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PURIFICATION AND ACTIVE SITE CHARACTERIZATION OF L-PHENYLALANINE AMMONIA-LYASE FROM SPOROBOLOMYCES PARAROSEUS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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DOCTOR OF PHILOSOPHY

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JAMES RODNEY PARKHURST

Oklahoma City, Oklahoma

1972

PURIFICATION AND ACTIVE SITE CHARACTERIZATION OF L-PHENYLALANINE AMMONIA-LYASE FROM SPOROBOLOMYCES PARAROSEUS

APPROVED BY

DISSERTATION COMMITTEE

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TO SHARON AND MY ENTIRE FAMILY

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LIST OF ABBREVIATIONS

$NaBH_4$	Sodium borohydride
NaCN	Sodium cyanide
$NaHSO_3$	Sodium hydrogen sulfite
TNBS	Trinitrobenzene sulfonic acid
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
SDS	Sodium dodecyl sulfate
NaH_2PO_4	Sodium phosphate
Na Citrate	Sodium citrate
$(NH_4)_2SO_4$	Ammonium sulfate
K_2HPO_4	Dipotassium hydrogen phosphate
$MgSO_4$	Magnesium sulfate
NH_4OH	Ammonium hydroxide
KCl	Potassium chloride
HCl	Hydrochloric acid
Tris	Tris (hydroxymethyl) amino methane HCl
PAL	Phenylalanine ammonia-lyase
TAL	Tyrosine ammonia-lyase
Rh.	Rhodotorula
S.	Sporobolomyces
OD	Optical Density
Km	Michaelis constant
Ki	Dissociation constant of EI complex or inhibition constant (Michaelis kinetics)
Vmax	Maximal velocity

LIST OF ABBREVIATIONS--Continued.

mamp	Milliampere
M	Molar
mM	Millimolar
μM	Micromolar
pM	Picamolar
l	Liter
ml	Milliliter
g	Gram
mg	Milligram
μg	Microgram
$^{w}/_{w}$	Weight to weight
cm	Centimeter
nm	Nanometer (1 nm = 10^{-7}cm)
min	Minute
hr	Hour(s)
E.C.	Enzyme Commission
S	Substrate concentration
V	Observed initial velocity of enzyme reaction
S	Svedberg unit
Ci	Curie

PURIFICATION AND ACTIVE SITE CHARACTERIZATION OF L-PHENYLALANINE AMMONIA-LYASE FROM SPOROBOLOMYCES PARAROSEUS

CHAPTER I

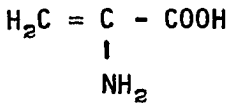
INTRODUCTION

There are two categories of amino acids known to undergo enzymatic deamination to the corresponding alkene compounds. In one category are the dicarboxylic acids, L-aspartic acid and L-β-methyl-aspartic acid and in the other are L-phenylalanine, L-tyrosine, and L-histidine which possess aromatic rings.

The mechanisms of these enzymatically catalyzed deaminations may also be categorized into two types, one for dicarboxylic and one for the aromatic acids. The mechanism for enzymatic deamination of β-methylaspartate and aspartate probably involves β-carbanion (a negatively charged carbon) formation, followed by the rate limiting expulsion of ammonia from the substrate (4,5,6,10). The mechanism for deamination of histidine (3,8,43) and phenylalanine (18,54) apparently involves: (a) the formation of an enzyme ammonia intermediate, (b) elimination of a β-proton, (c) expulsion of the deaminated product, and (d) expulsion of ammonia. At present the sequence of these events is not known conclusively, or if these are all of the reaction steps involved. In the case of the dicarboxylic acids, the deamination

reaction is readily reversible. For the aromatic amino acids phenylalanine and tyrosine the equilibrium lies far infavor of the deaminated product, but these reactions are reversible (54). In contrast histidine, also an aromatic amino acid, is apparently irreversibly deaminated (45).

The first enzyme discovered containing a catalytically essential non-pyridoxal phosphate carbonyl group was D-proline reductase which reductively deaminates D-proline to 5-aminovalerate and ammonia (22,23,25). Further investigation of D-proline reductase has provided a new enzymatic function for non-pyridoxal phosphate carbonyl groups, that of combining with the amino group of substrate; thereby making it a better leaving group due to electron withdrawal. Other important non-pyridoxal carbonyl type enzymes are S-adenosylmethionine decarboxylase, histidine decarboxylase and urocanase. Similarly, the aromatic amino acid ammonia lyases have in common a non-pyridoxal phosphate carbonyl-like group which is catalytically essential dehydroalanine, whose structure is seen below:

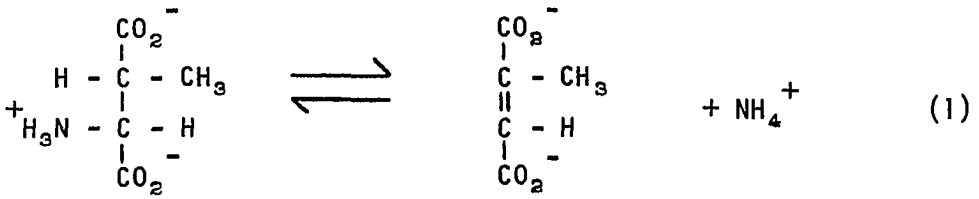


dehydroalanine

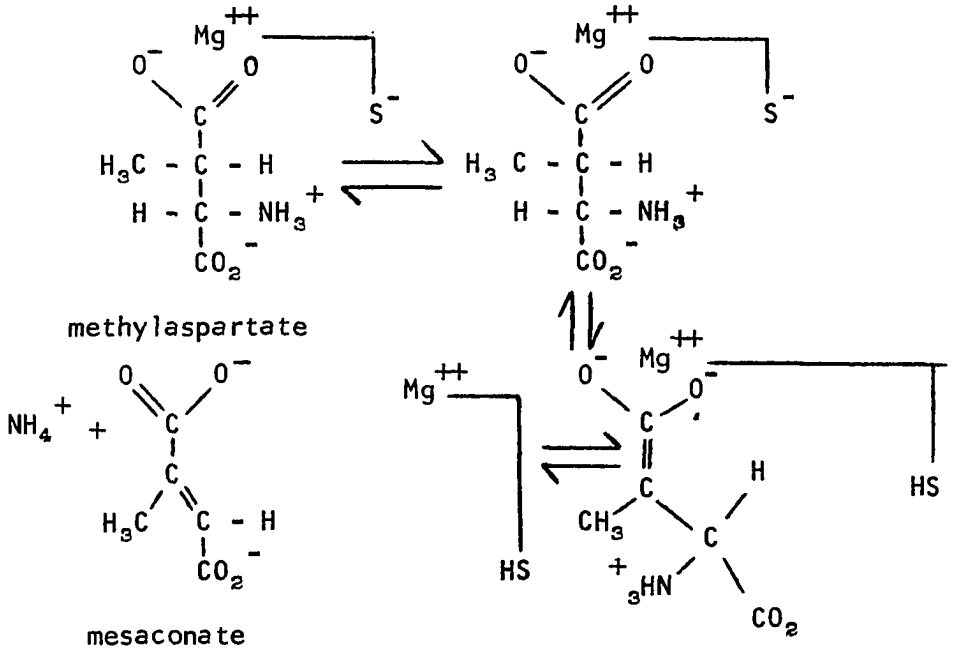
β-Methylaspartate Ammonia-Lyase

β-Methylaspartate ammonia-lyase (E.C. 4.3.1.2) catalyzes the reversible conversion of methylaspartate to mesaconate and ammonia (equation 1). Bright and co-workers have done extensive studies on the enzyme from Clostridium tetranomorphum and its catalytic properties (4-7,13). They have concluded that the mechanism most consistent with

their data involves extraction of the β-proton to form a carbanion intermediate, followed by the rate determining elimination of ammonia (equation 2).



