is the result of attack by a nucleophile in solution on the conjugated allene, the λ_{max} of the observed product varied with added nucleophile in the incubation. In 100 mM Hepes, pH 8 (a non-nucleophilic buffer) a λ_{max} of 300 nm (ε 12,000) was observed. Addition of 10 mM ethanethiol shifted the λ_{max} to 312 nm, addition of 200 mM butylamine HCl shifted the λ_{max} to 318 nm.

By varying the concentration of butylamine in Hepes buffer from 0 mM to 200 mM, the partition between the formation of the 300 nm and 318 nm

could be demonstrated (Figure 3). It was further shown that the 300 nm species is not a precursor of the 318 nm species; addition of 100 mM butylamine HCl caused only a very slow shift (over 24 hours) of the absorbance to higher wavelengths.

A comparison was then made between the absorbance of the product in non-nucleophilic buffer (thought to be the result of the attack of water at carbon 4 of (3)) and acetopyruvate. Authentic acetopyruvate exhibits a λ_{max} 295 nm, ε 12,000 at pH 8.[107] The discrepancy between these values and the 300 nm absorbance of the accumulating product was possibly attributable to the 300 nm absorbance being comprised of a mixture of components, a major one being acetopyruvate, in the enzymatic incubation. Therefore, direct chromatographic comparison of the enzymatic product with authentic material was attempted.

Identification of acetopyruvic acid in the reaction mixture.

2.5 μmoles DL-propargyl[2-^{14}C]glycine (500 cpm/nmole) was incubated with 100 μg L-amino acid oxidase for 90 minutes. The D-propargylglycine (and any unreacted L-propargylglycine) was removed from the incubation by passage through a column of DOWEX 50 H^+. After passage through the column, the aqueous solution was evaporated to a small volume and aliquots of the concentrate were spotted on Silica gel 1B plates. In two solvent systems, the material migrated an identical distance as authentic acetopyruvic acid and gave the same color test with the detection reagent. Furthermore, over 80% of the radioactivity spotted on the plate migrated with acetopyruvic acid.

Figure 3. Extinction coefficient of the product of enzymatic oxidation of propargylglycine as a function of the concentration of butylamine HCl in solution.

In 1 ml of buffer at 30°C, the solution contained:

125 µg L-amino acid oxidase

25 µg catalase

0.1 µmole L-propargylglycine

Complete oxidation required approximately 2 hours.

Buffers used:

a: 0.1 M Hepes, pH 8.

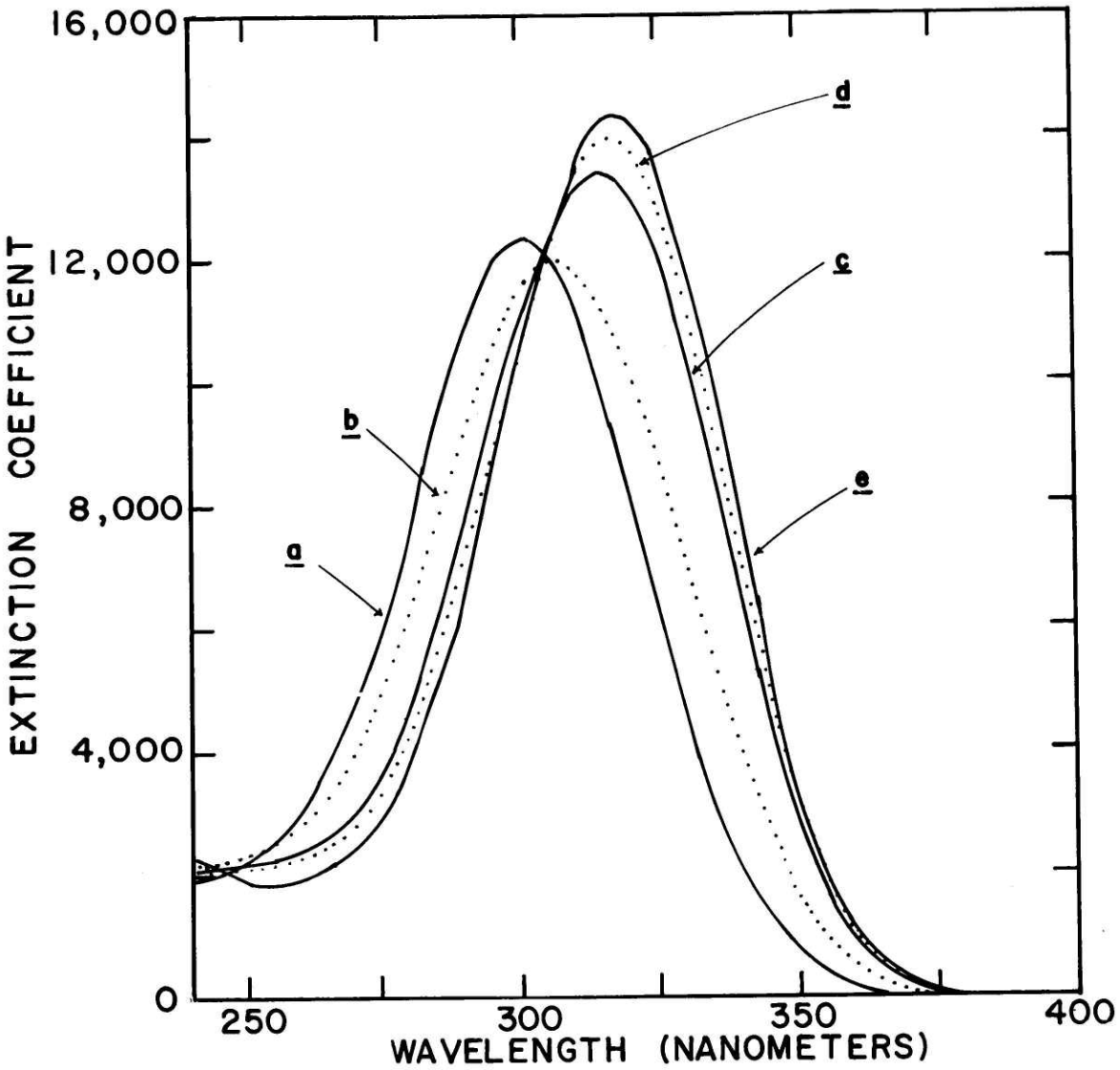
b: 0.1 M Hepes / 0.01 M butylamine , pH 8

c: 0.1 M Hepes / 0.05 M butylamine , pH 8

d: 0.1 M Hepes / 0.10 M butylamine , pH 8

e: 0.1 M Hepes / 0.20 M butylamine , pH 8

FIGURE 3


16,000
12,000
8,000
4,000
0
EXTINCTION COEFFICIENT
250
300
350
400
WAVELENGTH (NANOMETERS)
a
b
c
d
e

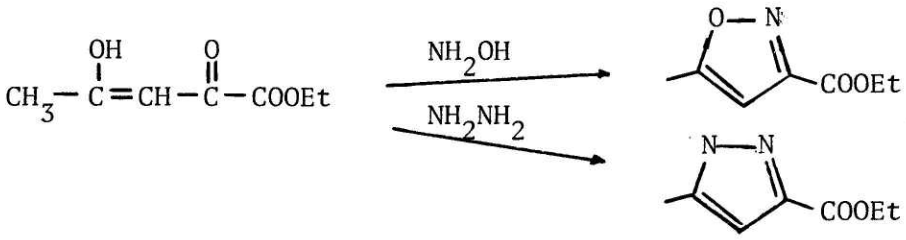
The results of the thin layer chromatographic comparison of the reaction product with authentic material was taken as evidence that the pathway of Scheme III represented a reasonable model of the reaction. However, further examination of the accumulating product showed that is was not comprised mostly of acetopyruvate. The first indication that a more complicated pathway was involved than had initially been considered was the finding that the product of propargylglycine oxidation at pH 8 is inert to sodium borohydride--at most 10% of the absorbance is reduced in 90 minutes. In contrast, a solution of acetopyruvate at pH 8 is reduced in seconds under the same conditions. The elucidation of the probable sturcture of the accumulating product was the subject of a great deal of work; as an aid in this structure determination, it is necessary to outline some properties of two compounds, acetopyruvic acid and the oxidation product of 2-amino-4-ketovaleric acid.

Properties of acetopyruvic acid.

The following data on authentic acetopyruvate were collected for comparison with the oxidation product. The absorption spectrum of the compound as a function of pH has been reported;[107] from the spectra pK_a's of 2.5 and 7.8 can be derived. Acetopyruvate is a poor substrate for lactic dehydrogenase catalyzed reduction by NADH[108], but it was found that addition of 250 μg commercial enzyme allows reduction of 50 nmoles of acetopyruvate in 90 minutes at pH 8.

Syntheses of 5-methyl-3-carboxyisoxazole and 5-methyl-3-carboxypyrazole are accomplished by treatment of ethyl acetopyruvate with hydroxylamine or

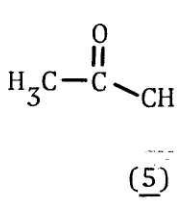
hydrazine, followed by saponification.[109 110]



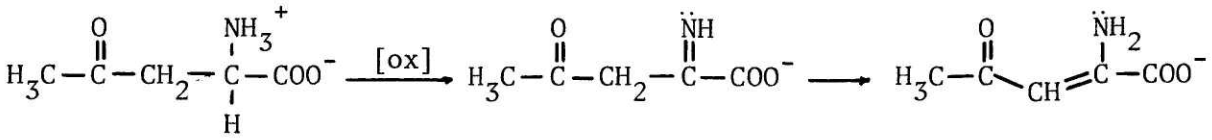
This was the rationale for addition of 10 mM hydrazine or 10 mM hydroxylamine to a buffered (pH 8) solution of 100 μM acetopyruvate. Both reagents cause the rapid (in seconds) bleaching of the spectrum. No product absorbance above 250 nm was observed in either case, consistent with the reported spectrum of the 3-carboxypyrazole nucleus.[111]

The oxidation product of 2-amino-4-ketovaleric acid.

After it was determined that acetopyruvic acid was not the accumulating product during the course of the oxidation, a possible alternative was the eneamine 2-amino-4-ketopentenoate (5). Although the properties of this material had not been reported, it was considered likely that the stabilization produced by conjugation and internal hydrogen bonding would make its hydrolysis to acetopyruvate slow in neutral solutions, and the same factors might make the compound inert to borohydride. The simplest route to this species is the enzymatic oxidation of 2-amino-4-ketovaleric acid with an amino acid oxidase.



(5)



The synthesis of the racemic amino acid via the acid hydrolysis of the condensation product of diethylacetamidomalonate and bromoacetone had been reported and was reproduced.[105]

Exhaustive oxidation of the amino acid with L-amino acid oxidase yielded a product with absorbance maximum 321 nm, ε 12,000 at pH 8. The prediction of the hydrolytic stability of this eneamine was proven correct, as no spectral change was observed over 20 hours at pH 8 and 4 hours at pH 12. However, it is acid labile; 10 minutes at pH 2 followed by neutralization yielded a spectrum identical to that of acetopyruvate.

This species is also stabilized relative to acetopyruvate in its reactions with hydrazine and hydroxylamine. On addition of 10 mM of these reagents, the eneamine spectrum (50 μM) is slowly bleached in a first order manner. At room temperature, the observed rate constant for hydrazine was 0.87 hr^{-1}; for hydroxylamine, 0.53 hr^{-1}. These properties will be found useful in later characterizations.

It is perhaps unnecessary to point out that this material is not the accumulated product of propargylglycine oxidation. However, the eneamine was found to have a role in the pathway, as will be discussed in the next section.

Properties of the 300 nm accumulated product.

Because of the small quantities (less than one μmole) of material available, characterization of the product was done in three ways.

1. Effect of various reducing agents on the absorbance.
2. Extractability of the material into organic solvents.

3. Acid or base titration of the product solution followed by neutralization and characterization of the products thereby produced.

A pH 8 solution of the material (λ_{max} 300 nm, ε 12,000) was prepared by exhaustive oxidation with catalytic L-amino acid oxidase of 50 nmoles of L-propargylglycine in 1 ml of 0.1 M Hepes, pH 8.0. A maxiumum of 10% of the material formed in this way is reducible with sodium borohydride. Likewise, only a small fraction is reduced by NADH/lactic dehydrogenase under conditions in which acetopyruvate is reduced.

The first positive data concerning the structure determination was found when the buffered solution containing the product was mixed with an equal volume of diethyl ether. Greater than 80% of the absorbance partitions into the organic phase under these conditions. As expected, both acetopyruvate and 2-amino-4-ketopentenoate were found to remain totally in the aqueous phase under identical conditions. The ether extractability of the product raised hopes that the isolation of the material could be accomplished. However, evaporation of the ether with a stream of dry argon resulted in the decomposition of the product, a yellowish residue with no defined absorption maxiumum when dissolved in buffer was obtained. Addition of a small amount of (fresh) buffer to the ether extract followed by evaporation as before was successful in producing an aqueous solution of the product fifty times the concentration of the enzymatic incubation without evident decomposition.

The acid and base labilities of the accumulated product were found to be of great value in determining its probable structure. Titration of the product solution to pH 2 resulted in changes which were time dependent and

irreversible. After 10 minutes, neutralization yielded a material the spectrum of which was identical to acetopyruvate in its pH dependence as well as being fully reducible by borohydride.

In view of the isolation of acetopyruvate after passage of the oxidation reaction mixture through DOWEX 50 H^+ as previously described, the acid lability of the material to acetopyruvate had been expected. It was the base lability of the accumulated product which yielded new information as to its structure.

Titration of a solution of the 300 nm product to pH 12 caused a rapid shift in the absorption maxiumum to 317 nm. This new absorbance did not change when the solution was returned to pH 8. The major contributor to this absorbance was identified as 2-amino-4-ketopentenoate (5) by the following criteria.

1. Addition of 10 mM hydroxylamine or 10 mM hydrazine caused the rapid (in seconds) bleaching of about 10% of the absorbance (presumably due to acetopyruvate content); the λ_{max} was also shifted by this treatment from 317 nm to 321 nm. The remainder of the absorbance bleached with the same first order rate constants reported earlier for the oxidation product of 2-amino-4ketovaleric acid.
2. Addition of solid sodium borohydride caused the bleaching of about 10% of the absorbance with a shift in λ_{max} from 317 nm to 321 nm. The remainder of the absorbance was stable to reduction.
3. Acidification of the 317 nm material to pH 2 followed by neutralization yielded a spectrum with properties identical to that of acetopyruvate.

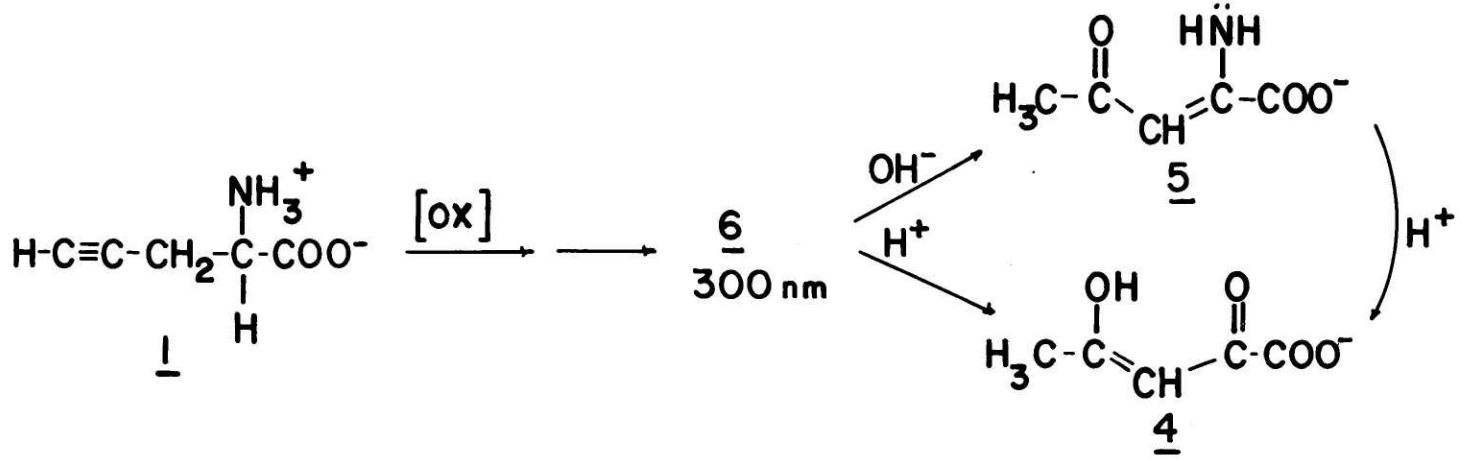
4. The 300 nm absorbing material recovered after ether extraction was quantitatively converted by acidification to acetopyruvate and to 2-amino-4-keto-2-pentenoate (the λ_{max} now shifting immediately to 321 nm) upon treatment with base.

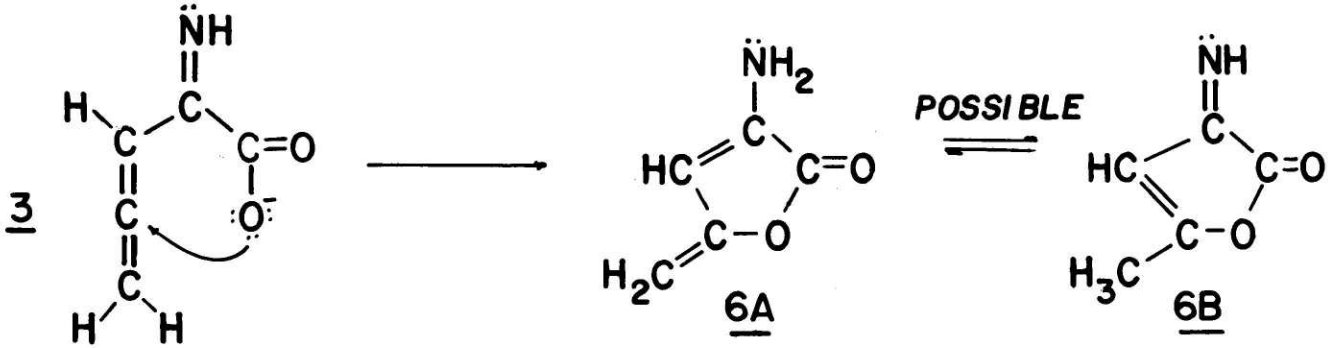
In Scheme IV is summarized the pathway which has been found to follow enzymatic oxidation of propargylglycine. At most 10% of the oxidation product is converted directly to acetopyruvate. The predominant reaction leads to the formation of a neutral species (required by its ether extractability) in which the nitrogen bound to carbon 2 has remained in place. The structure consistent with the experimental observations is the γ-lactone (6), produced by the attack of the carboxy oxygen on the electrophilic fourth carbon of the conjugated allene (Scheme IV). The lactone is neutral and accounts for the observed acid and base labilities of the 300 nm product.

Of the two possible lactones, the extended eneamine (6A) is believed to be the correct structure. There is no apparent reason that the imine (6B) should not be reducible by borohydride and should also be hydolyzed to the corresponding α-keto-γ-lactone. Spectral comparisons with the oxidation products of allylglycine and vinylglycine (to be presented) also favor the extended eneamine structure for the lactone.

Through an examination of the absorption spectrum of the product of propargylglycine, the probable structure of the accumulated product of the reaction has been deduced. While this study was in progress, another line of investigation was found to provide evidence on the mechanism of the formation of the 300 nm product, and led to identification of a transient

SCHEME IV





species which is a precursor of the eneamine lactone.