methylaspartate mesaconate (1)



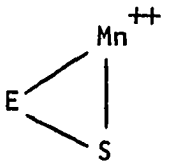
methylaspartate

mesaconate (2)

It was observed that both proton exchange into the β-carbon of substrate and the overall deamination reaction were similarly dependent on the nature of the divalent metal present. During the initial stages of a methylaspartate deamination reaction, neither ammonia nor mesaconate were converted into methylaspartate. Under these same conditions, solvent protons were incorporated into the methylaspartate. This eliminates the possibility of a rate determining breakdown of an enzyme-ammonia or

enzyme-mesaconate intermediate since reversal of the reaction at such a point would cause β-proton exchange and would be paralled by simultaneous ammonia or mesaconate incorporation into methylaspartate. This same data makes unlikely a concerted elimination of a proton and ammonia (6).

Evidence has been offered (5,13,53) for the participation of a thiol at the active site of β-methylaspartate ammonia-lyase which could act as the base necessary for proton abstraction. Furthermore, Fields (13) has suggested that a cyclic intermediate is formed in the process of catalysis (equation 3):



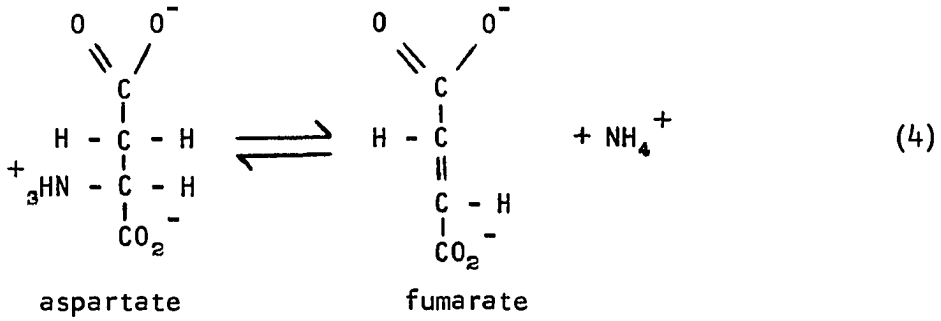
(3)

where E is enzyme, Mn^{++} is manganese, and S is substrate. From electron paramagnetic resonance measurements it has been concluded that a random-order addition of β-methylaspartate and Mn^{++} to the enzyme seems most likely (13).

L-Aspartate Ammonia-Lyase

L-Aspartate ammonia-lyase, (E.C. 4.3.1.1) an enzyme catalyzing an analogous deamination of aspartate to fumarate and ammonia (equation 4) has been partially purified (10,52) and studied (10,12).

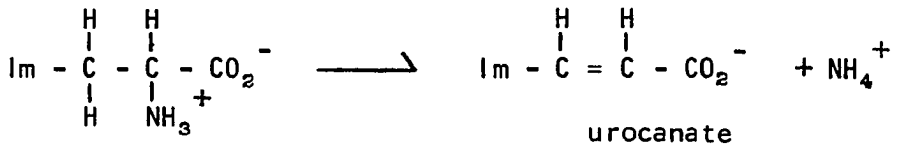
The enzyme requires divalent metals and thiols for maximal activity (10). The similarity of activators and substrates, along with the ready reversibility of both reactions, makes it likely that aspartate ammonia-lyase and β-methylaspartate ammonia-lyase utilize a similar mechanistic pathway.



aspartate fumarate (4)

L-Histidine Ammonia-Lyase

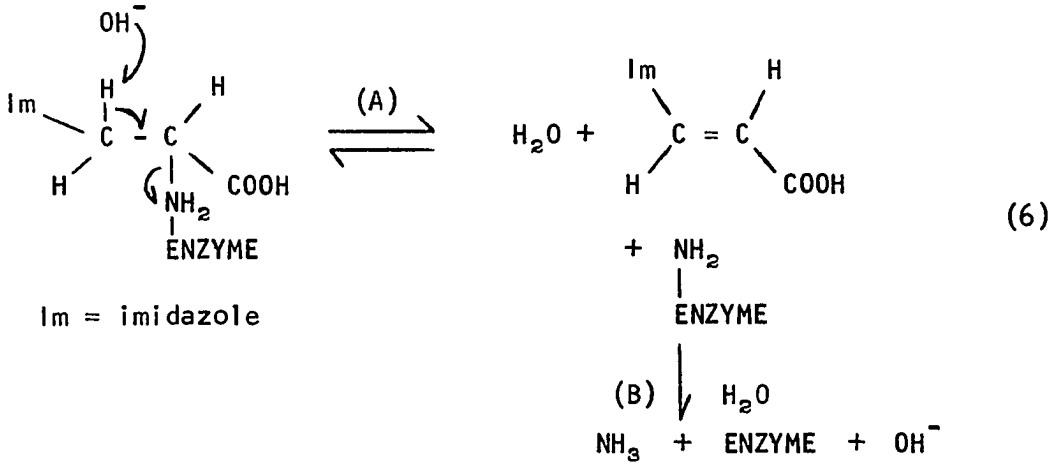
L-Histidine ammonia-lyase (E.C. 4.3.1.3) catalyzes the apparently irreversible (40) deamination of histidine to urocanic acid and ammonia (equation 5).



histidine urocanate (5)

Im = imidazole

The elegant studies of Peterkofsky (40) have offered evidence for the formation of an enzyme-ammonia intermediate, whose breakdown is the rate limiting step in the histidine ammonia-lyase reaction. He has postulated the reaction scheme on the following page (equation 6). When deamination of histidine was allowed to proceed in the presence of ^{14}C-urocanate, radioactivity was incorporated into the unreacted histidine. This is consistent with a reversal of Step (A) in equation (6). Urocanate and ammonia, incubated in the presence of histidine ammonia-lyase produced no histidine. This is consistent with an irreversible nature for Step (B), the breakdown of the enzyme-ammonia intermediate, and also with the postulated mode of urocanate exchange.



Im = imidazole

(6)

Also, when deamination of histidine was allowed to proceed in tritium oxide, unreacted histidine incorporated a tritium atom at the β-carbon [consistent with a reversal of Step (A)]. Both tritium incorporation and the overall deamination reaction was dependent on the presence of mercaptoethanol. When the deamination reaction proceeded in the presence of tritium oxide and $^{15}NH_4$; tritium, but no $^{15}NH_4$ was incorporated into histidine [again consistent with the irreversible nature of Step (B)]. Furthermore when the reaction proceeded in the presence of both tritium oxide and ^{14}C-urocanate, incorporation of both radioactive labels into histidine occurred at approximately the same rate. This would be the case if the exclusive path of proton exchange was a reversal of Step (A).