Formation of a D-amino acid oxidase charge transfer complex upon oxidation of D-propargylglycine.

On incubation with a large excess of D-propargylglycine, D-amino acid oxidase undergoes covalent modification after catalyzing several hundred oxidations. It had been assumed that incubation of the enzyme with many fewer equivalents of the suicide substrate than required for inactivation would result in rapid catalytic oxidation of the amino acid without demonstrable effect on the enzyme. Therefore, it was quite unexpected that, upon addition of 1-10 equivalents of D-propargylglycine to a spectroscopic quantity of D-amino acid oxidase, intense long wavelength absorbance characteristic of a charge-transfer complex is immediately (within seconds) evidenced. (Figure 4).

Addition of less than a stoichiometric quantity of D-propargylglycine to the enzyme resulted in the formation of an intensity of charge transfer absorbance equivalent to the amount of propargylglycine added. This demonstrates that the species responsible for the complex is produced quantitatively from propargylglycine and is bound very tightly to the enzyme (K_d < 10 μM). The species was also shown to be in rapid, non-covalent equilibrium with the enzyme by addition of 10 mM sodium benzoate (Figure 5). Since the rate of binding of this concentration of benzoate is much faster (it is greater than 200 sec^{-1}[112]) than the observed rate of decay of the 580 nm absorbance, this experiment demonstrates that the rate of release of the species responsible for the charge transfer complex is approximately

Figure 4. Spectrum of the complex between the oxidation product of D-propargylglycine and D-amino acid oxidase.
In 0.4 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5, the solution contained 13 nmoles of D-amino acid oxidase (a). To the enzyme solution was added 1 µl of 0.1 M D-propargylglycine. The spectrum of the complex was recorded immediately (b)

Figure 5. Rate of release from the enzyme of the propargylglycine product responsible for the charge transfer complex.
In 0.5 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5 at 10°C, the solution contained 21 nmoles of D-amino acid oxidase. At 0 seconds, 1 µl of 0.025 M D-propargylglycine (25 nmoles) was added to the solution. The absorbance change at 580 nmeters was observed. At 120 seconds, 10 µl 0.5 M sodium benzoate was added to the solution and the decay of the absorbance at 580 nmeters was observed.

FIGURE 4

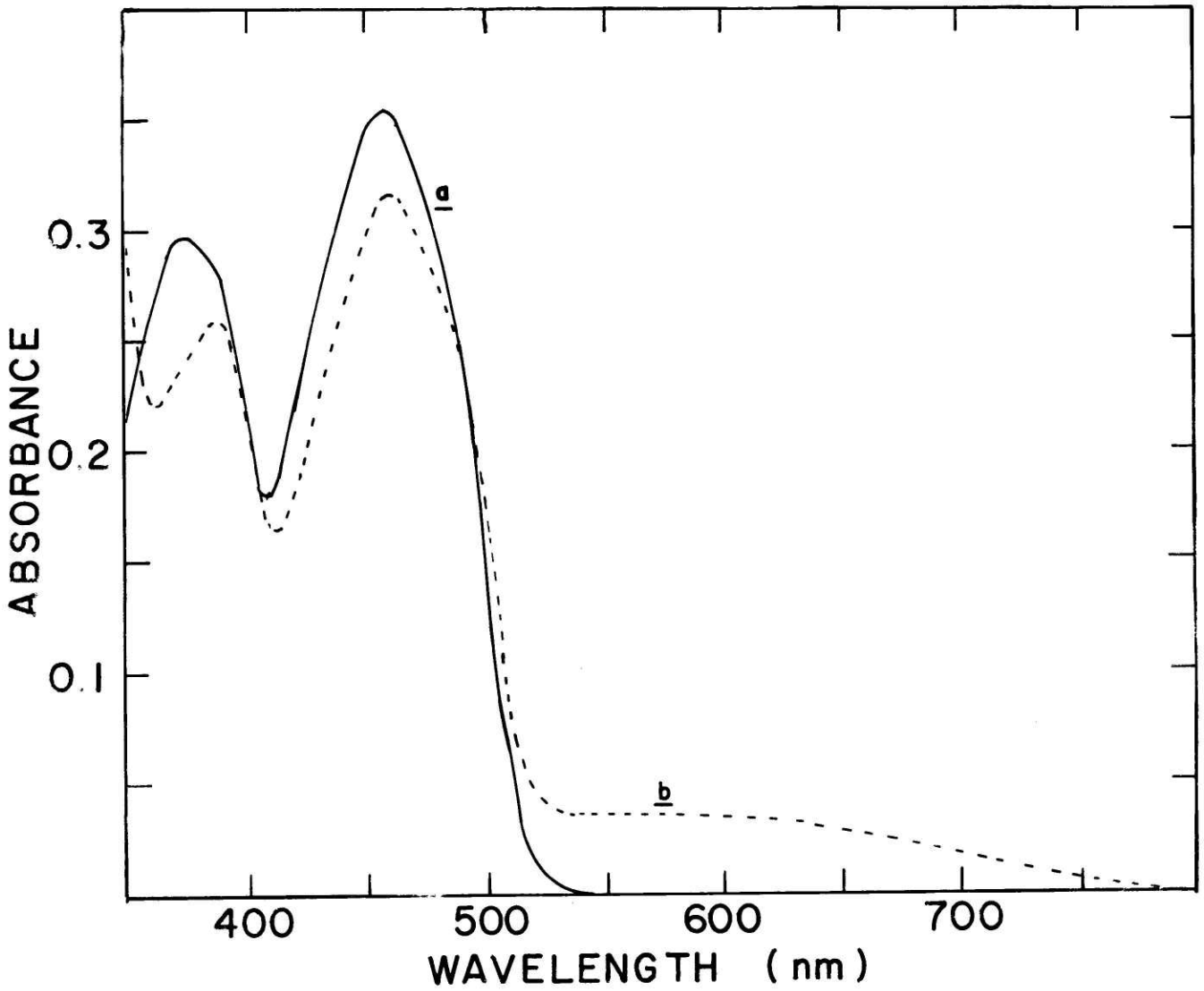
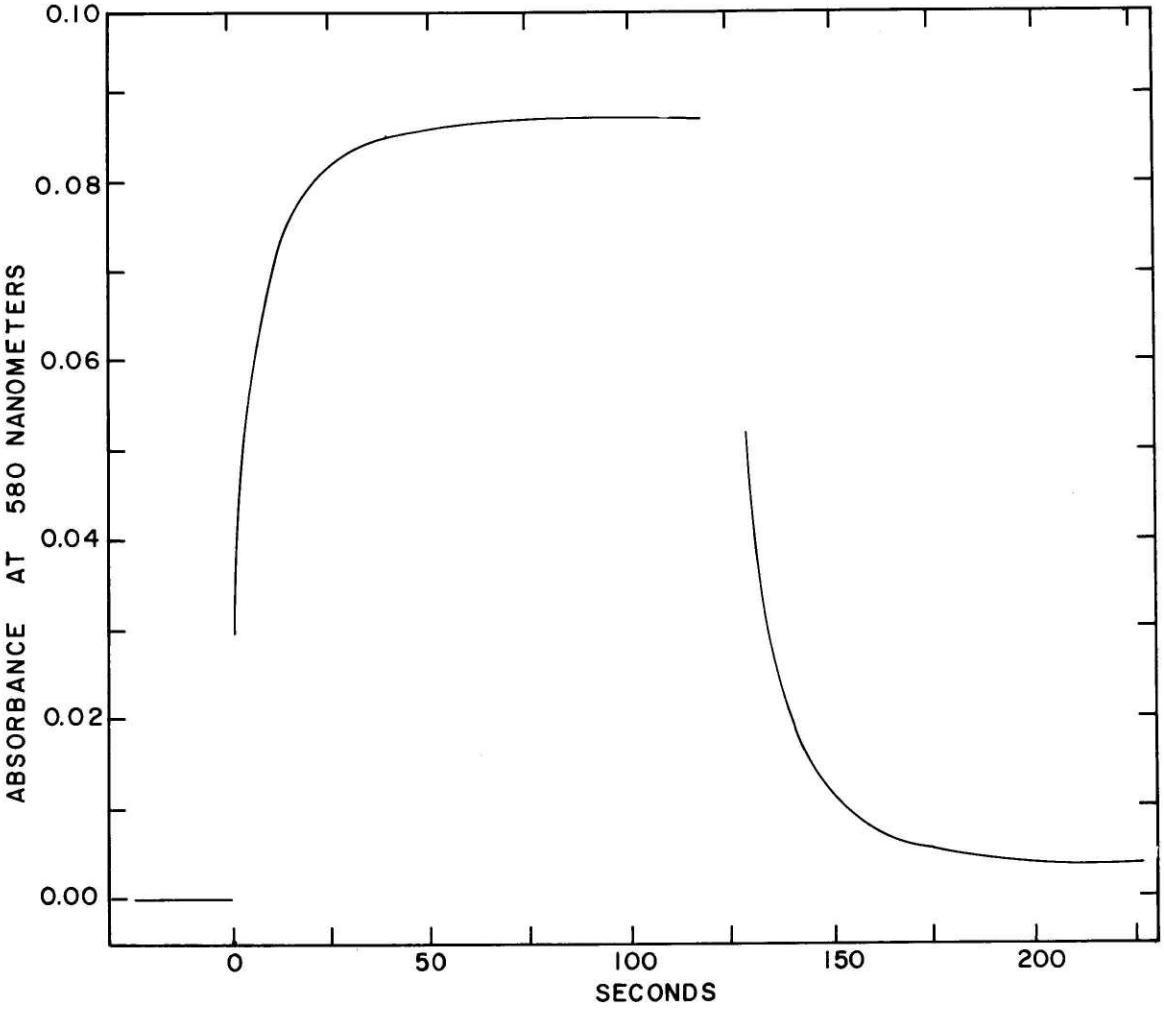


FIGURE 5


0.10
0.08
0.06
0.04
0.02
0.00
ABSORBANCE AT 580 NANOMETERS
0
50
100
150
200
SECONDS

$0.08\ sec^{-1}$.

Complexes produced by 2-amino-4-keto-2-pentenoate and acetopyruvate.

Addition of a slight stoichiometric excess of 2-amino-4-ketovaleric acid to D-amino acid oxidase also results in the rapid formation of a charge transfer complex. The visible spectrum of the species resulting from enzymatic binding of 2-amino-4-keto-2-pentenoate is shown in Figure 6. This eneamine is also bound very tightly (K_d < 10 μM) and was found to be in rapid equilibrium with the enzyme by the methods previously described for the propargylglycine product complex. Acetopyruvate also induces perturbations in the visible spectrum of D-amino acid oxidase upon binding (Figure 7). However, the changes are much less pronounced and it was found that acetopyruvate is bound much less tightly than the other inhibitors (K_i = 120 μM versus D-alanine).

Properties of the D-amino acid oxidase / propargylglycine product complex.

The charge transfer complex of D-amino acid oxidase and the propargylglycine product is not indefinitely stable. Over 60-90 minutes at 10°C, the long wavelength absorption decays (Figure 8). It does not return to the original spectrum; the residual long wavelength absorbance is that similar to that expected from the production in solution of a small quantity of 2-amino-4-keto-2-pentenoate.

Since addition of high concentrations of butylamine changes the ultraviolet spectrum of the accumulating products of propargylglycine oxidation, the affect of amines on the formation and decay of the charge

Figure 6. Spectrum of the complex between the oxidation product of 2-amino-4-keto-valeric acid and D-amino acid oxidase.

In 0.4 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5, the solution contained 13 nmoles of D-amino acid oxidase (a). To the enzyme solution was added 2 µl of 0.1 M DL-2-amino-4-keto-valeric acid (100 nmoles). The spectrum of the complex was recorded immediately. (b)

Figure 7. Spectrum of the complex between acetopyruvate and D-amino acid oxidase.

In 0.4 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5, the solution contained 13 nmoles of D-amino acid oxidase (a). To the enzyme solution was added 10 µl of 0.01 M acetopyruvate (100 nmoles). The spectrum of the complex was recorded immediately. (b)

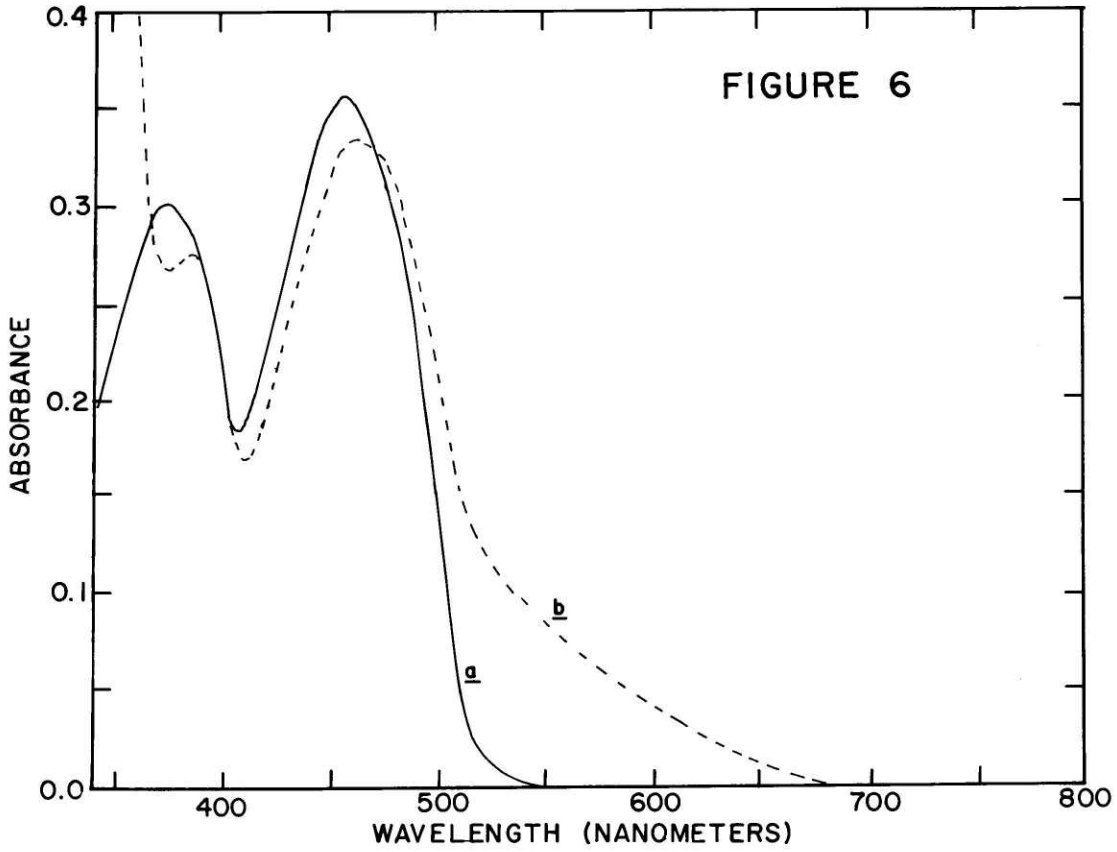


FIGURE 6

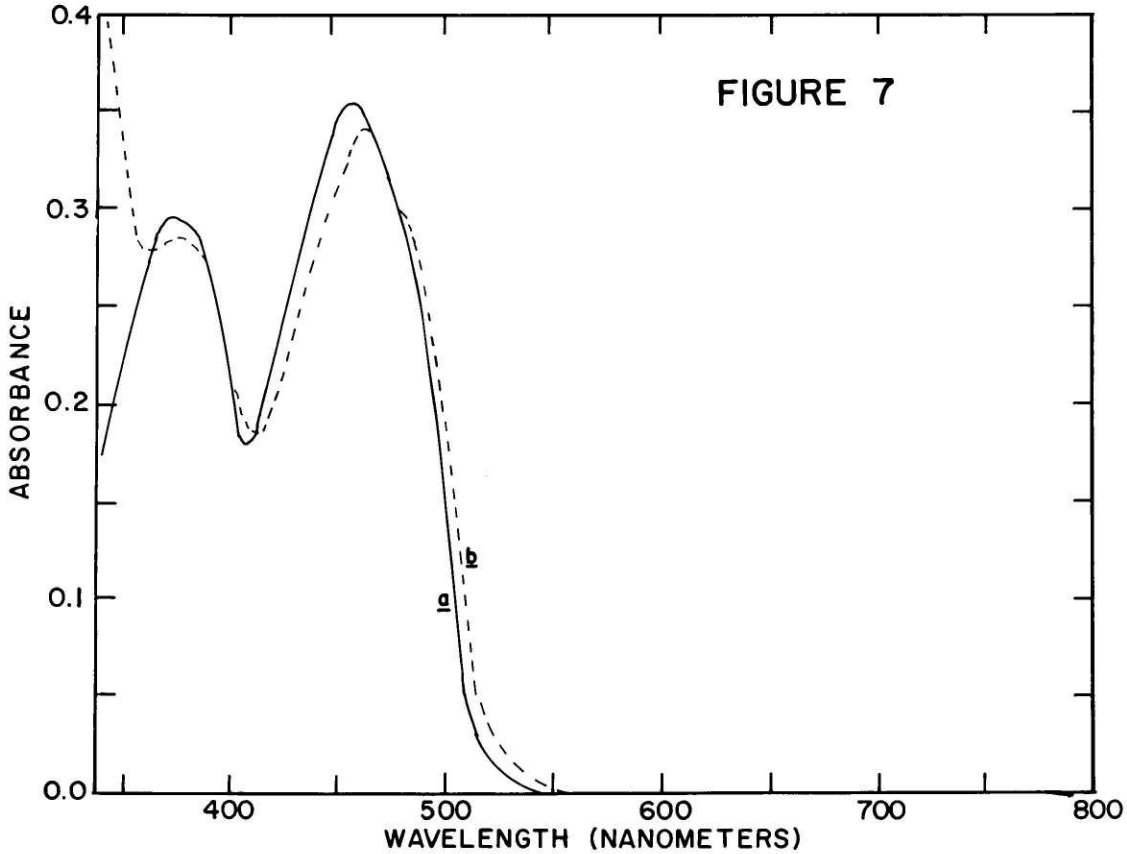


FIGURE 7

Figure 8. Stability of the D-amino acid oxidase / propargylglycine product complex.

In 0.5 ml 0.1 M Hepes, pH 8 at 10°C, the solution contained 8.5 nmoles of D-amino acid oxidase (a).

1 µl 0.0125 M D-propargylglycine (12.5 nmoles) was added to the solution and the spectrum recorded immediately (b).

Spectrum (c) was recorded 15 minutes after addition of propargylglycine.

No change in the spectrum was observed after 60 minutes, spectrum (d) was recorded 90 minutes after addition of propargylglycine.

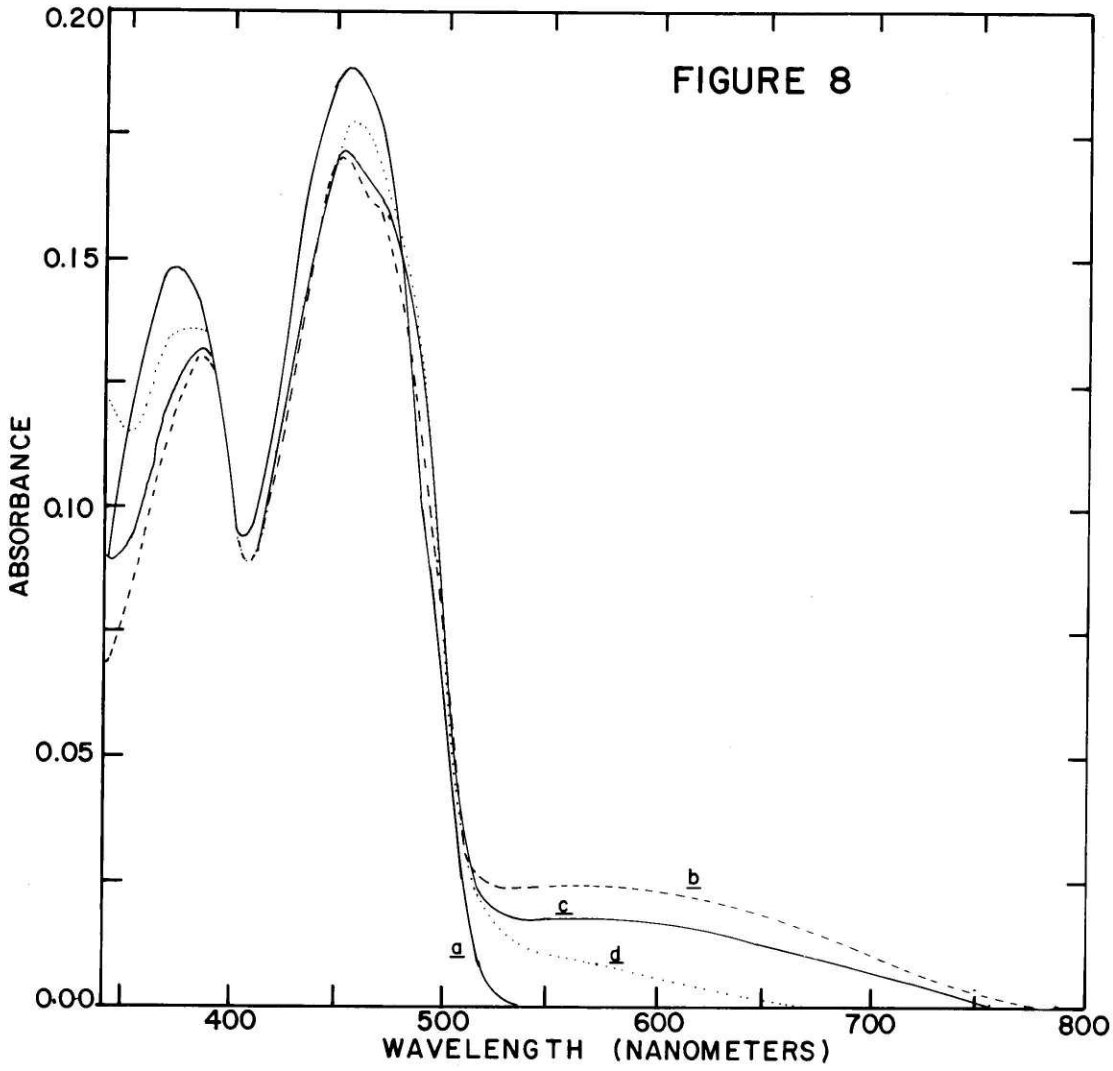


FIGURE 8
0.20
0.15
0.10
0.05
0.00
ABSORBANCE
400
500
600
700
800
WAVELENGTH (NANOMETERS)
a
b
c
d

transfer complex was examined. 160 mM butylamine HCl did not have any effect on the formation of the complex; the presence of amine did cause the spectrum to return over time to its original baseline, presumably by preventing the formation in solution of 2-amino-4-ketopentenoate.

The production of the D-amino acid oxidase / propargylglycine product charge transfer complex does not require the participation of the enzyme in the oxidation. This point was demonstrated by incubation of D-amino acid oxidase with a slight molar excess of L-propargylglycine (with no result) followed by a catalytic amount (1%) of L-amino acid oxidase. The identical charge transfer spectrum was generated over 20 minutes, demonstrating that finite amounts of the product species can diffuse through solution to the D-amino acid oxidase active site.

Should the species responsible for the charge transfer complex be the alkylating agent which causes the covalent modification, or be in equilibrium with that agent at the enzyme active site, it is possible that in experiments with high concentrations of enzyme and only a slight excess of propargylglycine, inactivation following many fewer than the several hundred in catalytic assays might be observed . This would be a consequence of the charge-transfer inducing species being bound to the enzyme for longer periods, instead of being replaced (competitively) at the active site by another molecule of propargylglycine as in catalytic assays with excess substrate.

The charge transfer complex was found not to be in any demonstrable way connected with the covalent modification of the enzyme--no kinetic inactivation (by assay of withdrawn aliquots) was observed during the lifetime

of the long wavelength absorbance. An effort was made to produce the modified enzyme by incubation of D-amino acid oxidase, L-propargylglycine, and L-amino acid oxidase. Under conditions in which L-propargylglycine was oxidized continually over a two hour period, no alkylation of D-amino acid oxidase was observed. A strong competitive inhibitor(s) was produced in the incubation, but following dialysis enzyme with native kinetic parameters was recovered.

The relatively short lifetime of the charge transfer complex, and the evidence that the species responsible is formed before the species subject to attack by amines, made it unlikely that the 300 nm accumulating product was the cause of the observed complex. Addition of the ether extracted product (recovered into buffer as previously described) to a solution of D-amino acid oxidase resulted in no change in the visible spectrum of the enzyme. Since both the species responsible for the charge transfer complex and the 300 nm product are formed in high yield, it is necessary to postulate that the charge transfer inducing species is a precursor of the 300 nm product.

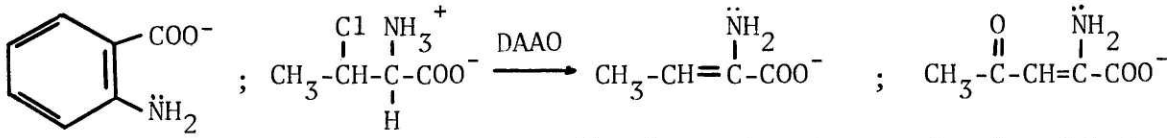
Direct observation of the initial product of propargylglycine oxidation.

All of the spectral evidence on the products of propargylglycine oxidation thus far discussed involved the exhaustive oxidation of propargylglycine with a catalytic quantity of enzyme. Therefore, several hours at room temperature was necessary for complete oxidation to be accomplished. To directly observe a species produced early in the reaction, a change in experimental procedure was required.

The procedure developed involved the use of a large quantity (4 nmoles) of D-amino acid oxidase incubated at 8°C with D-propargylglycine (50 nmoles) for 30 seconds. This interval is not sufficient for complete oxidation; therefore, 1 μmole of the tight competitive inhibitor (K_d = 3 μM)[113] sodium benzoate was added, which slowed continued oxidation sufficiently that spectral changes with time of the products could be observed (Figure 9).

The absorbance maximum which characterizes the product of propargylglycine oxidation is dependent on the nucleophilic species present in solution. As shown in Figure 9A and 9B, the same species appears to be the precursor of both the 300 nm species (in Hepes buffer) and the 318 nm species (in Hepes/butylamine HCl buffer); it has a λ_{max} 290 nm, $\varepsilon \simeq 10{,}000$ and has a lifetime of 5-10 minutes under these conditions.

A number of stable and metastable molecules have previously been found to induce intense charge transfer bands upon binding to D-amino acid oxidase. Two such species are anthranilate,[113] a stable molecule, and 2-aminocrotonate,[114] a transient species produced from D-amino acid oxidase catalyzed elimination of HCl from β-chloro-α-aminobutyrate.



anthranilate ; 2-aminocrotonate ; 2-amino-4-keto-2-pentenoate

These molecules have the common feature of being electron rich eneamino acids, and serve as donor components to the electron deficient oxidized flavin coenzyme. A third such eneamine, 2-amino-4-keto-2-pentenoate, has been

Figure 9. Direct observation of the initial product of the oxidation of propargylglycine.

In 2 ml of buffer the solution contained:

8.5 nmoles of D-amino acid oxidase

25 µg catalase.

0.9 ml of this solution was placed in each of two matched silica cells at 8°C. 10 µl 0.1 M soldium benzoate was added to the reference cell.

At T = 0 sec., 1 µl 0.05 M D-propargylglycine added to sample; after 30 sec., 10 µl 0.1 M sodium benzoate was added to sample. Spectra were recorded as rapidly as possible (240 nm scan/min, about 1 spectrum/min).

Buffers used:

A: 0.1 M Hepes, pH 8. Spectra at the following time points are reproduced.

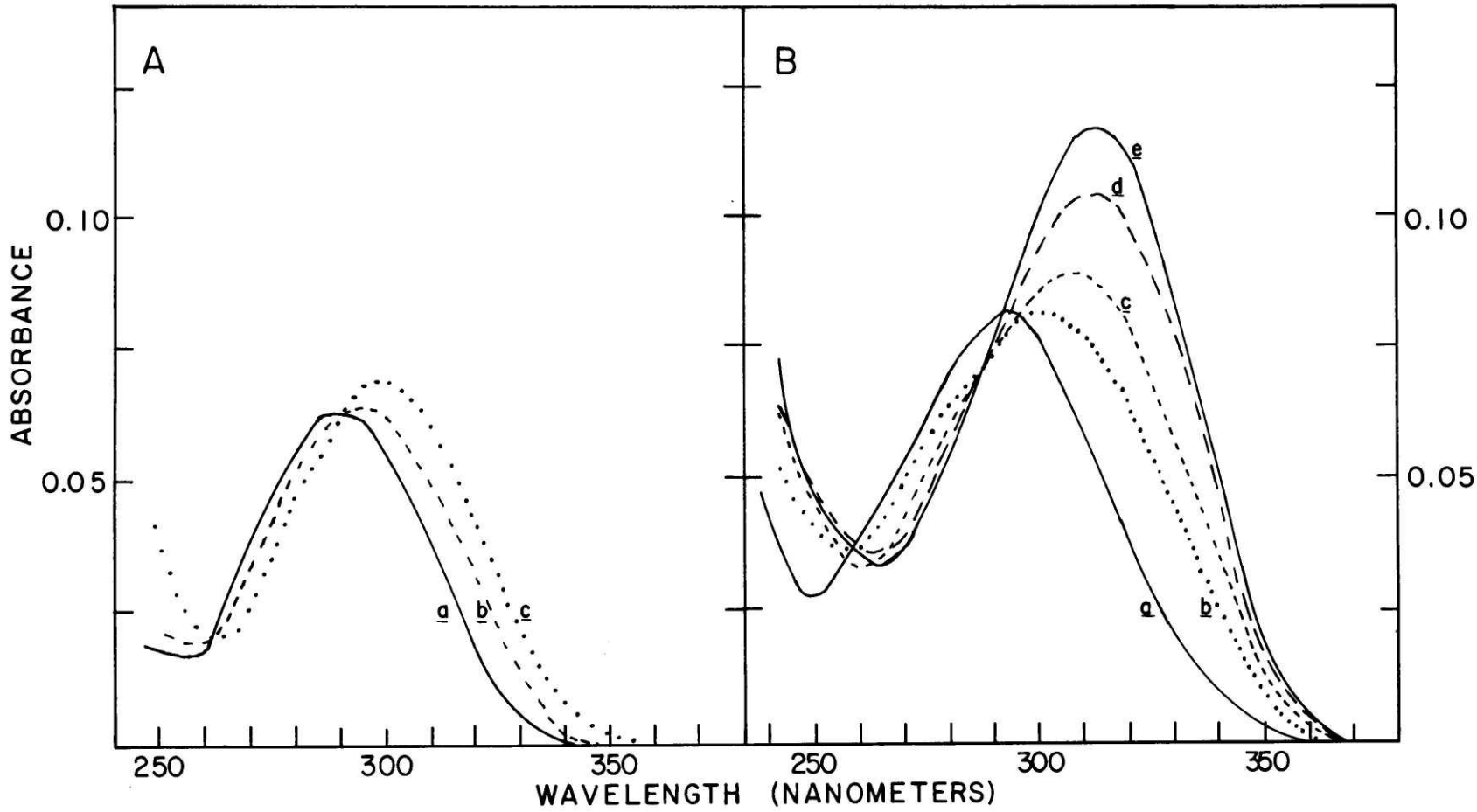
(a): 1 minute (b): 7 minutes (c): 15 minutes

B: 0.1 M Hepes / 0.1 M butylamine HCl pH 8. Spectra at the following time points are reproduced.

(a): 1 minute (b): 3 minutes (c): 5 minutes

(d): 10 minutes (e): 15 minutes

FIGURE 9


A
B
0.10
0.05
ABSORBANCE
a
b
c
d
e
250
300
350
WAVELENGTH (NANOMETERS)

shown in this work to be the cause of a charge transfer complex.

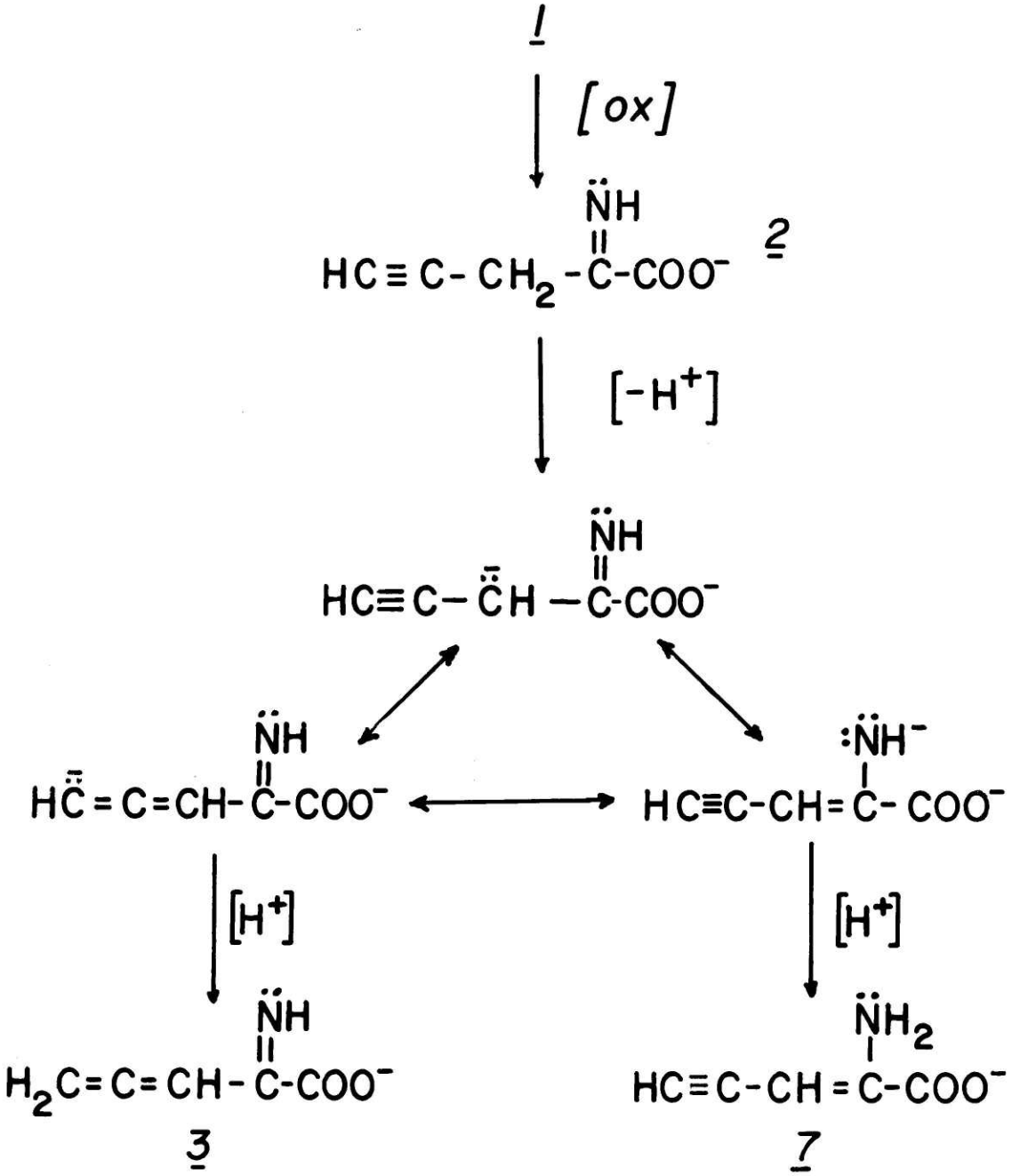
Therefore, the probable identity of the initial propargylglycine product is the acetylenic eneamino acid, (7) of Scheme V. It is derived from protonation at nitrogen of one resonance form of the carbanion intermediate produced by the removal of an acidic β-proton of the acetylenic imine oxidation product (2). The probable inactivator (3) is produced by the protonation at carbon 5 of the allenic anion which is another resonance form of the carbanion. There is no evidence available as to the geometrical isomerism of the acetylenic eneamine; therefore it is depicted in Scheme V without prejudice on this point.

Should the assignment of structure (7) to the species responsible for the charge transfer complex be correct, the evidence requires that protonation of the anion at nitrogen be much faster than that at carbon. Furthermore, the rearrangement of (7) to (3) which must occur to account for the observed accumulated products, must be a relatively slow process. The long lifetime of the charge transfer complex relative to the lifetime of the species free in solution can be explained by the very low dissociation of the complex, which reduces to a very small fraction the amount of the species which is free in solution.

The products of enzymatic oxidation of allylglycine and vinylglycine.

The tentative assignment of the acetylenic eneamine structure (7) to the species responsible for the charge transfer complex, led to the examination of the oxidation of allylglycine by the amino acid oxidases for corroboration of the reaction pathway postulated for propargylglycine. Oxidation

SCHEME V

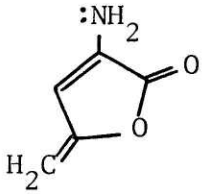

I
[ox]
NH
HC≡C-CH2-C-COO⁻
2
[-H⁺]
NH
HC≡C-CH-C-COO⁻
NH
HC=C=CH-C-COO⁻
:NH⁻
HC≡C-CH=C-COO⁻
[H⁺]
[H⁺]
NH
H2C=C=CH-C-COO⁻
3
NH2
HC≡C-CH=C-COO⁻
7

of this olefinic amino acid (Scheme VIa) could yield a product which might rearrange to a similar conjugated eneamine, albeit olefinic rather than acetylenic. Upon incubation of D-amino acid oxidase with allylglycine, a charge transfer complex is formed, characterized by intense absorbance from 600-800 nm (Figure 10).

It was also found that upon oxidation of allylglycine by L-amino acid oxidase, a species is produced with λ_{max} 285 nm, ε 9000 (Figure 11). This species has a short lifetime, the absorbance decays with a $t_{1/2}$ = 9 minutes at 30°C. This evidence is consistent with the extended eneamine from the oxidation of propargylglycine and allylglycine being the cause of the charge transfer complexes and ultraviolet product absorbance.

Another possibility was that the conjugated keto or imino acids are responsible for the charge transfer complexes. However, the oxidation of vinylglycine (Scheme VIb) does not lead to a charge transfer complex with D-amino acid oxidase (Figure 12). Also, it was found that the product from the oxidation of vinylglycine has no absorbance at wavelengths above 250 nm. This makes it unlikely that the conjugated keto or imino acids are responsible for the charge transfer spectrum of allylglycine and (by inference) propargylglycine.