These observations are all consistent with the concerted elimination of ammonia and a proton from histidine as an early step in the reaction sequence. (If a carbanion-histidine intermediate were extant, urocanate exchange would be slower than proton exchange.) The α-amino group of substrate is presumably bound to the enzyme prior to the elimination step. After the formation and dissociation of urocanate,

the enzyme-ammonia intermediate must either react with urocanate or be degraded to native enzyme and ammonia.

Further evidence as to the likelihood of this mechanism was provided by Smith et al. (43) and Givot et al. (16) who investigated the possible nature of the ammonia-enzyme bond. They noted that histidine ammonia-lyase was irreversibly inactivated by compounds known to react with carbonyl groups (e.g., phenylhydrazine, hydroxylamine and sodium borohydride). When histidine ammonia-lyase was treated with ^{3}H-$NaBH_4$, radioactivity was non-exchangeably incorporated into the enzyme. This would be consistent with the reduction of an electrophilic center. Further work by Givot et al. (16) showed a loss of catalytic activity upon exposure of histidine ammonia-lyase to nitromethane. The inactivation produced by nitromethane could be reduced by addition of a sufficient level of substrate analogs, suggesting that nitromethane reacts at the active site of histidine ammonia-lyase. Chemical reduction and acid hydrolysis of ^{14}C-nitromethane inactivated enzyme followed by high voltage paper electrophoresis (pH 3.5) revealed radioactive 2,4-diaminobutyric acid, 4-amino-2-hydroxybutyric acid, and β-alanine. From these results they concluded that a dehydroalanine moiety was probably located at the active site of the enzyme.

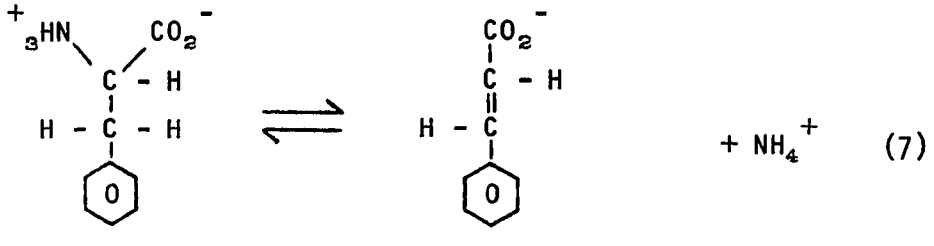
At present, it is not understood if the enzyme-ammonia intermediate postulated by Peterkofsky (40) is associated with the dehydroalanine moiety, but it appears most probably that this is the case.

Givot et al. (16) also characterized the active site of histidine ammonia-lyase suggesting that maximal binding occurs when the imidazole, carboxyl, and amino groups of substrate all interact

with enzyme. Some of the inhibitors reported by these investigators (16) were: L-histidine (Km = 7.0 mM), D-histidine (Ki = 15.6 mM), glycine (Ki = 6.0 mM), L-alanine (Ki = ∞), imidazole (Ki = 26.7 mM), histamine (Ki = 75 mM), urocanic acid (Ki = 9.7 mM), and dihydrourocanic acid (Ki = 130 mM). The most effective inhibitors contained three groups corresponding to those of the substrate, (D and L histidine), while those having two binding sites were bound less tightly. Glycine was an exception to this observed fact (16).

Phenylalanine Ammonia-Lyase

L-Phenylalanine ammonia-lyase (E.C. 4.3.1.5) catalyzes the deamination of L-phenylalanine to trans-cinnamic acid and ammonia (equation 7).



phenylalanine cinnamic acid (7)

This enzyme has been found in a wide variety of plants and fungi (8,11, 19,20,24,30,31,33-37,44,54). Some examples of the distribution of this enzyme are: tobacco leaves, sweet potato root, potato tubers, maize seedlings, barley and several strains of yeast most notably Rhodotorula glutinus and Sporobolomyces pararoseus, and one bacterial species Streptomyces verticillatus. In plants the product of phenylalanine deamination, cinnamic acid, is used to make cell wall structures. Briefly the scheme is as follows: L-phenylalanine ——> cinnamic

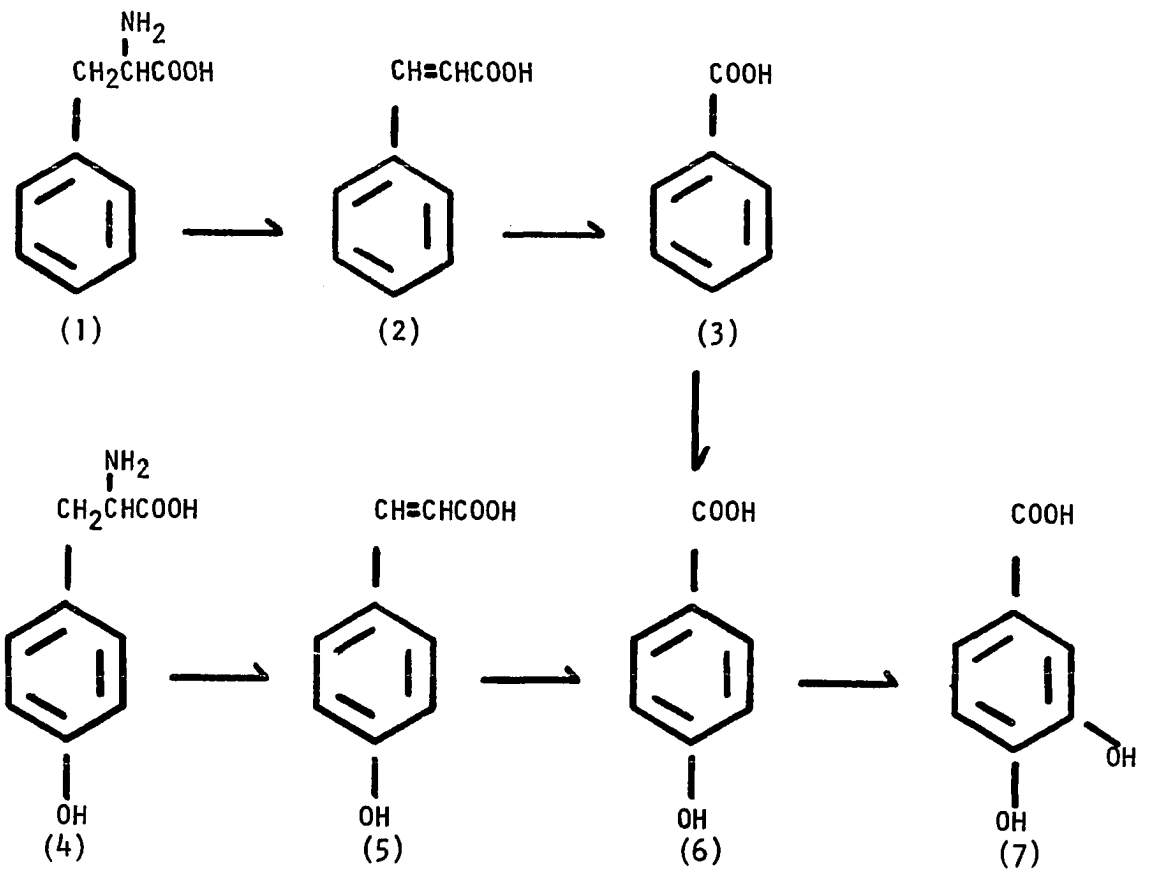
acid ——> p-coumaric acid ——> p-hydroxy-cinnamyl alcohol ——> 3-methoxy-p-hydroxy cinnamyl alcohol. A polymer formation results when a bond is formed between the hydroxyl group in the para position of 3-methoxy-p-hydroxy cinnamyl alcohol and either the α or β carbon of 3-methoxy-p-hydroxy cinnamyl alcohol (14); this scheme is generally referred to as lignin formation. Whereas, the fate of cinnamic acid in the Rhodotorula yeast strains follows a different course as is depicted in Fig. 1 after Uchiyama et al. (47). Protocatechuate is thought to undergo ring fission leading to the formation of acetyl-CoA.