The ultraviolet absorbance of the oxidation products of allylglycine and vinylglycine also corroborate the proposed structure of the accumulated product as the extended eneamine lactone (6A) instead of the conjugated

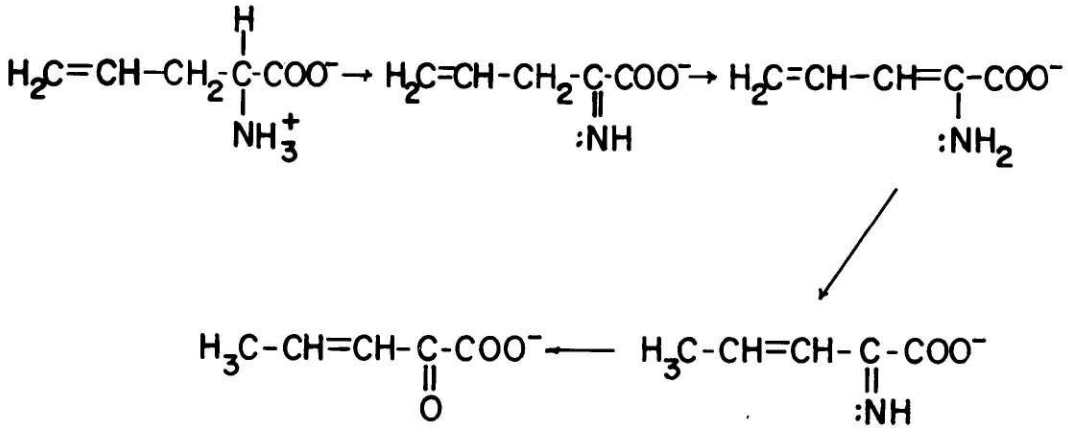


6A

tautomer (6B). 6A has a chromophore not dissimilar from the allylglycine oxidation product. On the other hand, the vinylglycine oxidation

SCHEME VIa

ENZYMATIC OXIDATION OF ALLYLGLYCINE



SCHEME VIb

ENZYMATIC OXIDATION OF VINYLGLYCINE

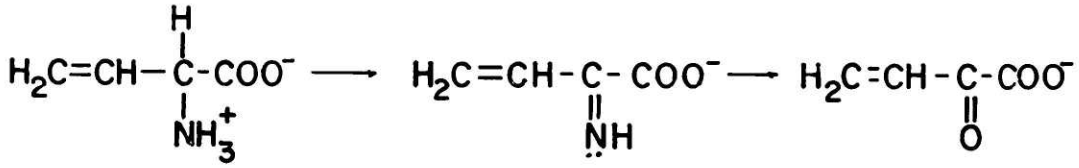


Figure 10. Spectrum of the complex between the oxidation product of allylglycine and D-amino acid oxidase.

In 0.5 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5, the solution contained 8.7 nmoles of D-amino acid oxidase (a). To the enzyme solution was added 2 µl of 0.1 M DL-allylglycine. The spectrum of the complex was recorded immediately (b).

Figure 11. Spectrum of the product of enzymatic oxidation of allylglycine.

In 1 ml of 0.1 M Hepes, pH 8 at 0°C, the solution contained:

125 µg L-amino acid oxidase

25 µg catalase

The reaction was initiated with the addition of 1 µl of 0.1 M DL-allylglycine, pH 8. The spectrum of the solution was recorded every two minutes after the addition of the amino acid. The reference cell contained all species except allylglycine.

A. Spectra taken at the following times are reproduced:

(a): 3 minutes

(b): 9 minutes

(c): 17 minutes

(d): 27 minutes

B. A plot of log A_{285} versus time for all of the time points taken in this experiment.

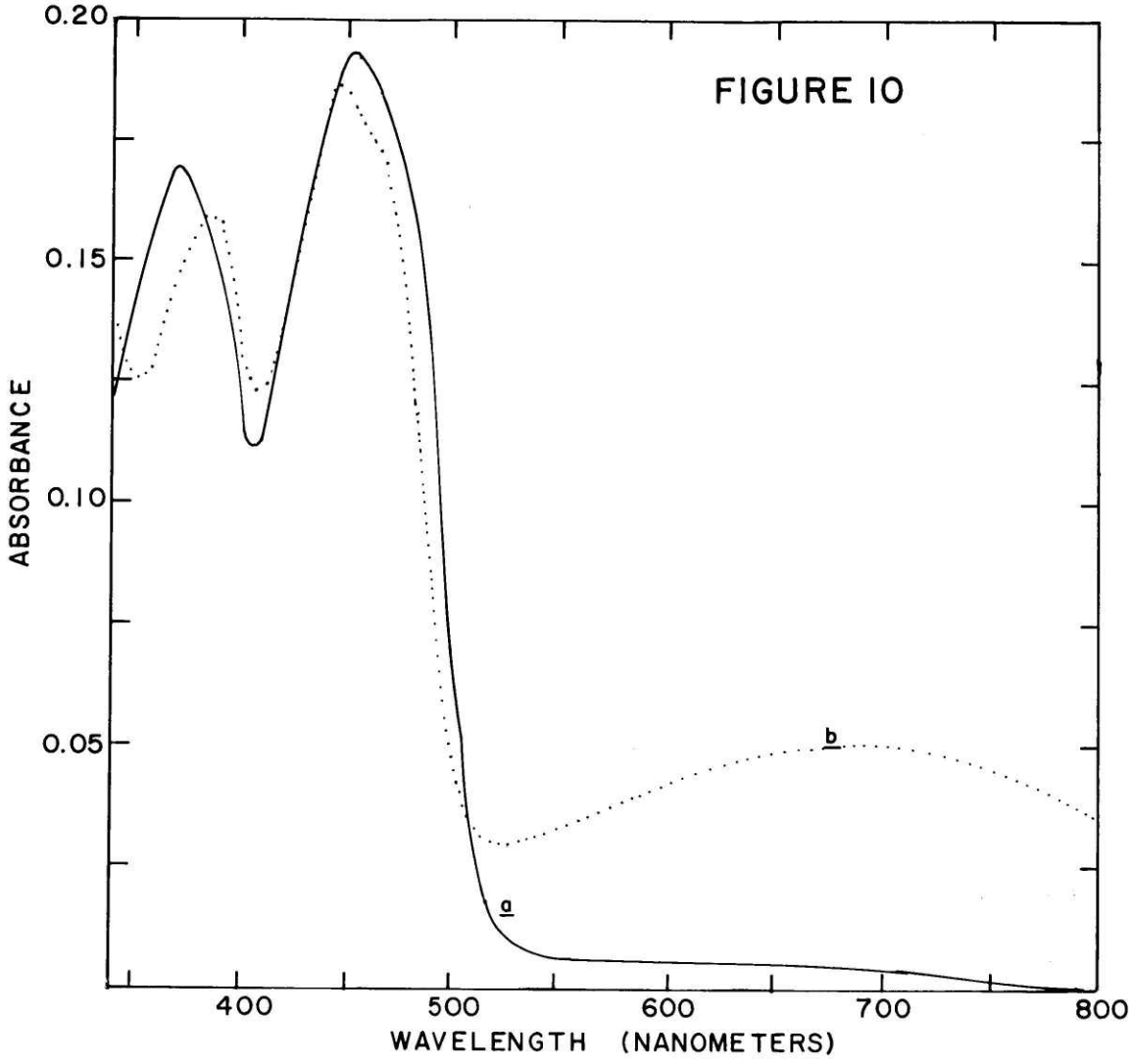


FIGURE 10
0.20
0.15
0.10
0.05
ABSORBANCE
400
500
600
700
800
WAVELENGTH (NANOMETERS)
a
b

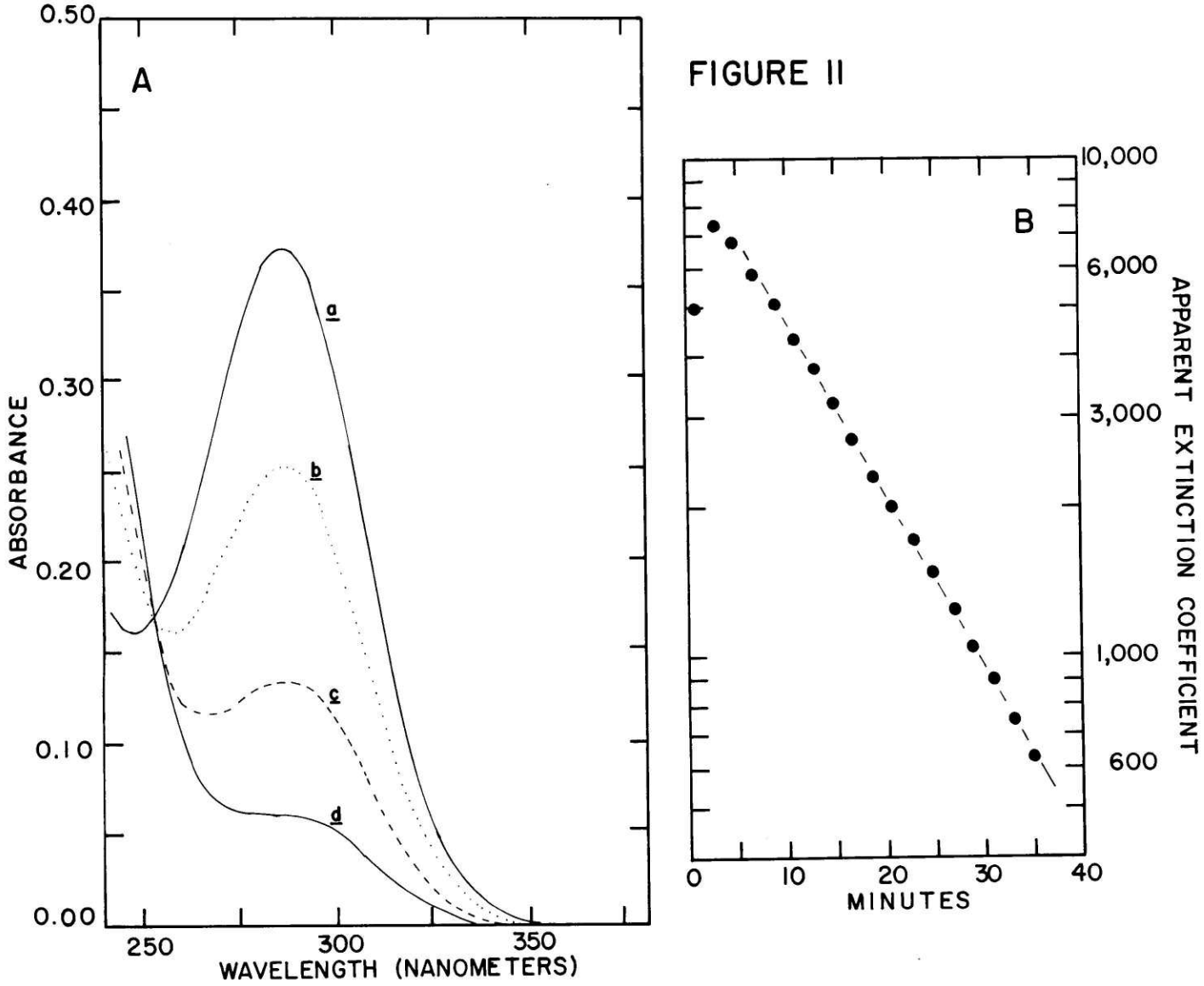

A
B
0.50
0.40
0.30
0.20
0.10
0.00
ABSORBANCE
250
300
350
WAVELENGTH (NANOMETERS)
a
b
c
d
10,000
6,000
3,000
1,000
600
APPARENT EXTINCTION COEFFICIENT
0
10
20
30
40
MINUTES

FIGURE II

Figure 12: Spectrum of the complex between the oxidation product of vinylglycine and D-amino acid oxidase.
In 0.5 ml of 0.05 M sodium pyrophosphate buffer at pH 8.5, the solution contained 8.7 nmoles of D-amino acid oxidase (a). To the enzyme solution was added 2 µl of 0.1 M DL-vinylglycine. The spectrum of the complex was recorded immediately (b).

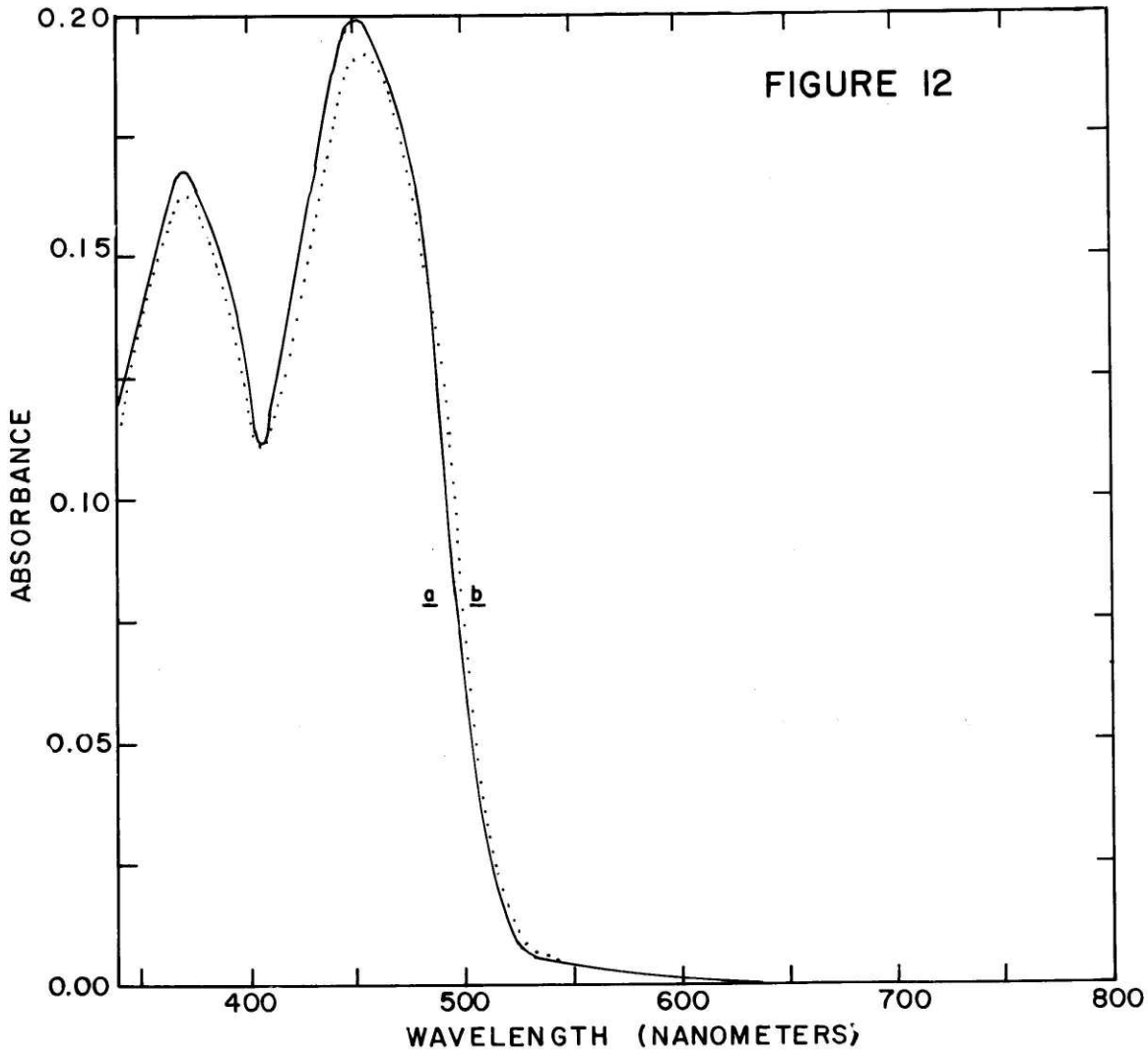
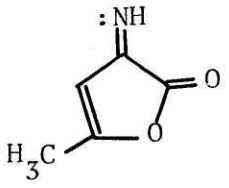


FIGURE 12
0.20
0.15
0.10
0.05
0.00
ABSORBANCE
a
b
400
500
600
700
800
WAVELENGTH (NANOMETERS)



6B

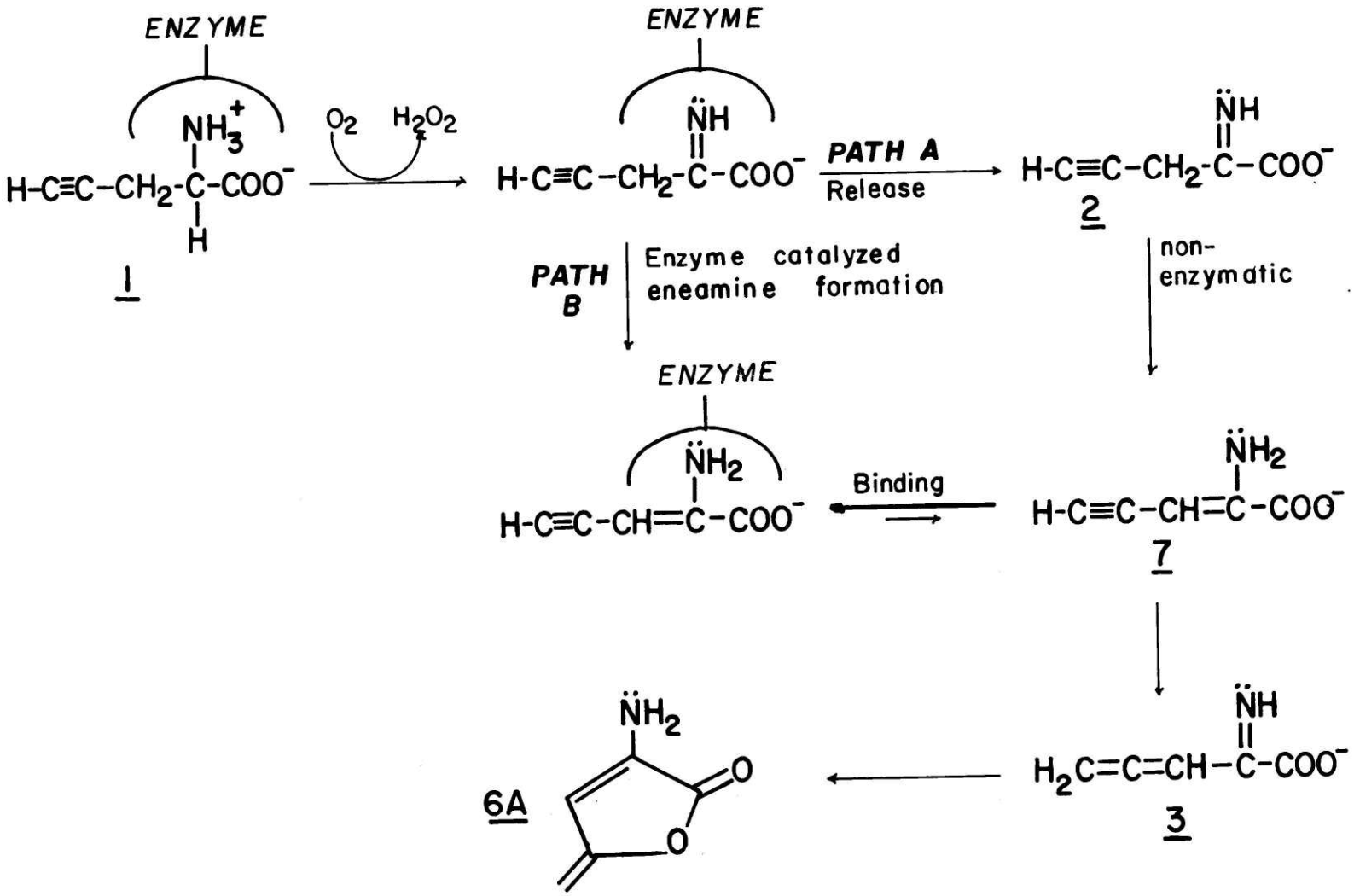
demonstrates that the structure 6B would not be expected to exhibit a λ_{max} 300 nm, ε 12,000.

Evidence pertaining to the first step of the pathway.

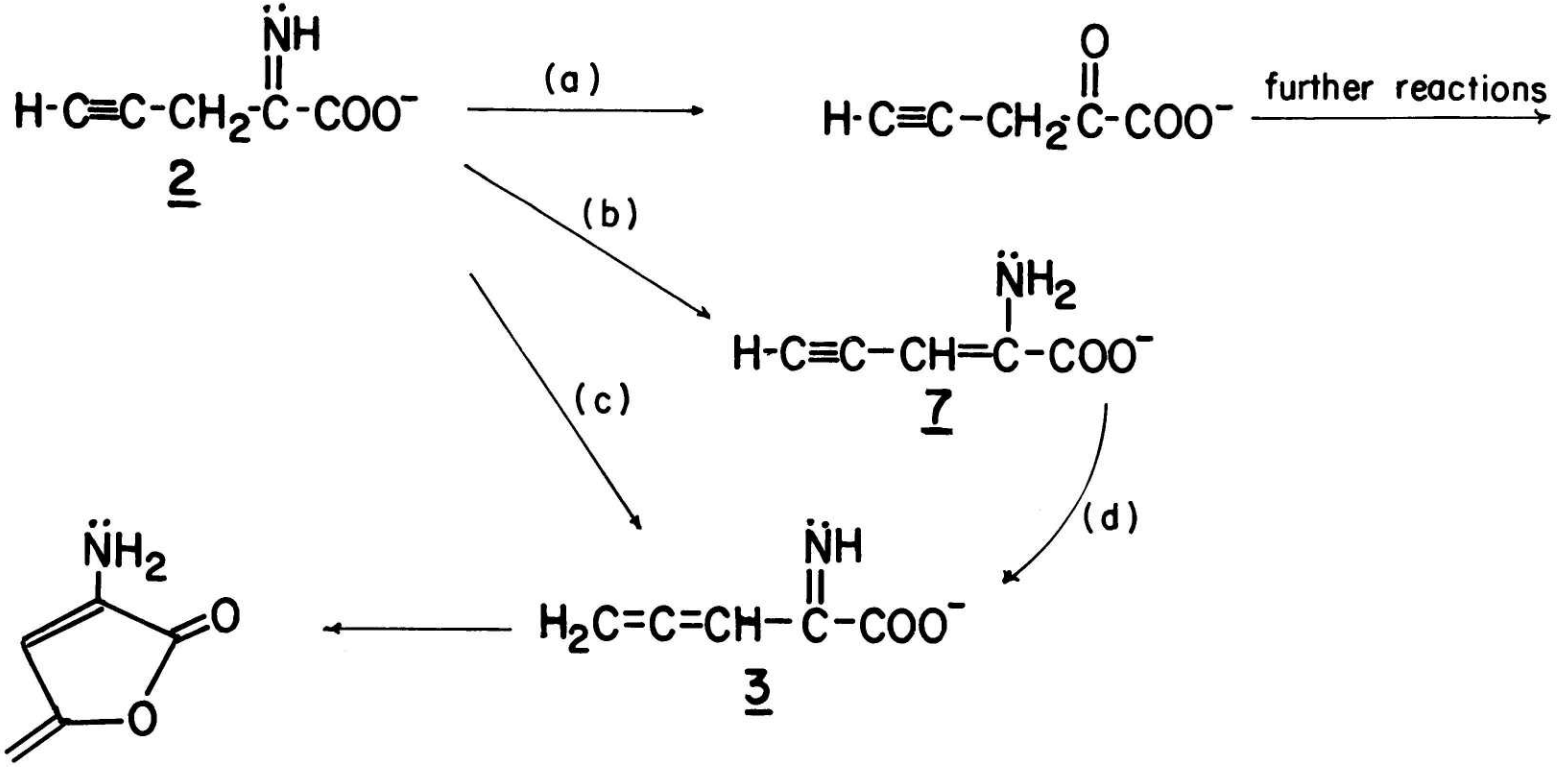
With the probable assignment of the acetylenic eneamine as the species responsible for the charge transfer complex, the question arises (Scheme VII) as to whether (path A) the enzyme catalyzes the conversion of the acetylenic imine to the eneamine or whether (path B) the enzyme releases the imine followed by its rapid non-enzymatic conversion to the eneamine and rebinding to the enzyme. This question can be answered by direct comparison of the maximal initial turnover number of catalytic propargylglycine oxidation with the rate of release of the acetylenic eneamine. The former constant can be measured by direct observation of the initial rate of increase in product absorbance for a given quantity of enzyme, and has been found to be 2 sec^{-1} at 10°C. The latter constant as already been determined (Figure 5), and was found to be 0.08 sec^{-1} at 10°C. This rules out enzyme catalyzed production of the eneamine as a normal step in the catalytic oxidation of propargylglycine.

In view of the preceding discussion, a more complete analysis of the first step in the pathway following oxidation of propargylglycine can be made. Following release of the acetylenic imino acid product (2), three non-enzymatic processes can be imagined (Scheme VIII): (a), hydrolysis of (2) to the corresponding acetylenic keto acid followed by further reactions; (b), direct conversion of (2) to (7); or (c), conversion of (2)to the conjugated allene (3). The evidence presented conclusively

SCHEME VII


ENZYME
NH3+
H-C≡C-CH2-C-COO-
H
1
O2
H2O2
ENZYME
NH
H-C≡C-CH2-C-COO-
PATH A
Release
H-C≡C-CH2-C-COO-
2
PATH B
Enzyme catalyzed eneamine formation
non-enzymatic
ENZYME
NH2
H-C≡C-CH=C-COO-
Binding
NH2
H-C≡C-CH=C-COO-
7
NH2
O
O
6A
NH
H2C=C=CH-C-COO-
3

SCHEME VIII


NH
H-C≡C-CH2-C-COO⁻
2
(a)
O
H-C≡C-CH2-C-COO⁻
further reactions
(b)
NH2
H-C≡C-CH=C-COO⁻
7
(c)
(d)
NH
H2C=C=CH-C-COO⁻
3
NH2
O
O
6A

proves that (b) is by far kinetically predominant. The further reactions of the pathway require that (7) be converted to (3) in a subsequent slower step (d). This step must also have no enzymatic catalysis, otherwise covalent modification of the enzyme would be a consequence of the charge transfer complex--this has been found not to be the case.

The complete pathway following enzymatic oxidation of propargylglycine has now been presented. Because of the short lifetimes of a number of species involved and the low concentrations available for characterization, a number of the points in the mechanism must be considered speculative. This analysis of the pathway will be of value in the discussion in the following sections on other features of the reaction of propargylglycine and D-amino acid oxidase.

2. PREPARATION AND PROPERTIES OF D-AMINO ACID OXIDASE COVALENTLY MODIFIED UPON ITS OXIDATION OF D-PROPARGYLGLYCINE

EXPERIMENTAL SECTION

Materials. DL-propargyl[2-^{2}H]glycine was prepared by acid hydolysis of propargyldiethylacetamidomalonate[45] in $^2HCl/^2H_2O$ purchased from Stohler isotopes. D-[2-^{2}H]alanine was prepared by an adaptation of the $CuSO_4$/salicylaldehyde mediated exchange procedure of Ikawa and Snell.[115] DL-[2-^{2}H]phenylalanine was purchased from MSD Isotopes. All other amino acids were purchased from Sigma chemical.

Incubation of D-amino acid oxidase and D-propargylglycine. D-propargylglycine (68 mg, 0.6 mmole) was dissolved in 3.0 ml 0.1 M sodium pyrophosphate, pH 8.0. To this solution was added 25 µg catalase and 0.25 µmole FAD. The reaction was started by addition of 66 nmoles D-amino acid oxidase in 0.75 ml buffer. The solution was protected from light and gently stirred in a 25 ml erlenmeyer flask for up to 8 hours. When the incubation was completed, the solution was dialyzed twice against 500-1000 volumes of 1 M KBr/0.1 M pyrophosphate, pH 8.5 at 0°C for 24 hours each dialysus. The salt was removed by dialysis against 0.02 M pyrophosphate buffer. The enzyme was reconstituted with excess commercial FAD and the excess removed by dialysis against two changes of 0.02 M pyrophosphate buffer.

Assays of oxidase activity (both native and modified enzyme). The oxidation of all amino acids, except D-alanine and D-propargylglycine, was assayed

oxygen consumption using a Clarke-type electrode (Yellow Springs Instruments model 53 oxygen monitor) in a 0.4 ml chamber thermostated at 30°C. D-alanine oxidation was monitored in the same buffer by coupled assay of pyruvate reduction by NADH and L-lactic dehydrogenase. Oxidation of D-propargylglycine was measured directly from the absorbance at 317 nm of the product of propargylglycine oxidation in 0.1 M Hepes/0.1 M butylamine HCl, pH 8. 10 μM FAD and 25 μg/ml catalase were added to all assay solutions and termperature was maintained at 30°C.

Spectral characterization of the modified D-amino acid oxidase. All spectra were recorded while the sample was maintained at 8-10°C to minimize turbidity in the enzyme samples. 0.5 ml of enzyme preparation was used. Aliquots of 0.5 M sodium benzoate, 0.5 M sodium anthranilate, and 0.1 M D-phenylalanine were added to the modified enzyme solution to determine the effects induced by these compounds.

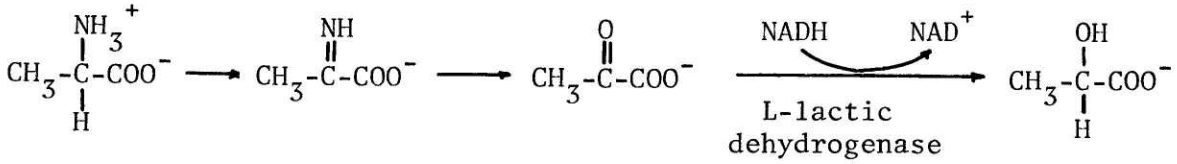
Incubation of D-amino acid oxidase with propargyl[2-^{14}C]glycine. In 0.45 ml 0.5 M potassium phosphate pH 8 containing 0.2 μg catalase and 0.02 mM FAD, 21 nmoles of D-amino acid oxidase was incubated with 7 mg DL-propargyl[2-^{14}C]-glycine for three hours. The solution was dialyzed twice against 500 ml 1 M KBr/ 0.05 M pyrophosphate pH 8.3, followed by dialysis against pyrophosphate buffer. The solution was passed through a Sephadex G-25 column (1.2 x 50 cm). Fractions containing protein were located by their absorbance at 280 nm; protein was assayed by the method of Lowry.[84] The radioactivity associated with the protein was measured by diluting an aliquot of the fraction into scintillation fluid.

RESULTS

Catalytic inactivation of D-amino acid oxidase.

The inactivation of D-amino acid oxidase which is a consequence of its oxidation of D-propargylglycine can be monitored in a number of ways. The simplest procedure is the direct observation of the ultraviolet absorbance of the product of enzymatic oxidation of propargylglycine. The rapid loss of activity is evident in such an assay; also apparent is the residual activity of the enzyme which is insensitive to inactivation. This method has the advantage of allowing the direct calculation of the number of catalytic oxidations before the modified enzyme is produced; in Figure 13 the number of turnovers is demonstrated to be ∿1800.

The amino acid most commonly used to assay the activity of D-amino acid oxidase is D-alanine. Although not the optimal substrate for the enzyme, the oxidation of D-alanine to pyruvate is easily followed by the coupled reduction of pyruvate with NADH catalyzed by (commercially available) L-lactic dehydrogenase.



The decrease in absorbance at 340 nm (ε 6220) as NADH is consumed provides a sensitive assay of pyruvate formation.

The propargylglycine induced inactivation of D-amino acid oxidase can thus be follwed by removal of aliquots of an incubation and determining the enzyme activity by diluting the aliquot into a solution of D-alanine (Figure 14A). This was the method the original workers, Horiike et al,[51] used to monitor

the inactivation.

At first, the work of Horiike et al could not be reproduced; approximately 2-4% residual activity with D-alanine was observed on prolonged incubation with propargylglycine, compared to the previously reported 20%. The contradiction is resolved by the fact that the residual activity observed is strongly dependent on the concentration of D-alanine used to assay the incubation. Since the concentration of D-alanine initially used in this work (10 mM) is well above the K_m of D-alanine oxidation by native enzyme (1.2 mM), a possible explanation was that the modified enzyme, in addition to having a much reduced V_{max}, has a greatly elevated K_m over that of the native enzyme. That this is, in fact, the explanation is demonstrated in Figure 14B. Upon modification, the observed K_m has been elevated by a factor of 40.

Figures 13 and 14 demonstrate that the inactivation of a catalytic amount of enzyme is complete in 60-90 minutes. A more concentrated solution of the modified enzyme was desired for two reasons. The first was so that the spectral characterizations of the modified enzyme described in the next section could be performed. The second reason was to have a solution of the modified enzyme dialyzed free of all small molecules, so that the substrate specificity and residual activity of the modified enzyme could be determined without the ambiguity caused by the presence of inhibitors in the incubation. When the inactivation of higher concentrations

Figure 13: Inactivation of D-amino acid oxidase with D-propargylglycine observed by the formation of the product at 300 nanometers.

In 1 ml of 0.1 M Hepes pH 8, the solution contained:

25 mM D-propargylglycine

0.05 mM FAD

2.5 µg catalase

The reaction was initiated by addition of 0.021 nmole D-amino acid oxidase.

Figure 14: Inactivation of D-amino acid oxidase with D-propargylglycine observed by the assay of aliquots of an incubation with D-alanine.

Preincubation: In 0.25 ml of 50 mM sodium pyrophosphate, pH 8.5, the solution contained: 40 mM D-propargylglycine, 2.5 µg catalase, and 0.02 mM FAD. The reaction was initiated by addition of 2.1 nmoles of D-amino acid oxidase.

A: Activity of a 10 µl aliquot of the enzyme as assayed with 0.1 M D-alanine in 1 ml of 50 mM sodium pyrophosphate, pH 8.5.

B: Apparent K_m of D-alanine of the propargylglycine modified D-amino acid oxidase.

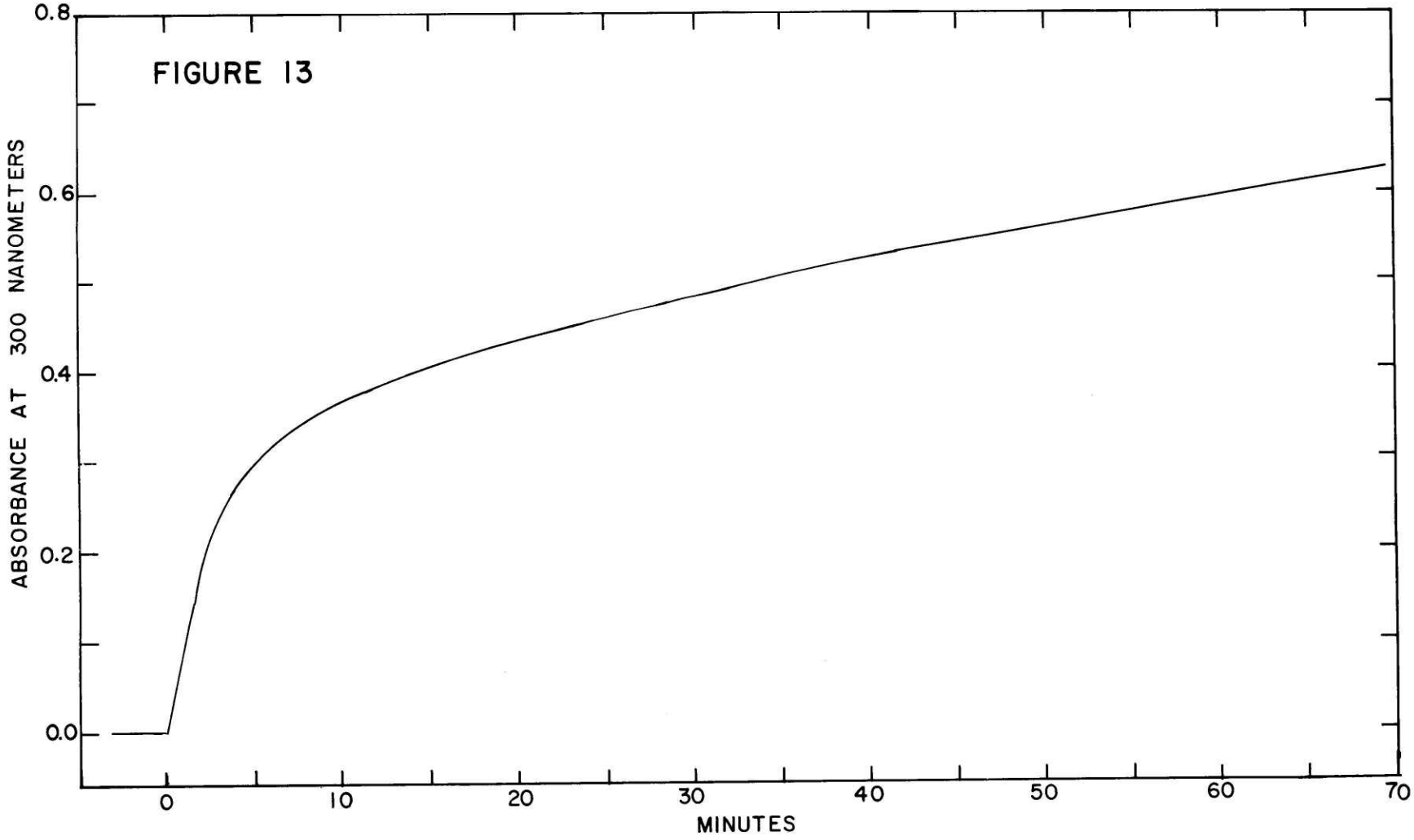
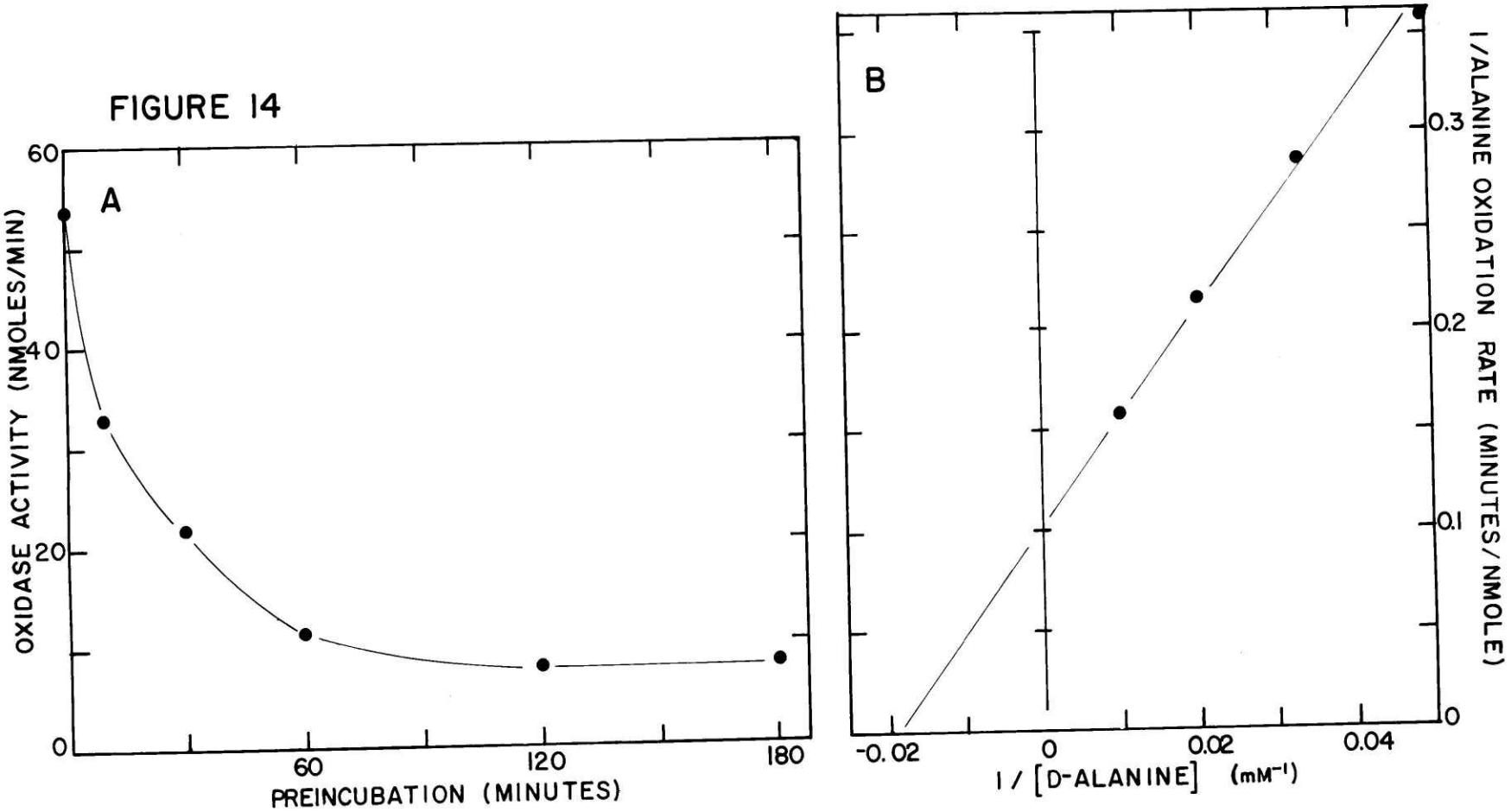