Phenylalanine ammonia-lyase from potato tubers (18-20), Rhodotorula glutinus (24,26,27) and Sporobolomyces pararoseus (39) has been purified to near homogeneity and characterized as to its physical and active site properties. Havir and Hanson (19) have purified the enzyme more than 300-fold from extracts of potato tubers. Using the techniques of sucrose density gradient centrifugation and molecular sieve chromatography the molecular weight of the enzyme was estimated as 330,000 (19). They found that sulfhydryl reagents failed to inhibit the enzyme whereas carbonyl reagents (e.g., $NaBH_4$) were potent inhibitors, whose action could be blocked by cinnamate. Also a carbonyl-ammonia intermediate was postulated from radioactive exchange experiments (20).

Similarily, Hodgins (26) showed phenylalanine ammonia-lyase from Rhodotorula glutinus to be an enzyme of approximately 275,000 molecular weight as estimated by G-200 Sephadex chromatography and sucrose density gradient centrifugation (26). This yeast ammonia-lyase was also sensitive to carbonyl reagents such as NaCN, $NaBH_4$, and $NaHSO_3$ (24).

Both Hodgins (26) and Hanson (18) have demonstrated a catalytically

Figure 1. Proposed metabolic pathway of L-phenylalanine and L-tyrosine metabolism in Rhodotorula. (1) L-phenylalanine; (2) cinnamic acid; (3) benzoic acid; (4) L-tyrosine; (5) p-coumaric acid; (6) p-hydroxybenzoic acid; (7) 3,4-dihydroxybenzoic acid (protocatechuic acid) (Uchiyama et al., 47).


NH2
CH2CHCOOH
CH=CHCOOH
COOH
(1)
(2)
(3)
NH2
CH2CHCOOH
CH=CHCOOH
COOH
COOH
OH
OH
OH
OH
(4)
(5)
(6)
(7)

essential dehydroalanine moiety as the active site of phenylalanine ammonia-lyase. Hodgins inactivated the yeast enzyme with ^{14}C-NaCN. This inactivation could be prevented with cinnamic acid indicating participation of the active site. Inactive ^{14}C-CN-enzyme was hydrolyzed at 110^{o} for 24 hr in 6 M HCl, after which time the acid was removed by rotary evaporation under partial vacuum. The radioactive hydrolysate was subjected to high voltage electrophoresis at pH 1.9 and 8.9 with appropriate standards. After drying, the high voltage electrophoresis strips were scanned for radioactivity. The majority of the radioactivity observed corresponded with the migration of the standard L-aspartic acid. These observations by Hodgins (26) are in accord with the presence of catalytically essential dehydroalanine at the active site of the yeast Rh. glutinus. Similarly, Hanson (18) using tritiated $NaBH_4$ inactivated potato tuber phenylalanine ammonia-lyase and after acid hydrolysis and high voltage electrophoresis at various pH's identified the radioactive product as tritiated alanine. This also is in accord with a dehydroalanyl residue at the active site of potato tuber phenylalanine ammonia-lyase.

Table 1 after Hodgins (27) shows the wide specificity of the Rhodotorula glutinus enzyme for several substrate analogs. Of the compounds tested by Hodgins (27) 15 of 20 were deaminated by the enzyme. Thus, the yeast enzyme appears to deaminate a wide range of compounds. It is worthy to note that neither L-histidine nor DL-tryptophane were deaminated but L-tyrosine was. This indicates that although there is a wide deaminating specificity for this enzyme it does not include all of the aromatic amino acids. Table 2 compares several of the properties of

TABLE I

Rhodotorula PHENYLALANINE AMMONIA-LYASE AND SUBSTRATE ANALOGS[a]

Compound	Concentration mM	Substrate Activity
L-Tyrosine	0.83	+
DL-3-Hydroxyphenylalanine	0.83	+
L-3-Nitrotyrosine	0.83	+
DL-4-Nitrophenylalanine	0.166	+
L-3,5-Dinitrotyrosine	0.83	+
L-3-Iodotyrosine	0.83	+
L-3-Aminotyrosine	0.83	+
L-3-Methoxytyrosine	0.83	+
DL-4-Aminophenylalanine	0.166	+
DL-4-Methylphenylalanine	0.83	+
L-4-Iodophenylalanine	0.83	+
L-2-β-Thienylalanine	0.83	+
DL-4-Fluorophenylalanine	1.6	+
DL-3-Fluorophenylalanine	1.6	+
DL-2-Fluorophenylalanine	1.6	+
L-Histidine	0.83	-
DL-β-Phenyllactic Acid	1.76	-
DL-Tryptophane	1.7	-
DL-α-Methyl-3-hydroxyphenylalanine	1.71	-
DL-2-Hydroxyphenylalanine	1.76	-

[a]Hodgins (27)

TABLE 2

COMPARISON OF L-PHENYLALANINE AMMONIA-LYASE FROM POTATO TUBERS[a] AND *Rhodotorula glutinus*[b]

	Molecular weight	Inactivated by sulfhydryl reagents	Inactivated by carbonyl reagents	Contains a dehydroalanine moiety
Potato tuber	330,000	no	yes	yes
Rh. glutinus	275,000	yes	yes	yes

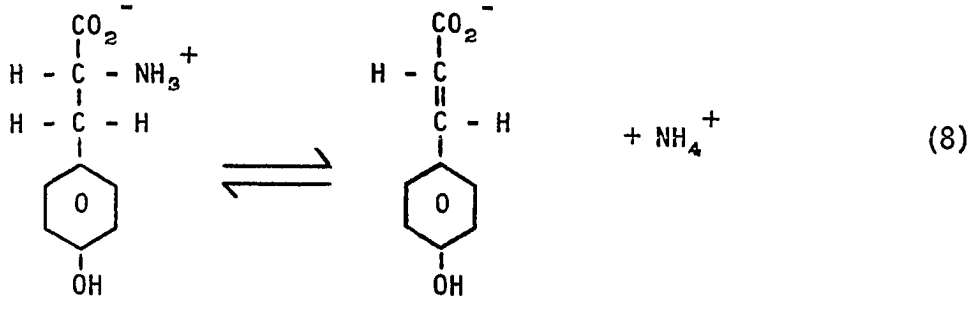
[a] Havir and Hanson (18,19,20)

[b] Hodgins (24,26)

the potato tuber and Rh. glutinus enzymes from Havir and Hanson (18-20) and Hodgins (24,26).

Tyrosine Ammonia-Lyase

Tyrosine ammonia-lyase catalyzes the deamination of L-tyrosine to p-coumaric acid and ammonia (equation 8).



(8)

tyrosine p-coumaric acid

Thus far no tyrosine ammonia-lyase has been purified separate from phenylalanine ammonia-lyase activity. It is for this reason that at present there is no enzyme classification number for this enzyme. Although most phenylalanine ammonia-lyases have bisubstrate activity for L-phenylalanine and L-tyrosine not all do as is exemplified by the potato tuber enzyme, which deaminates only phenylalanine.

Specific Aims

The aim of this research endeavor was to isolate, purify, and characterize phenylalanine ammonia-lyase from S. pararoseus. More specifically, the aim was to ascertain certain properties of the enzyme such as: substrate specificity, molecular weight, subunit composition, catalytically essential residues, whether the enzyme was regulatory or non-regulatory, and if the enzymic deamination of phenylalanine proceeded via an enzyme-ammonia intermediate. Several other properties of the

enzyme were also investigated during the course of this research such as: requirements for the enzyme assay, spectrum of the enzyme, and stability of the enzyme after purification. Furthermore, the existance of separate tyrosine and phenylalanine ammonia-lyases has been postulated to exist in the related organism S. roseus. An initial objective of this research was to ascertain if two such enzymes were present in S. pararoseus.

CHAPTER II

MATERIALS AND METHODS

Organism

The primary organism used in these studies was Sporobolomyces pararoseus (ATCC 11386); other organisms were as follows:

1. Saccharomyces rosei (ATCC 10664)
2. Rhodotorula rubra (ATCC 9449)
3. Rhodotorula glutinus rubescens (ATCC 6495)
4. Rhodotorula glutinus (ATCC 15385)
5. Rhodotorula flava (ATCC 14551)
6. Serratia marcesens

All of the above mentioned organisms were obtained from the American Type Culture except for Serratia marcesens which was obtained from the Department of Microbiology of the University of Oklahoma Health Sciences Center.

Sporobolomyces pararoseus was maintained at 4^{o} on agar slants containing 0.3% yeast extract, 1.0% malt extract, and 3.0% agar.

Media

Three types of media were employed in the growth of the above organisms. Medium 1, a salts medium, was modified after the medium proposed by Vogel (48) and contained per liter:

$MgSO_4$. . . . . 0.2 g

K_2HPO_4	10.0 g
glucose	1.0 g
L-tyrosine	1.0 g
$(NH_4)_2SO_4$	1.0 g
Na Citrate	2.0 g

Medium II contained per liter:

Malt extract	7.0 g
Yeast extract	0.5 g
L-tyrosine or L-phenylalanine	1.0 g

The medium used most often for growth of Sporobolomyces pararoseus was Medium III and contained per liter:

Malt extract	7.0 g
Yeast extract	1.0 g
D,L-phenylalanine	1.0 g

All solutions were sterilized by autoclaving.

Enzyme Assay

The deamination of L-phenylalanine was measured by following the increase in optical density at 290 nm (56) where the molar extinction coefficient is 10,000 for cinnamic acid. The standard assay mixture contained 5 mM L-phenylalanine, 0.1 M Tris HCl, pH 8.5, and enzyme at 30°. Tyrosine deamination was measured similarly, except that a wavelength of 310 nm was used (a molar extinction coefficient of 12,400 was utilized in activity calculations). In this latter assay L-tyrosine replaced L-phenylalanine in the standard system. An enzyme unit is

defined as that amount of protein catalyzing the appearance of 1 μmole of product per min at 30°. For purified enzyme protein concentrations were measured by the modified Lowry method of Zak and Cohen (55). For crude protein determinations the Warburg-Christian spectrophotometric method was employed (49).

Quantitative Phenylalanine Determinations

L-phenylalanine standards (0.0 to 45 μM) were incubated at 25° for 4 hr in a volume of 2.2 ml containing 0.025 units of enzyme. L-phenylalanine unknowns were incubated under the same conditions. At the end of the incubation period the optical density (OD) at 290 nm was measured for standard and unknown L-phenylalanine samples. A linear relationship was found to exist between the OD at 290 nm and the L-phenylalanine concentrations. Unknown L-phenylalanine concentrations were determined graphically. Under these conditions greater than 90% of the L-phenylalanine standards were deaminated to trans-cinnamic acid and ammonia.

Polyacrylamide Disc Gel Electrophoresis

Disc gel electrophoresis was performed by the method of Davis (9) with apparatus and reagents purchased from Canalco. Samples were applied to the stacking gel in 50% sucrose. Gels (7.5% crosslinked polyacrylamide) were subjected to 3 m amp/tube for 3 hr at 4°. After removal, the protein in the gels was stained for 1 hr with 1% amido black in 7.5% acetic acid and then were electrophoretically freed of unbound dye in 7.5% acetic acid.

Molecular Weight Estimation by Gel Filtration

The molecular weight of the enzyme was determined by gel filtration using Sephadex G-200 (40-200 μ) from which the fine particles in suspension had been decanted. The Sephadex G-200 was equilibrated in 50 mM sodium pyrophosphate (pH 8.0) and 10 mM 2-mercaptoethanol and packed to give a column dimension of 3 x 100 cm. The column was calibrated with the following standards: blue dextran (Sigma; molecular weight (MW) = 2,000,000), urease (Sigma - Type IV; MW = 483,000), glutamate dehydrogenase (Sigma - Type I; MW = 340,000), xanthine oxidase (Boehringer; MW = 270,000), catalase (Sigma - 2x; MW = 240,000), fumarase (Sigma - crystalline; MW = 194,000), and lactic dehydrogenase (Sigma - Type IX; MW = 140,000). Urease was assayed by the method of Gorin and Chin (17). Bovine liver glutamate dehydrogenase was assayed by the method of Fridovitch (15), bovine liver catalase by the method of Beers and Sizer (2), pig heart fumarase by the method of Racker (41), and beef heart lactate dehydrogenase by the method of Kaloustian (29). All of the standards listed above plus phenylalanine ammonia-lyase, in a total volume of 1.0 ml, were layered on the gel column which was eluted in 1.9 ml fractions.

Molecular Weight Estimation by Sucrose Density Gradient Centrifugation

A 4.6 ml linear sucrose density gradient was prepared in a gravity flow mixing chamber using two solutions: first 2.2 ml of 20% ($^{w}/_{w}$) sucrose in 50 mM Tris HCl, pH 8.5, and bromphenol blue (0.3 mg/ml) and second, 2.4 ml of 5% sucrose in the same buffer without bromphenol blue. The gradients were stored for 4 hr at 4^{o} before use. A mixture of either

catalase, xanthine oxidase, or fumarase and phenylalanine ammonia-lyase was layered on the sucrose gradient, and the tubes centrifuged at 99,972 x g for 10 hr at 4^{o} in a SW 50L rotor with a Beckman-Spinco L-265-B ultracentrifuge. Fractions of 0.13 ml were collected from the gradient tubes, and bromphenol blue concentrations were measured in samples diluted 1 to 10 with 50 mM Tris HCl, pH 8.5, at an optical density of 590 nm. The enzyme activities were measured employing the assay methods previously described.