FIGURE 13
ABSORBANCE AT 300 NANOMETERS
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
MINUTES

FIGURE 14


A
OXIDASE ACTIVITY (NMOLES/MIN)
60
40
20
0
60
120
180
PREINCUBATION (MINUTES)
B
I/ALANINE OXIDATION RATE (MINUTES/NMOLE)
0.3
0.2
0.1
0
-0.02
0
0.02
0.04
1 / [D-ALANINE] (mM⁻¹)

of enzyme was attempted, incubation for much longer time periods was required to achieve complete modification. Two reasons are probable for this difficulty, the first is in keeping the solution air saturated during the course of the incubation. The second arises from the formation of those same competitive inhibitors from the oxidation products of propargylglycine discussed in the preceding section.

Preparation of modified D-amino acid oxidase.

A solution of D-amino acid oxidase was incubated with excess D-propargylglycine as described in the "Experimental Section." The solution was gently stirred in a 25 ml erlenmeyer flask for the desired time of incubation (up to 8 hours).

To remove all small molecules (including the FAD coenzyme), the incubation mixture was dialyzed twice against 500-1000 volumes of buffer containing 1 M KBr followed by dialysis against low salt buffer.[116]

As had been reported by Horiike et al,[51] the modified apoenzyme exhibits an absorbance maximum at 318 nm not seen in native apoenzyme--this characteristic absorbance is shown in Figure 15. The ratio of the absorbance maxima at 278 nm and 318 nm is a measure of the degree of alkylation of the enzyme and is a function of the time of incubation (Table I). The value of 1.35 appears to represent the alkylation of all susceptible enzyme nucleophiles in the preaparation.

The modified apoenzyme was reconstituted by addition of (commercial) FAD, the excess was removed by dialysis against low salt buffer. When the reconstituted enzyme is reincubated with D-propargylglycine in a catalytic

TABLE I

Progress of propargylglycine induced alkylation of D-amino acid oxidase measured by the ratio of absorbance at 278 nm and 318 nm of the apoenzyme.

Time of incubation	A_{278}/A_{318}†
2 hours	2.86
4 hours	2.01
6 hours	1.56
8 hours	1.35

† the data presented are not corrected for the use of enzyme ∿70% homogeneous in these experiments

Figure 15. Spectral properties of the propargylglycine/modified D-amino acid oxidase.

(a): spectrum of the apoenzyme following exhaustive dialysis *versus* 1 M KBr.

(b): after reconstitution of the apoenzyme with FAD and dialysis to remove the excess (the spectrum is normalized at 318 nm).

(c): addition of 10 µl of 100 mM D-phenylalanine to 0.5 ml reconstituded enzyme under anaerobic conditions.

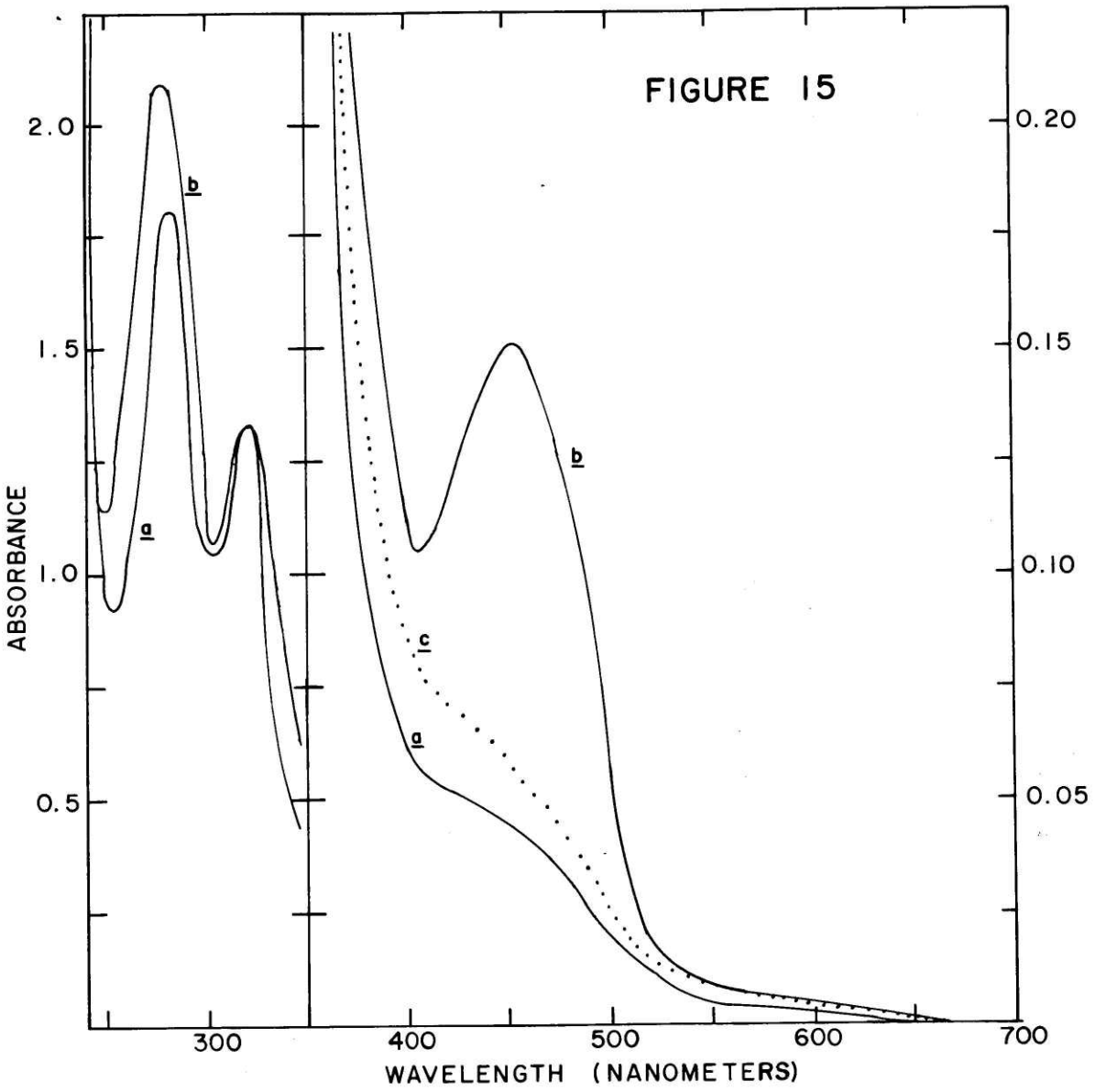


FIGURE 15
ABSORBANCE
2.0
1.5
1.0
0.5
0.20
0.15
0.10
0.05
a
b
c
300
400
500
600
700
WAVELENGTH (NANOMETERS)

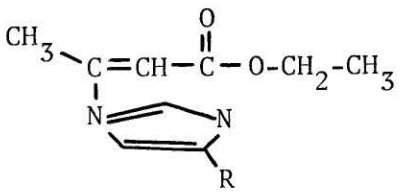
assay, the degree of linearity in product formation with time is a measure of the extent of modification. It is of interest that modified enzyme preparations with A_{278}/A_{318} = 1.5 were found to be fully kinetically modified (i.e., reincubation with propargylglycine results in linear production of product) demonstrating that not all alkylations which contribute to the absorbance are responsible for the modification of kinetic properties. Also, even after 48 hours of high salt and 24 hours of low salt dialysis, enzyme which had been incubated for greater than 6 hours with D-propargylglycine remains fully kinetically modified, pointing out the stability of the alkylation under these conditions.

Physical properties of modified D-amino acid oxidase

The nature of the alkylation: Two features of the apoenzyme spectrum of Figure 15 require further discussion. Even though the conditions of [116] dialysis to which the modified enzyme preparations was subjected is sufficient to remove all traces of the coenzyme from the native enzyme, the modified apoenzyme retains significant visible absorbance. This absorbance was not reduced by addition of D-phenylalanine, nor was it removed by 24 hours of further dialysis against 1M KBr. This evidence demonstrates that should this absorbance be due to FAD, a fraction of the coenzyme must have been placed in a severely changed environment by the modification, or perhaps has itself been covalently modified. Experiments with [^{14}C-FAD] will provide certain answers to these questions.

The identity of the amino acid residue(s) which have undergone alkylation is a matter open to speculation. The 318 nm absorbance of the fully modified

apoenzyme has an extinction coefficient of approximately 70,000. Because of the juxtaposition of this absorbance with that of the butylamine trapped product of propargylglycine oxidation, it is tempting to implicate lysine residues as the species which have undergone alkylation. However, Morasaki and Bloch have characterized a species formed upon addition of histidine methyl ester to 2,3 butadienyl esters and have shown it to have the probable structure



This species exhibits a λ_{max} 319 nm, ε 25,000.[117] Carrying out the catalytic oxidation of L-propargylglycine with L-amino acid oxidase in the presence of 100 mM imidazole resulted in the accumulation of a product with λ_{max} 299 nm, ε 12,000, i.e., not significantly different from the product in non-nucleophilic buffers. The probable explanation is that this concentration of imidazole does not react rapidly enough with the conjugated allene in solution to prevent the intramolecular lactonization.

Therefore, both the number and nature of alkylations is open to question at this time. A determination of the stoichiometry of alkylation with radioactive propargylglycine showed 1.7 nmoles incorporated per nmole enzyme, but this experiment was performed before the problems of producing fully modified enzyme were realized. Therefore, this number may underestimate the true stoichiometry by more than a factor of two.

A form of modified D-amino acid oxidase in which a single lysine residue has been specifically alkylated has been previously prepared by addition of sodium borohydride to a solution of the enzyme while it is

carrying out the oxidation of D-alanine.[118] It was possible that enzyme modified in this way might be unsusceptible to propargylglycine. Enzyme modified by the method of Hellerman[118] and Massey[119] was found to be inactivated fully by D-propargylglycine, evidence that the lysine residue blocked by that process is not the site of the deleterious modification induced by D-propargylglycine.

Spectral properties of the reconstituted modified enzyme.

Whenever the treatment of an enzyme with an inactivating agent results in the fomation of a catalyst with an activity unsusceptible to further loss, it is important to determine whether or not all of the enzyme molecules are participating in the residual activity. Therefore, D-phenylalanine was added under anaerobic conditions to a solution of the reconstituted modified enzyme; this resulted in the rapid bleaching of essentially all of the reconstituted flavin absorbance (Figure 15), demonstrating the catalytic competence of nearly all of the enzyme molecules in the preparation.

In kinetic studies (which will be detailed in the next section), it was found that the K_i's of the competitive inhibitors benzoate and anthranilate were much elevated in the modified enzyme. The binding of these inhibitors to the native D-amino acid oxidase induces characteristic changes in the visible absorbance spectrum of the enzyme. Figure 16 (compared with Figure 17 for native enzyme) demonstrates that the manner of binding of the enzyme and inhibitors has been significatly altered upon modification.

Figure 16: Effect of benzoate and anthranilate complexation of the modified D-amino acid oxidase.

(a): visible spectrum of the reconstituted propargylglycine/ modified D-amino acid oxidase in 0.5 ml 0.05 M sodium pyrophosphate, pH 8.5.

(b): addition of 5 µl 0.5 M sodium benzoate to (a).

(c): addition of 5 µl 0.5 M sodium anthranilate to (a).

Figure 17: Spectra of the complexes between D-amino acid oxidase and benzoate and anthranilate.[113]

(a): visible spectrum of 8 nmoles of D-amino acid oxidase in 0.5 ml 0.05 M sodium pyrophosphate, pH 8.5.

(b): addition of 5 µl 0.1 M sodium benzoate to (a).

(c): addition of 5 µl 0.1 M sodium anthranilate to (a).

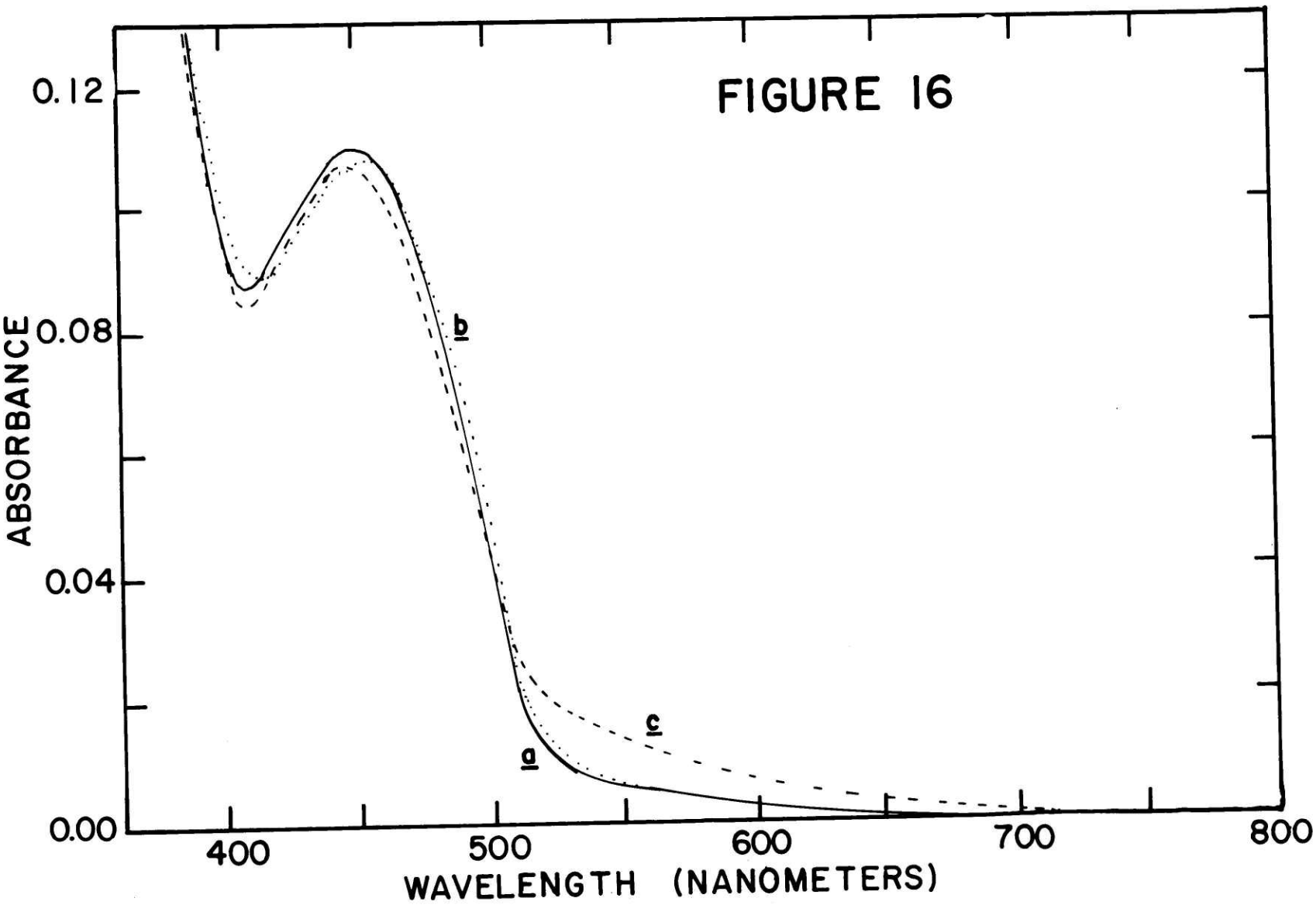


FIGURE 16
0.12
0.08
0.04
0.00
ABSORBANCE
400
500
600
700
800
WAVELENGTH (NANOMETERS)
b
c
a

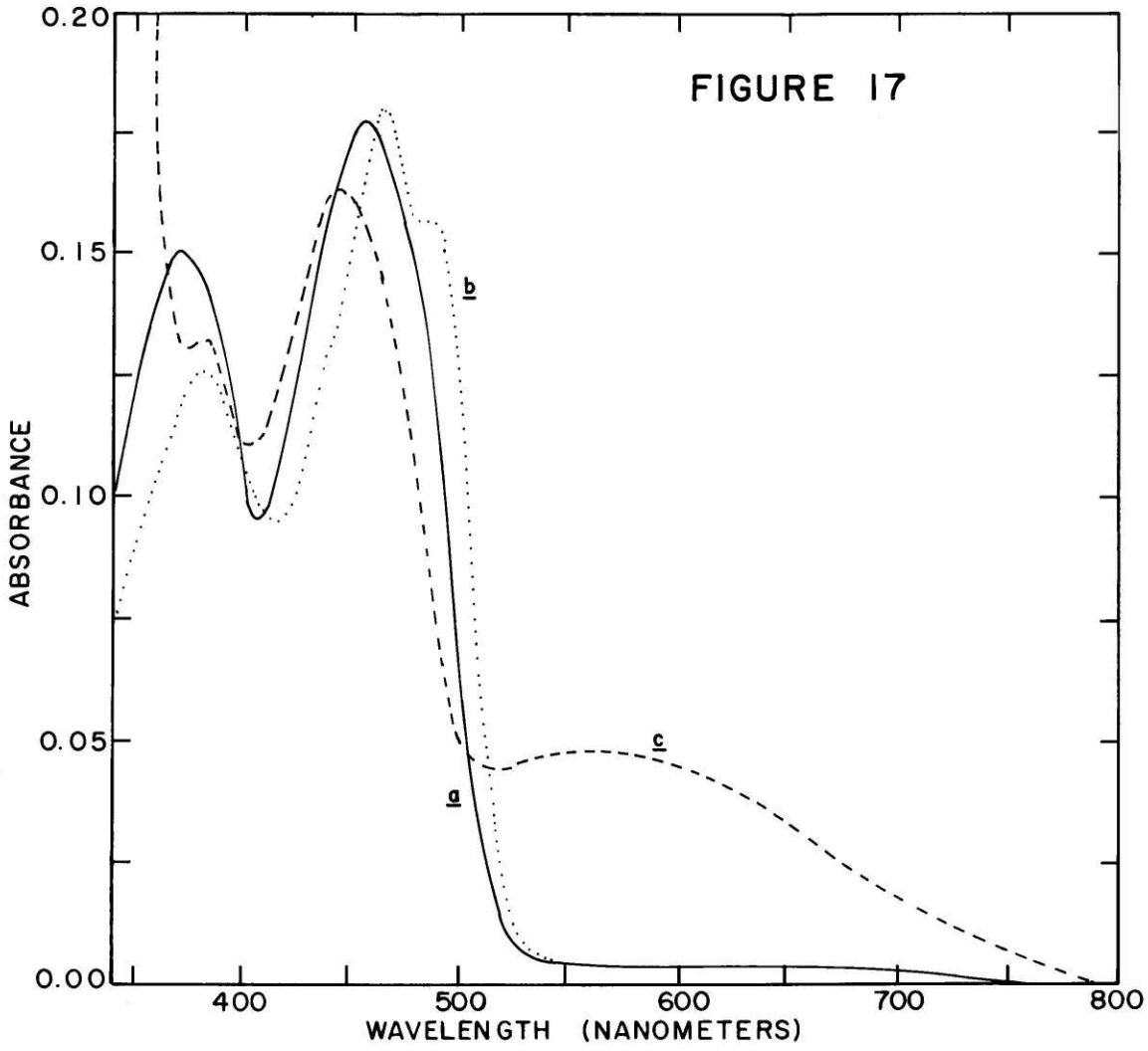


FIGURE 17
0.20
0.15
0.10
0.05
0.00
ABSORBANCE
400
500
600
700
800
WAVELENGTH (NANOMETERS)
a
b
c

Catalytic properties of the modified D-amino acid oxidase.

Substrate specificity: As stated earlier, it was first realized that the enzyme had undergone marked changes in its catalytic properties when it was found that the K_m of D-alanine had been much elevated upon alkylation. Other D-amino acids were then tested as substrates for the modified D-amino acid oxidase, with the finding that certain amino acids are good substrates of the modified enzyme (Table II). The apparent K_i (vs. D-alanine) of the competitive inhibitors benzoate and anthranilate are included in Table II. It was found that the enzyme retains its enantiomeric specificity -- L-alanine and L-phenylalanine are not detectably oxidized.

One way of quantitating the relative preference of the native and modified enzyme for a substrate is to compare the V_{max}/K_m ratio for the oxidation of an amino acid by the two enzyme forms; this ratio is provided in the last column of Table II. By this criterion, both D-alanine and D-propargylglycine are very poor substrates for the modified enzyme. Also, D-proline, the substrate with the highest V_{max} of any amino acid for native holoenzyme, is a poor substrate for the alkylated enzyme. In contrast, D-methionine and (especially) D-phenylalanine retain a significant fraction of their activity by the V_{max}/K_m estimation. The manner in which the alkylation results in this reordering of substrate specificity is as yet unclear; it could be related to the hydrophobicity of the amino acid substrate, but information on the exact geometry of the active site before and after modification would be required before a conclusive argument can be made.

Another point of interest is whether a single modified enzyme species is responsible for the oxidation of all of the amino acids. It was possible

TABLE II

Substrate specificity of native and propargylglycine modified D-amino acid oxidase

	Native Enzyme			Modified Enzyme		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
D-alanine	100*	1.2 mM	83	20	50 mM	0.4
D-phenylalanine	220	1.7	129	130	2.0	65
D-proline	310	1.4	221	27	11	2.5
D-methionine	120	0.3	400	100	2.9	35
D-valine	72	0.7	103	6	5.4	1.1
D-propargylglycine	70	2.0	35	4	13	0.3
Benzoate (K_i)†		0.006			0.3	
Anthranilate (K_i)†		0.08			1.5	

* all maximal velocities relative to oxidation of D-alanine by native enzyme

† as a competitive inhibitor of D-alanine oxidation

that different enzyme forms in a microinhomogeneous population of modified enzyme molecules contribute to the observed variation in substrate specificity. Therefore, D-phenylalnine was examined as a competitive inhibitor of D-alanine oxidation; it was found that D-phenylalanine is a potent inhibitor, exhibiting a K_i of 2 m$\underline{M}$, identical to the K_m for its oxidation by the modified enzyme. Although it is possible that the preparation used was microheterogeneous, the observed kinetic behavior appears to be largely attributable to a single catalytic species.

Rate determining step: With the observation that the binding of certain amino acid substrates and competitive inhibitors are altered by the propargylglycine-induced modification, the question was raised as to whether the binding of the imino acid product would also be impeded. Since ample evidence exists that imino acid product release is rate determining in the oxidation of amino acids by native enzyme[40], an acceleration of product release in the modified enzyme could make some other step, e.g., flavin reduction, partially or fully rate determining. One consequence of such a development might be the appearance of substrate kinetic isotope effects upon removal of the hydrogen at carbon 2.

In Table III are summarized the results of the examination of the isotope effects (substituting deuterium for hydrogen at carbon-2) upon the oxidation of three amino acids, D-alanine, DL-propargylglycine, and DL-phenylalanine by native and modified D-amino acid oxidase. The breakage of the C_2-H bond is clearly substantially rate determining in catalytic oxidation of these amino acids by the modified enzyme. In view of the wide variation in substrate specificity, it is of interest to note that

the isotope effect on phenylalanine and propargylglycine oxidation is increased and is exhibited only on V_{max}, in contrast to the findings with native enzyme. These data support the supposition that a change in rate determining step is a consequence of the modification.

TABLE III

Isotope effect on oxidation of amino acids by native and propargylglycine modified D-amino acid oxidase

	Native Enzyme			Modified Enzyme		
	on V_{max} *	on K_m †	on V_{max}/K_m	on V_{max} *	on K_m †	on V_{max}/K_m
D-alanine	1.0	1.0	1.0	2.7	1.0	2.7
DL-phenylalanine	1.6	0.5	3.2	1.9	1.0	1.9
DL-propargylglycine	1.7	0.6	2.9	4.3	1.0	4.3

* maximal velocity of enzymatic oxidation of α-[^{1}H]amino acid divided by maximal velocity of oxidation of α[^{2}H] amino acid.

† K_m of α-[^{1}H]amino acid divided by K_m of α-[^{2}H]-amino acid.

3. THE MECHANISM OF INACTIVATION OF D-AMINO ACID OXIDASE UPON ITS OXIDATION OF D-PROPARGYLGLYCINE

EXPERIMENTAL SECTION

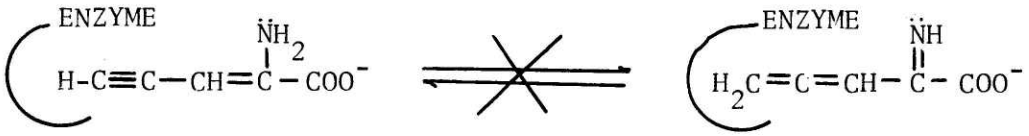
Methods

Effect of reaction conditions on the number of catalytic oxidations observed before inactivation of D-amino acid oxidase with D-propargylglycine. In 0.1 M Hepes pH 8 or 0.1 M Hepes / 0.2 M butylamine HCl pH 8 at 30°C, 0.21 nmole of D-amino acid oxidase was added to a solution containing 0.05 mM FAD and 5, 7.5, 12.5, or 25 mM D-propargylglycine. The increase in ultraviolet absorbance of the product was monitored for 2 hours, after which time a linear increase in absorbance was observed. The number of turnovers was estimated by extrapolating the final linear rate back to the time of addition of enzyme.

RESULTS

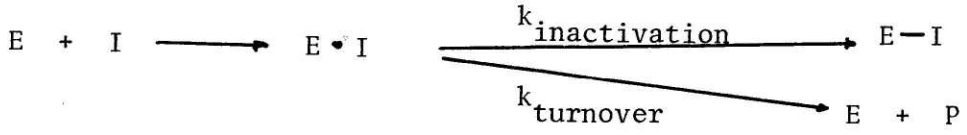
This chapter has thus far discussed the reactions which follow enzymatic oxidation of propargylglycine and the properties of D-amino acid oxidase which has been alkylated upon carrying out that oxidation. The question of the mechnism of the modification process remains to be answered.

This work has previously demonstrated that in catalytic oxidation of propargylglycine by D-amino acid oxidase, it is the acetylenic imine (2) not the acetylenic eneamine (7) which is the released product. Therefore, the enzyme does not, in normal catalysis, abstract the β-proton of the imine before its release. It has further been shown that although bound to the enzyme for a number of minutes, the eneamine is not converted to the species which causes the covalent modification. Therefore, the path-



way responsible for the observed inactivation must be different from the pathway presented earlier for the catalytic oxidation.

A simple model for the kinetics of active site directed inactivation (such as that described in the Chapter I of this paper) makes the following prediction: the number of catalytic events before inactivation of the enzyme is governed only by the relative rates of the release of the inactivating



species ($k_{turnover}$) and attack on it by an enzyme nucleophile ($k_{inactivation}$).[120] Therefore, the number of turnovers before inactivation should be independent of factors which should affect steps prior to this partition (such as substrate concentration of competitive inhibitors of the substrate) or after the release of the electrophile (such as its entrapment by nucleophiles in solution).

The number of catalytic oxidations of propargylglycine before inactivation of D-amino acid oxidase was measured as a function of substrate concentration in the presence and absence of butylamine. These values were determined by both oxygen consumption assay (Figure 18A) and, more quantitatively, by direct observation of the ultraviolet absorbance of the products. As is shown in Figure 18B, the number of turnovers is strongly dependent on both the concentration of substrate and nucleophiles in the incubation. The maximum number of turnovers before inactivation is observed is 2600.

A possible explanation of the observed dependence of the number of turnovers before inactivation on the conditions of the experiment could be the alkylation process taking place in part from molecules of 2-imino-3,4-pentadienoate free in soluion. By this mechanism, the inactivation process might be partially competitive with substrate and be slowed by conditions which prevent accumulation of the electrophile. However, 200 mM butylamine HCl offers only partial protection; therefore the susceptible enzyme nucleophile (present at 21 nM in catalytic assays) would be required to be very reactive towards the allene.

Experiments have been discussed in which incubations of D-amino acid

Figure 18: Effect of reaction conditions on the number of catalytic oxidations observed before inactivation of D-amino acid oxidase with D-propargylglycine.

A: Reaction of 2.5 mM D-propargylglycine with 30µg oxidase in a 0.4 ml oxygen electrode cell.

(a): in 100 mM Hepes pH 8.

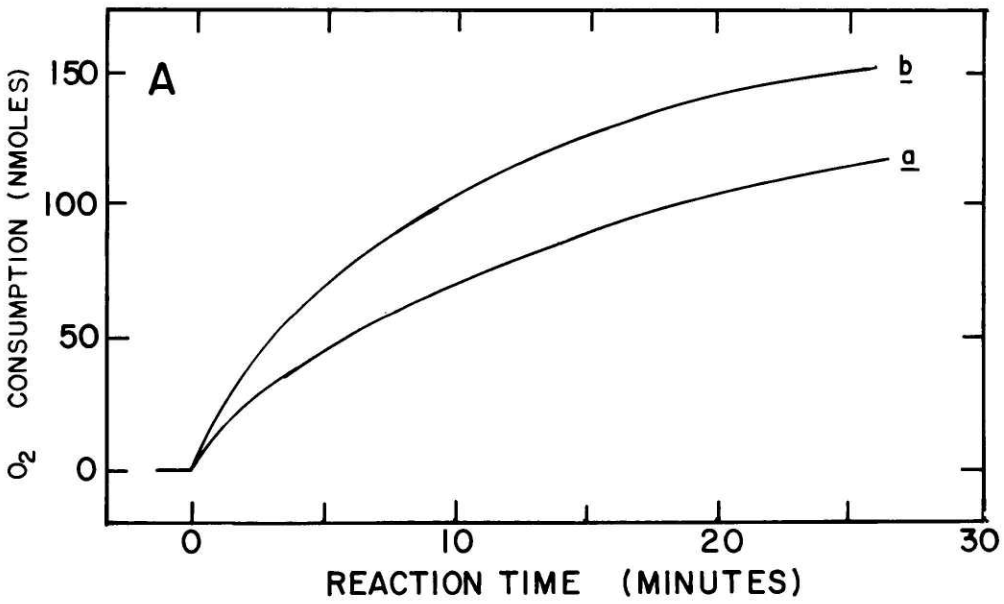
(b): in 100 mM Hepes / 100 mM butylamine pH 8.

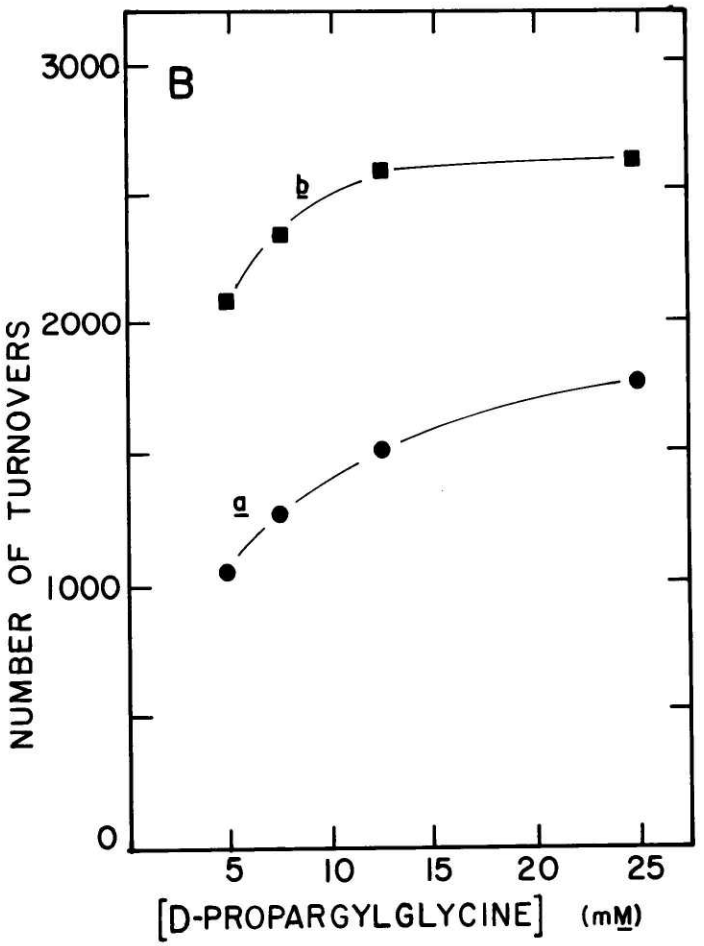
B: The number of catalytic oxidations observed before inactivation as a function of substrate concentration and solution nucleophile as monitored by the ultraviolet absorbance of the products.

(a): In 1 ml of 100 mM Hepes pH 8, the solution contained 2.5 µg catalase and 0.05 mM FAD. The reaction was initiated by addition of 0.021 nmole D-amino acid oxidase and the product monitored at 300 nm.

(b): Same conditions as (a) except done in 100 mM Hepes / 200 mM butylamine HCl pH 8 and the product was monitored at 318 nm.

FIGURE 18


A
150
100
50
0
O_2 CONSUMPTION (NMOLES)
b
a
0
10
20
30
REACTION TIME (MINUTES)


B
3000
2000
1000
0
NUMBER OF TURNOVERS
b
a
5
10
15
20
25
[D-PROPARGYLGLYCINE] (mM)

oxidase, L-propargylglycine, and L-amino acid oxidase have resulted only in the formation of strong competitive inhibitors--no alkyated D-amino acid oxidase could be produced in such incubations. Since the conjugated allene which must have transient existence in these mixtures does not induce the covalent modification, it follows that the catalytic action of D-amino acid oxidase on propargylglycine is required to produce the inactivator at the susceptible site.

The evidence is inconclusive; the following mechanism is presented as being consistent with the experimental observations. The first step in any mechanism must be the oxidation of propargylglycine to 2-imino-4-pentynoate (2) with concomitant reduction of the FAD coenzyme. Since the reoxidation of the enzyme by molecular oxygen is a fast process in catalysis by D-amino acid oxidase,[40] the enzyme will be considered to be in the oxidized form in all further steps. This assumption is not necessarily valid, however it is difficult to imagine substrate protection if the inactivation process does not involve the oxidized enzyme.

There has been no evidence which suggests that once a molecule of (2)is released by the enzyme that it ever takes part in the covalent modification. (2)is rapidly converted to the eneamine (7); it is necessary that 2-imino-3,4-pentadienoate (3) be formed transiently during the conversion of (7) to the lactone (6), but the lifetime of the allene in solution must be too short for it to be a factor in the observed modification.

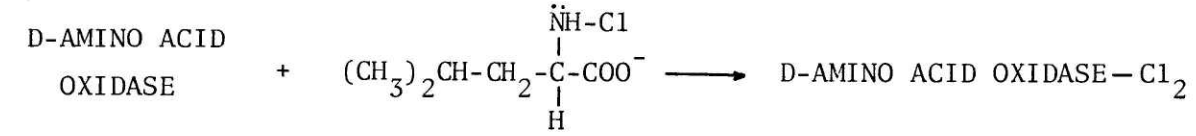
The proposed inactivation pathway involves enzyme catalyzed conver-

sion of (2) to (3) while still at (or near) the active site of the enzyme. This occurence, a relatively rare event (0.1 - 0.2%) can result in inactivation. Since the modification does not result in completely inactive enzyme, the site of alkylation must be removed from the site of substrate binding. Thus the binding of another molecule of propargylglycine can result in a protein conformational change which facilitates diffusion of the allene away from the enzyme. The position of the bound allene must also be open to diffusion of solution nucleophiles, thus explaning the observed partial protection provided by high concentrations of amines.

In order for there to be a more complete understanding of the mechanism of propargylglycine induced modification of D-amino acid oxidase, information as to which residues are modified and the position of these residues in the three dimensional configuration of the enzyme will be necessary. The amino acid sequence of D-amino acid oxidase is not yet known, nor is the three dimensional structure. Therefore, it is impossible for there to be more than conjecture at this time on the mechanism of the modification process.

DISCUSSION

The modification of D-amino acid oxidase upon its oxidation of D-propargylglycine was the first active site directed reagent found which covalently modifies an amino acid residue of D-amino acid oxidase.[51] Recent work from the laboratory of Porter and Bright reports the modification of D-amino acid oxidase with an affinity reagent , N-chloro-D-leucine.[13] Incubation of the enzyme with this substrate analogue results in the incor-



poration of two atoms of chlorine into the enzyme, possibly a specific tyrosine residue. Enzyme thus modified has changed kinetic parameters -- the V_{max} of D-alanine oxidation is reduced to 28%, the K_m is increased from 1.2 mM to 6.1 mM. By using rapid kinetic techniques,[13] Porter and Bright demonstrated that modification of one fundamental step, i.e., a 1000 fold reduction in the specific rate of flavin reduction, is the cause of the change in steady state kinetic parameters. Such stopped-flow apparatus was not available for the examination of propargylglycine modified D-amino acid oxidase; therefore, indirect methods were used to gather information on the mechanism of its catalysis.