The mathematical assumptions of Martin and Ames (32) were employed for the determination of the molecular weight of phenylalanine ammonia-lyase. Martin and Ames (32) demonstrated that the migration of proteins through a sucrose gradient was linear with time. This observation allows one to estimate the sedimentation coefficient of an unknown substance if a similar standard of known sedimentation coefficient is also in the gradient, according to the following proportionality

$$\frac{Sx}{Sy} = \frac{dx}{dy}$$

where dx and dy represent the migrated distances from the top of the gradient and Sx and Sy represent the sedimentation coefficients. Once the sedimentation coefficient is known for the experimental molecule its molecular weight can be estimated by comparison with a standard of known molecular weight and sedimentation coefficient by the following equation

$$\frac{S_1}{S_2} = \frac{(MW_1)^{2/3}}{(MW_2)^{2/3}}$$

where S_1 and S_2 represent the sedimentation coefficients of the standard and experimental compounds and MW_1 and MW_2 represent the molecular weights of the standard and unknown compounds. It is also assumed that the standard has the same shape as the unknown compound and that the reported sedimentation coefficient and molecular weight of the standard are accurate.

Subunits

The method of Weber and Osborn (50) was followed for polyacrylamide disc gel electrophoresis of proteins in sodium dodecyl sulfate (SDS). The separation of native proteins on polyacrylamide gels was shown by Davis (9) to be dependent on the charge and size of the molecules. The binding of dodecyl sulfate ions to proteins confers a large net negative charge to these molecules which allows all SDS-proteins to migrate as anions (42, 50). Shapiro, et al. (42) reported that because of the large net anionic charge the migration of SDS-protein complexes is solely a function of their molecular weight. In experiments using SDS to determine the subunit molecular weight of phenylalanine ammonia-lyase, standards of known subunit molecular weight and the experimental molecules were treated as described below. The subunit molecular weights of the standards were plotted logarithmically versus their mobility towards the anode after disc gel electrophoresis. The resultant graph described a linear relationship which was used to interpret the mobility of phenylalanine ammonia-lyase subunits in terms of their molecular weight.

Preparation of Protein Solutions

The proteins were incubated at 37° for 2-3 hr in 50 mM $NaPO_4$ buffer, pH 7.0, 1% in SDS and 1% in mercaptoethanol. Protein concentrations were between 0.4 and 0.6 mg per ml. After incubation the protein solutions were dialyzed for several hours against 500 ml of 10 mM $NaPO_4$ buffer, pH 7.0 containing 0.1% SDS and 0.1% mercaptoethanol.

Preparation and Electrophoresis of Gels

The gel buffer contained 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g of $Na_2HPO_4 \cdot 7H_2O$, 2.0 g of SDS per liter. Gels contained 10% acrylamide. All other steps in the preparation of the gels were performed as described by Weber and Osborn (50).

Electrophoresis was performed for 6 hr at a constant current of 8 milliampere per gel with the positive electrode in the lower chamber.

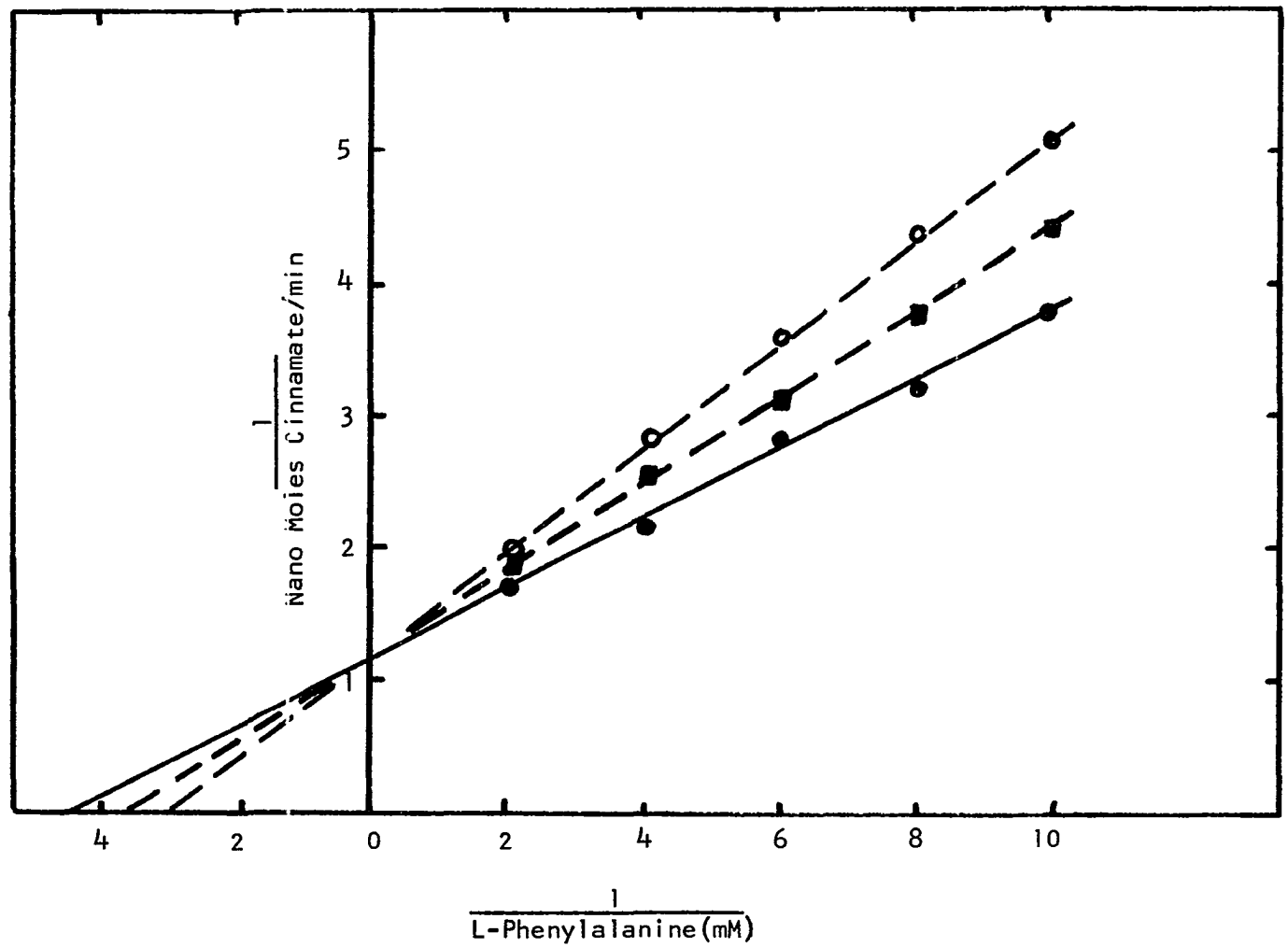
Staining and Destaining

Gels were stained for 6 hr in a 1% Amido-black solution, and destained in a 7.5% glacial acetic acid solution.

Inhibition Studies

Michaelis constants (K_m) and inhibition constants (K_i) were determined by performing duplicate assays at all concentrations of either L-phenylalanine or L-tyrosine tested. This data was plotted on a Lineweaver-Burk plot (51). Figure 2 illustrates a typical Lineweaver-Burk plot for L-phenylalanine and L-phenylalanine plus two levels of a competitive inhibitor, cinnamic acid. From this type of plot was

Figure 2. Lineweaver-Burk plot of L-phenylalanine ammonia-lyase. Phenylalanine control (●) and two levels of cinnamic acid (■,o), a competitive inhibitor.


5
4
3
2
1
Nano Moles Cinnamate/min
1
4
2
0
2
4
6
8
10
1
L-Phenylalanine(mM)

obtained: (a) the Michaelis constant (K_m), (b) the maximal velocity (Vmax) of the enzyme reaction, and (c) the inhibition constant (K_i) for the inhibitor tested (51).

Chemicals

All chemicals used in this research project were of reagent grade. U-^{14}C-cinnamic acid was purchased from Schwartz/Mann. Urease, glutamate dehydrogenase, catalase, fumarase and lactic dehydrogenase were purchased from Sigma Chemical Company. Xanthine oxidase was obtained from Boehringer. Disc electrophoresis chemicals were purchased from Canalco.

Instruments and Equipment

Spectrophometric measurements were made on either an automated Gilford 240 recording spectrophotometer or a Beckman DU-2. Other instruments or equipment used in the process of this research were:

1. Sorvall RC2-B refrigerated centrifuge
2. Beckman-Spinco L-265-B ultracentrifuge with a SW 50 L rotor
3. Mettler H 20 balance
4. Corning pH meter model 12
5. Sonifier Cell Disrupter model W 185 D Heat Systems
6. Radioactive strip scanner by Atomic Accessories model RSC310
7. High voltage electrophoresis by Savant
8. Liquid scintillation spectrometer by Packard
9. Sharples continuous flow centrifuge
10. Canalco disc gel electrophoresis apparatus

CHAPTER III

RESULTS

Presence of Phenylalanine Ammonia-Lyase in Several Yeast Strains

Six strains of yeasts and one of bacteria were cultured on two different media (Medium I and II) to determine if any of these organisms contained L-phenylalanine ammonia-lyase. The organisms were grown for 24, 48 and 72 hours. At the end of these times the cells were collected and washed twice with 0.5% KCl and 0.5% NaCl after which they were disrupted by sonic oscillation. This solution was centrifuged and the supernatant solution was assayed for tyrosine and phenylalanine ammonia-lyase activity. Table 3 shows the results obtained. Four of the seven organisms cultured had both phenylalanine and tyrosine ammonia-lyase activity. Two of these four yeasts contained more enzyme activity than the others, namely Rh. glutinus and S. pararoseus. Because Camm and Towers (8) reported the existence of two separate ammonia-lyases for phenylalanine and tyrosine in the organism S. roseus, the author's initial objective was to ascertain if the yeast S. pararoseus contained two separate ammonia-lyases for these amino acids.

Yeast Culture

Phenylalanine ammonia-lyase was obtained from S. pararoseus grown in batch culture at 30° with a medium of 0.7% Difco malt extract, 0.1% Difco yeast extract, and 0.1% D,L-phenylalanine (Medium III). The

TABLE 3

TYROSINE AND PHENYLALANINE AMMONIA-LYASE ACTIVITY
OF VARIOUS ORGANISMS

Organism	TAL[a] Activity	PAL[b] Activity
1. Saccharomyces rosei (ATCC 10664)	-	-
2. Rhodotorula rubra (ATCC 9449)	+	+
3. Rhodotorula glutinus rubescens (ATCC 6495)	+	+
4. Sporobolomyces pararoseus (ATCC 11386)	+	+
5. Rhodotorula glutinus (ATCC 15385)	+	+
6. Rhodotorula flava (ATCC 14551)	-	-
7. Serratia marcesens (Bacteria)	-	-

[a]TAL is Tyrosine ammonia-lyase

[b]PAL is Phenylalanine ammonia-lyase

organism was initially inoculated in 125 ml flasks containing approximately 40-50 ml of medium. After 18 hr of growth on a rotary shaker, the culture was transferred to a 1 liter flask having 500 ml of the above media. Approximately 18 hr later this flask was used to inoculate a 20 liter carboy containing 15 liters of media with Antifoam A (Dow-Corning) to prevent excess foaming. The carboys were aerated with compressed air. Under these conditions maximum phenylalanine ammonia-lyase activity occurred during the late logarithmic phase of growth which corresponded to 29 hr after inoculation. The yeast cells (336 g from 5 carboys) were collected with a Sharples continuous flow centrifuge, washed with 1.5 l of 0.5% NaCl-0.5% KCl and frozen.

Purification of Phenylalanine Ammonia-Lyase

Step 1. Crude Enzyme

Table 4 summarizes a typical enzyme purification. All operations were performed at 0-5° except where otherwise stipulated. The washed cells were suspended with 600 ml of 50 mM Tris HCl, pH 8.5. This mixture was divided into two 450 ml aliquots and each was sonicated with a Branson model W-185-w sonifier for one and one-half hours at maximum power, generating a temperature of 12°. The solutions were then centrifuged at 19,600 x g for 10 min, and the supernatant solutions (900 ml) retained.

Step 2. Treatment with Protamine Sulfate

One ml of 5% protamine sulfate suspension (Calbiochem) pH 8.5 (adjusted with NH_4OH) was added to the Step 1 supernatant solution for every 200 mg of protein present as measured spectrophotometrically (49).

TABLE 4

PURIFICATION PROCEDURE FOR PHENYLALANINE AMMONIA-LYASE FROM *S. pararoseus*

Purification Step	Volume ml	Total mg protein	Total enzyme units	Specific Activity (Enzyme units/mg protein)
1. Crude enzyme sonicate	900	46,700	126	0.003
2. Protamine sulfate supernatant	920	17,450	227	0.013
3. Ammonium sulfate fractionation	45	2,923	134	0.046
4. Sodium citrate fractionation	20	892	92	0.103
5. 30% ammonium sulfate saturation	8	328	70	0.213
6. G-200 Sephadex	80	61	72	1.180
7. Third ammonium sulfate fractionation	10	45	59	1.310

The protein-protamine sulfate mixture was stirred for 1 hr and then centrifuged at 19,600 x g for 10 min. The pellet was discarded.

Step 3. Ammonium Sulfate Fractionation

Ammonium sulfate (46 g, 7% $(NH_4)_2$ SO_4 saturation) was added to the protein solution (920 ml) and stirred for 20 min. The mixture was then centrifuged at 19,600 x g for 10 min. The precipitate was dissolved in a minimum amount of 50 mM Tris HCl, pH 8.5. The supernatant solution was brought to 14% saturation with 46 g $(NH_4)_2SO_4$. Similar $(NH_4)_2SO_4$ additions were continued until all the enzyme activity was present in the redissolved precipitates. Phenylalanine ammonia-lyase precipitated between 21 and 28% $(NH_4)_2SO_4$ saturation. The most active fractions were pooled (45 ml).

Step 4. Sodium Citrate Fractionation

The same procedure was employed for sodium citrate fractionation as described for the $(NH_4)_2SO_4$ fractionation. With sodium citrate phenylalanine ammonia-lyase precipitated when 25-35 g of salt per 100 ml of solution had been added.

Step 5. Second Ammonium Sulfate Precipitation

A second ammonium sulfate fractionation at 30% saturation (35 g salt/100 ml solution) served to concentrate phenylalanine ammonia-lyase prior to G-200 Sephadex chromatography.