"Wounded enzyme" species have also been produced by thiol reagents (not necessarily active site directed); two enzymes have been so modified upon reaction with methylmethanesulfonate. In the case of rabbit muscle creatine kinase,[121] the modified enzyme was found to retain 28% of its initial activity with no change in the Michaelis constants of its substrates.[122] In

contrast, pig heart lactic dehydrogenase exhibits no change in V_{max} upon modification, but the K_m of pyruvate is increased from 40 μM to 12 mM.[123] The reaction of pig heart cytoplasmic aspartate aminotransferase in syn-catalytic manner with a variety of thiol reagents have been reported to yield enzyme species with a considerable fraction of their initial activity.[124] More detailed kinetic properties of these modified catalysts were not reported.

The present work is the first documented case of a modified enzyme being found to have dramatically changed substrate specificity. The configuration of the active site following alkylation is likely to be more complex than simple steric interferance at the active site. The introduction of a five carbon, probably negatively charged, residue induces a conformational change such that D-phenylalanine, a large and hydrophobic amino acid, is the best substrate. It is surprising that the oxidation of the smallest amino acid substrate, D-alanine, is the most severly affected. Because of the residual activity, the site of the modification cannot be exactly "at the active site" in the sense that the phrase is commonly used. However, it is clear that the modification severly alters the structure of the active site, in a manner as yet undetermined.

The rate determining step of the enzymatic oxidation process has also been changed upon modification. The appearance of a primary kinetic isotope effect implies that a proton transfer step is at least partially rate determining, rather than product release as in native enzyme.[40] The binding of the competitive inhibitors benzoate and anthranilate is even more severely impaired upon modification than that of any amino acid;

that is consistent with enzyme recognition of these compounds as product analogues.

The pathway following the oxidation of each molecule of propargylglycine has been studied in detail. The assignment of the acetylenic eneamino acid structure as the initially observed product is based on the charge transfer complex induced, the short lifetime of the ultraviolet absorbance which characterizes it, and model studies of a similar transient substance produced upon the oxidation of allylglycine.

In the process of its catalytic oxidation of propargylglycine, covalent modification of the enzyme occurs infrequently (<0.1%). It was possible that the alkylation might proceed from the occasional conversion of the acetylenic eneamine to the conjugated allene while bound at the active site as the charge-transfer complex. However, no inactivation via this process was observed; evidence against the direct conversion. The second possibility was that modification proceeds from the allene produced by rearrangement in solution. All efforts to demonstrate this by incubation of D-amino acid oxidase, L-propargylglycine, and L-amino acid oxidase were unsuccessful. This evidence, together with the failure of nucleophiles in solution to prevent inactivation, makes it unlikely that alkylation proceeds to any extent from allene formed in solution.

Because of the partial protection of high concentrations of substrate and/or amine nucleophiles, it is necessary to postulate that the inactivator is loosely associated with the enzyme before inactivation takes place. Once in many turnovers, the bound imine undergoes propargylic rearrangement to the allene while still at (or near) the active site (presumably catalyzed

by some general base on the enzyme). The resultant conjugated allene is attacked by a nucleophile proximal to, but not inside, the active site of the enzyme. The allene must be partially accessible to nucleophiles from solvent; furthermore, binding of substrate must cause the more rapid diffusion of the allene away from the susceptible nucleophiles. Once in solution, the allene has a short lifetime, its decomposition is a unimolecular reaction, preventing its accumulation in sufficient concentration to alkylate the enzyme.

The product which accumulates in solution is most likely the eneamine lactone designated by structure 6A. Although the amounts of material available have prevented proper structural identification to date, the characterization of this compound as a neutral species with acid and base labilities yielding known compounds makes the assignment of structure 6A a reasonable probability. Acetopyruvate (4) and 2-amino-4-keto-2-pentenoate (5) appear to be the end products of the pathway. Except for the acid lability of 5 to 4, they are stable in solution.

Propargylglycine and other acetylenic amino acids have been used as inactivating agents of a number of enzymes. Simple kinetic and mechanistic hypotheses have often been used in the examination of these processes. This work has demonstrated that simple models may not be adequate to describe the action of these compounds as "active site directed" inactivators. The examination of this enzyme modification is not yet complete; with continued work on the physical and catalytic properties of the modified D-amino acid oxidase, a greater understanding of how the enzyme carries out its catalysis may be achieved.

REFERENCES

1. B. R. Baker, Design of Active Site Directed Irreversible Enzyme Inhibitors, J. Wiley and Sons, New York (1967)

2. R. Rando, Science 185, 320 (1974)

3. R. H. Abeles and A. L. Maycock, Acc. Chem. Res. 9, 313 (1976)

4. C. Walsh, Horizons in Biochemistry and Biophysics, in press.

5. L. Weinstein in The Pharmacological Basis of Theraputics, 5th Ed., (L. Goodman and A. Gilman, eds.) Macmillan, New York (1975) p. 1210

6. M. Nickerson and J. Ruedy, ibid, p. 715

7. D. J. Tipper and J. L. Strominger, Proc. Nat. Acad. Sci. U.S.A. 54, 1133 (1965).

8. M. Hamberg, J. Svensson, and B. Samuelsson, Proc. Nat. Acad. Sci. U.S.A. 71, 3824 (1974)

9. B. Samuelsson, unpublished work, revealed at a seminar at M.I.T., March, 1977.

10. E. Shaw in The Enzymes, 3rd Ed., (P.D. Boyer, ed.) Vol. I, Academic Press, New York (1970) p. 91.

11. R. Rando, Biochemistry 13, 3859 (1974).

12. W. Lawson and H. Schram, Biochemistry 4, 377 (1965)

13. D. J. T. Porter and H. J. Bright, J. Biol. Chem 251, 6150 (1976)

14. J. Mackworth and E. Webb, Biochem J. 42, 91 (1948)

15. I. B. Wilson and S. Ginsburg, Biochim. Biophys. Acta 18, 168 (1955)

16. J. H. Wills, A. M. Kunkel, R. V. Brown, and G. E. Groblewski, Science 125, 743 (1957)

17. A. R. Main, Science 144, 992 (1964)

18. Q. R. Bantz, C. C. Elder, R. P. Frohardt, S. A. Fusari, T. H. Haskell, D. W. Johannsen, and A. Ryder, Nature 173, 72 (1954)

19. H. E. Skipper, L. L. Bennett, and F. M. Schabel, Fed. Proc. 13, 298 (1954)

20. B. Levenberg, I. Melnick, and J. M. Buchanan, J. Biol. Chem. 225, 163 (1957)

21. T. C. French, E. G. Dawid, and J. M. Buchanan, J. Biol. Chem. 238, 2171, 2178, 2186 (1963)

22. B. R. Baker, W. W. Lee, E. Tong, L. O. Ross., J. Amer. Chem. Soc. 83, 3713 (1961)

23. G. Schoellman and E. Shaw, Biochem. Biophys. Res. Comm. 7, 36 (1962)

24. E. Shaw, M. Mares-Gina, and W. Cohen, Biochemistry 4, 2219 (1965)

25. E. B. Org, E. Shaw, and G. Schoelling, J. Biol. Chem. 240, 694 (1965)

26. E. Shaw and S. Springhorn, Biochem. Biophys. Res. Comm. 27, 391 (1967)

27. D. M. Blow, J. J. Birktoft, and B. S. Hartley, Nature 221, 337 (1969)

28. R. Stroud, Scientific American 231, 74 (1974)

29. R. Rando, Acc. Chem. Res. 8, 281 (1975)

30. G. M. Helmkamp, R. R. Rando, D. J. H. Brock, and K. Bloch, J. Biol. Chem. 243, 3229 (1968)

31. F. Batzold and C. Robinson, J. Amer. Chem. Soc. 97 2576 (1975)

32. P. Holland, M. Clark, and D. Bloxham, Biochemistry 12, 3309 (1973)

33. Examples and references are detailed in the introduction to Chapter II.

34. Examples and references are detailed in the introduction to Chapter III.

35. Examples and references are detailed in the introduction to Chapter II.

36. R. Neumann, R. Hevey, and R. H. Abeles, J. Biol. Chem. 250, 6362 (1975)

37. E. Miles, Biochem. Biophys. Res. Comm. 64, 248 (1975)

38. A. Maycock, R. Suva, and Abeles, R. H., J. Amer. Chem. Soc. 97, 5613 (1975)

39. R. Kitz and I. B. Wilson, J. Biol Chem 237, 3245 (1962)

40. H. J. Bright and D. J. T. Porter in The Enzymes, 3rd Ed, Vol. XII, (P. D. Boyer, ed) Academic Press, New York (1975) p. 421

41. A. Meister, Biochemistry of the Amino Acids, Academic Press, New York (1965) p. 375.

42. T. Cromartie and C. Walsh, Biochemistry 14, 3482 (1975)

43. W. B. Lawson and H. Schram, J. Amer. Chem. Soc. 84, 2017 (1962)

44. H. Gershon, J. S. Meek, and K. Dittmer, J. Amer. Chem. Soc. 71, 3573 (1949)

45. A. C. A. Jansen, R. J. M. Weustink, K. E. T. Kerling, and E. Havinga, Rec. Trav. Chim. Pays-Bas. 88, 819 (1969)

46. J. P. Scannel, D. L. Preuss, T. C. Demny, F. Weiss, T. Williams, and A. Stempel, J. Antibiotics 24, 239 (1971)

47. H. Gershon, J. Shapira, J. S. Meek, and K. Dittmer, J. Amer. Chem. Soc. 76, 3484 (1954)

48. R. Abeles and C. Walsh, J. Amer. Chem. Soc. 95, 6124 (1973)

49. P. Marcotte and C. Walsh, Biochem. Biophys. Res. Comm. 62, 677 (1975)

50. S. Tanase and Y. Morino, Biochem. Biophys. Res. Comm. 68, 1301 (1976)

51. K. Horiike, Y. Nishina, Y. Miyake, and T. Yamono, J. Biochem (Tokyo) 78, 57 (1975)

52. P. Marcotte and C. Walsh, Biochemistry 15, 3070 (1976)

53. E. Younathan and E. Frieden, J. Biol. Chem. 220, 801 (1956)

54. P. Friis, P. Helboe, and P. Larsen, Acta Chem. Scan. Ser B 28, 317 (1974)

55. No experimental details are provided in the publication cited. Following a procedure supplied in a personal communication with Prof. Rando, a consistent yield of 15% vinylglycine from vinylglycolate was obtained.

56. G. Dardenne, J. Casimir, M. Marlier, and P. Larsen, Phytochemistry 13, 1897 (1974)

57. T. S. Soper, J. M. Manning, P. A. Marcotte, C. T. Walsh, J. Biol Chem. 252, 1571 (1977)

58. G. Kapke and L. Davis, Biochem. Biophys. Res. Comm. 65, 765 (1975).

59. E. W. Miles, Biochem. Biophys. Res. Comm. 66, 94 (1975)

60. J. Donovan, P. Marcotte, and C. Walsh, unpublishied observations.

61. W. Washtein, C. Walsh, and R. Abeles, unpublished observations.

62. H. R. Mahler and E. H. Cordes, Biological Chemistry, 2nd. ed, Harper and Row, New York (1971) p. 393

63. T. Oshima and N. Tamiya, J. Biochem. (Tokyo) 46, 1675 (1959)

64. M. Jung and B. Metcalf, Biochem. Biophys. Res. Comm. 67, 301 (1975)

65. P. Shannon and C. Walsh, unpublished observations.

66. R. Rando, Nature 250, 586 (1974)

67. I. Giovanelli, L. D. Owens, and S. H. Mudd, Biochim. Biophys. Acta 227, 671 (1971)

68. A. Phillips, Biochim. Biophys. Acta 151, 523 (1968)

69. R. A. John and P. Fasella, Biochemistry 8. 4477 (1969)

70. T. S. Soper, W. M. Jones, B. Lerner, M. Trop, and J. M. Manning, J. Biol. Chem. (1977) in press.

71. Y. Morino and M. Okamoto, Biochem. Biophys. Res. Comm. 47, 498 (1972)

72. Y. Morino, A. M. Osman, and M. Okamoto, J. Biol. Chem. 249, 6684 (1974)

73. R. B. Silverman and R. H. Abeles, Biochemistry 15, 4718 (1976)

74. L. J. Fowler and R. A. John, Biochem. J. 130, 569 (1972)

75. E. Wang and C. Walsh, unpublished results.

76. M. Saier and W. T. Jenkins, J. Biol. Chem. 242, 91 (1967)

77. A. Meister, Biochemistry of the Amino Acids, Vol I, Academic Press, New York (1965) p. 390

78. U. M. Babu and R. B. Johnston, Biochem. Biophys. Res. Comm. 58, 460 (1974)

79. A. J. L. Cooper, J. Biol. Chem. 251, 1088 (1976)

80. W. T. Jenkins, D. A. Yphantis, and I. W. Sizer, J. Biol. Chem. 234, 51 (1959)

81. J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, Vol III, J. Wiley and sons, New York (1961) p. 1834

82. K. Weber and M. Osborn, J. Biol. Chem. 244, 4406 (1969)

83. H. Wada and E. E. Snell, J. Biol. Chem. 236, 2089 (1961)

84. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193 265 (1951).

85. T. Oshima and N. Tamiya, Biochem J. 78, 116 (1961)

86. J. Harley-Mason, Arch. Biochem. Biophys. 93 178 (1961)

87. A. Golichowski, R. C. Harruff, and W. T. Jenkins, Archiv. Biochem. Biophys. 178, 459 (1977)

88. U. Walter, H. Luthe, F. Gerhart, and H. Soling, Eur. J. Biochem. 59, 395 (1975)

89. I. A. Rose in The Enzymes, 3rd. ed, Vol II (P. D. Boyer, ed), Academic Press, New York (1970) p. 281

90. W. Washtein and R. H. Abeles, Biochemistry (1977) in press

91. H. Lis, P. Fasella, C. Turano, and P. Vecchini, Biochim. Biophys. Acta 45, 529 (1960)

92. M. Saier and W. T. Jenkins, J. Biol. Chem. 242, 101 (1967)

93. The product of propargylglycine exhibits intense ultraviolet absorbance, as will be discussed in Chapter III.

94. W. F. Diven, J. J. Scholz, and R. B. Johnston, Biochim. Biophys. Acta 85, 322 (1964)

95. M. Martinez-Carrion and W. T. Jenkins, J. Biol. Chem. 240, 3538 (1965)

96. K. Soda, K. Yonaha, H. Misono, and M. Osugi, F.E.B.S. Lett. 46, 359 (1974)

97. D. H. Spackman, W. H. Moore, and S. Stein, Anal. Chem. 30, 1190 (1958)

98. S. Ghisla, H. Ogata, V. Massey, A. Schonbrunn, R. H. Abeles, and C. T. Walsh, Biochemistry 15, 1791 (1976)

99. A. Maycock, R. Abeles, J. Salach, and T. Singer, Biochemistry 15, 114 (1976)

100. A. Schonbrunn, R. H. Abeles, C. Walsh, S. Ghisla, H. Ogata, and V. Massey, in Flavins and Flavoproteins (T. P. Singer, ed.), Elsevier Publishing, Amsterdam (1976) p. 187

101. I am aware of efforts in the laboratories of R. H. Abeles, J. E. Baldwin, and T. H. Cromartie.

102. D. Wellner and A. Meister, J. Biol. Chem. 235, 2013 (1960)

103. P. Brumby and V. Massey, Biochem. Prep. 12, 29 (1968)

104. V. Massey and B. Curti, J. Biol Chem. 242, 1259 (1967)

105. O. Wiss and H. Fuchs, Helv. Chim. Acta 35, 407 (1952)

106. G. Sweig and J. Sherma, eds., Handbook of Chromatography, Vol II, CRC Press, Cleveland, OH, (1972) p. 111

107. A. Meister and J. Greenstein, J. Biol. Chem. 175, 573 (1948)

108. A. Meister, J. Biol. Chem. 184, 117 (1950)

109. Farbenfabriken Bayer A.-G., Fr. Demande 2,012,604 (C1.C 07d), 20 Mar 1970. Chem. Abs. 74, 13134 (1971)

110. J. Elguero, G. Guirard, and R. Jacquier, Bull. Soc. Chim. Fr., 619, (1966)

111. I. I. Grandberg, Zh. Obshch. Khim. 33, 519 (1963)

112. M. Nishikimi, M. Osamura, and K. Yagi, J. Biochem (Tokyo) 70, 457 (1971)

113. V. Massey and H. Ganther, Biochemistry 4, 1161 (1965)

114. C. Walsh, E. Krodel, V. Massey, and R. Abeles, J. Biol. Chem. 248, 1946 (1973)

115. M. Ikawa and E. Snell, J. Amer. Chem. Soc. 76, 653 (1954)

116. V. Massey and B. Curti, J. Biol. Chem. 241, 3417 (1966)

117. M. Morisaki and K. Bloch, Biochemistry 11, 309 (1972)

118. D. S. Coffey, A. H. Neims, and L. Hellerman, J. Biol. Chem. 240, 4058 (1965)

119. V. Massey, B. Curti, F. Muller, and S. G. Mayhew, J. Biol. Chem. 243, 1329 (1968)

120. H. P. Meloche, M. A. Luczak, and J. M. Wurster, J. Biol. Chem. 247, 4186 (1972)

121. D. J. Smith and G. L. Kenyon, J. Biol. Chem. 249, 3317 (1974)

122. E. T. Maggio, G. L. Kenyon, G. D. Markham, and G. H. Reed, J. Biol. Chem. 252, 1202 (1977)

123. D. P. Bloxham and D. C. Wilton, Biochem. J. 161, 643 (1977)

124. W. Birchmeier, K. J. Wilson, and P. Cristen, J. Biol. Chem. 248, 1751 (1973)

BIOGRAPHICAL NOTE

Patrick Allen Marcotte was born in New Orleans, Louisiana on July 26, 1952, the second child of Steven Stephen Marcotte and Gloria Catherine DeValcourt. After an uneventful childhood, he was graduated from Holy Cross High School in New Orleans in 1969, after which he enrolled as an undergraduate at the Massachusetts Institute of Technology. On receiving an S.B. in chemistry in 1973, he remained at M.I.T. as a candidate in the doctoral program in organic chemistry. A year as a postdoctoral fellow at M.I.T. will follow graduation, after which he maintains that he will finally leave M.I.T.

Publications

"Synthesis of α-Hydroxy-β-acetylenic Acids and their Oxidation by and Inactivation of Flavoprotein Oxidases," by Thomas Cromartie, Jed Fisher, Gregory Kaczorowski, Richard Laura, Patrick Marcotte, and Christopher Walsh, J. Chem. Soc. Chem. Comm., 597 (1974).

"Active Site-Directed Inactivation of Cystathionine γ-Synthetase and Glutamic Pyruvic Transaminase by Propargylglycine," by Patrick Marcotte and Christopher Walsh, Biochem. Biophys. Res. Comm. 62, 677 (1975).

"Vinylglycine and Propargylglycine: Complementary Suicide Substrate for L-Amino Acid Oxidase and D-Amino Acid Oxidase," by Patrick Marcotte and Christopher Walsh, Biochemistry 15, 3070 (1976).

"Inactivation of Bacterial D-Amino Acid Transaminases by the Olefinic Amino Acid D-Vinylglycine," by Thomas S. Soper, James M. Manning, Patrick A. Marcotte, and Christopher T. Walsh, J. Biol. Chem. 252, 1571 (1977)