Step 6. G-200 Sephadex Chromatography

The active phenylalanine ammonia-lyase (8.0 ml) was applied to a column (3 x 100 cm) of G-200 Sephadex which was equilibrated with 50 mM Tris HCl, pH 8.5. Fractions (8 ml) were collected every 16 min.

The enzyme was associated with the first major protein peak to be eluted. Those fractions which contained a specific activity greater than 0.5 units/mg were combined (80 ml).

Step 7. Third Ammonium Sulfate Precipitation

Phenylalanine ammonia-lyase precipitated between 18 and 22% saturation with ammonium sulfate during a third ammonium sulfate fractionation. The most active fractions were dialyzed against 50 mM Tris HCl, pH 8.5 and stored at -15°.

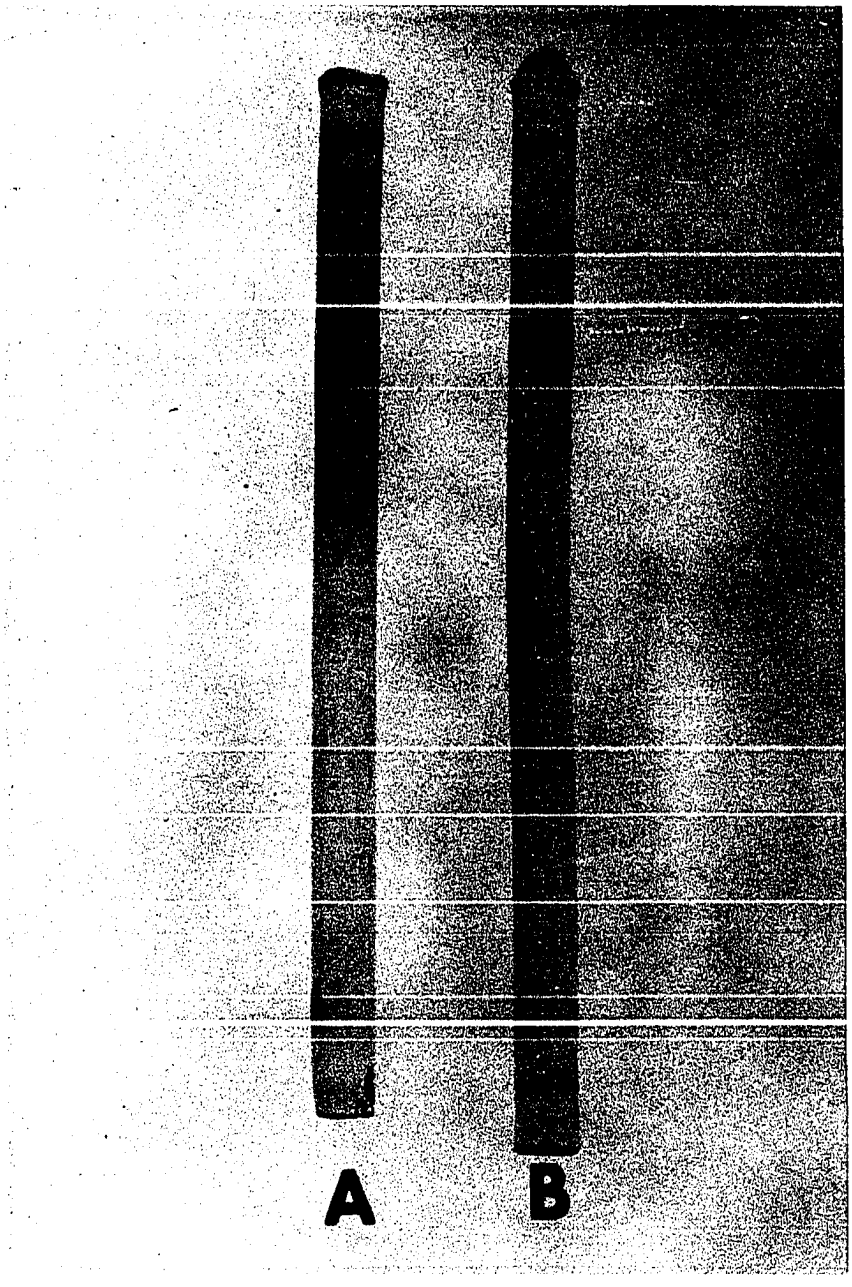
Polyacrylamide Disc Gel Electrophoresis

Gel electrophoresis was performed as described in "Materials and Methods" chapter. The polyacrylamide gels were subjected to 3.0 mamp/tube until the bromphenol blue dye marker reached the terminal portion of the gel. Figure 3 shows the electrophoretic patterns of purified phenylalanine ammonia-lyase. The polyacrylamide gels were run in duplicate, one gel being sliced and eluted in 0.5 ml of 50 mM Tris HCl, pH 8.5 buffer and the other stained with 1% Amido black. Phenylalanine ammonia-lyase activity corresponded with the intense stained protein band and with a smaller band at the top of the gel. The smaller band may be an aggregate form of phenylalanine ammonia-lyase.

Tyrosine Ammonia-Lyase Activity in S. pararoseus

Camm and Towers (8) have postulated that S. roseus contains two ammonia-lyases, one deaminating phenylalanine and the other tyrosine. An experiment was performed in which all but the last step in the purification procedure outlined for phenylalanine ammonia-lyase was carried out. During the purification the activity of phenylalanine ammonia-

Figure 3. Disc gel electrophoresis of L-phenylalanine ammonia-lyase. Gel (A) contains 93μg and Gel (B) 46μg of purified enzyme having a specific activity of 1.31 units/mg. The gels were run at 3.0 mamp/tube for 3.0 hr, at which time the bromphenol blue marker had reached the bottom.


A
B

lyase and tyrosine ammonia-lyase was monitored spectrophotometrically. The ratio of enzyme activities was recorded for each of these steps. Table 5 lists the data from this experiment.

As shows in Table 5 there is a large transient increase of phenylalanine and tyrosine ammonia-lyase activity following protamine sulfate treatment. This increased activity, which invariably occurs during this step is lost during the subsequent purification. Tyrosine ammonia-lyase activity appears to be increased more than the phenylalanine ammonia-lyase activity resulting in a decrease in the phenylalanine to tyrosine ammonia-lyase ratio. Both enzyme activities, however, precipitated in the same fractions during ammonium sulfate and sodium citrate fractionations. The enzyme unit ratios of phenylalanine to tyrosine ammonia-lyase were approximately the same for the major and minor fractions. This indicated that both enzymes precipitate at the same point during these salt fractionations.

Figure 4 shows the elution patterns of phenylalanine and tyrosine ammonia-lyase from G-200 Sephadex (3 x 100 cm). After chromatography the deaminating activity peaks for tyrosine ammonia-lyase and phenylalanine ammonia-lyase coincided, 66.7% of the phenylalanine ammonia-lyase activity and 49.0% of the tyrosine ammonia-lyase was recovered. The specific enzyme activity for phenylalanine ammonia-lyase after the G-200 Sephadex fractions were pooled was 0.934 units/mg protein. The ratio of phenylalanine to tyrosine ammonia-lyase units again was of a magnitude which indicated that there was little if any separation of these activities. To further ascertain whether tyrosine ammonia-lyase activity could be separated from phenylalanine ammonia-lyase activity

TABLE 5

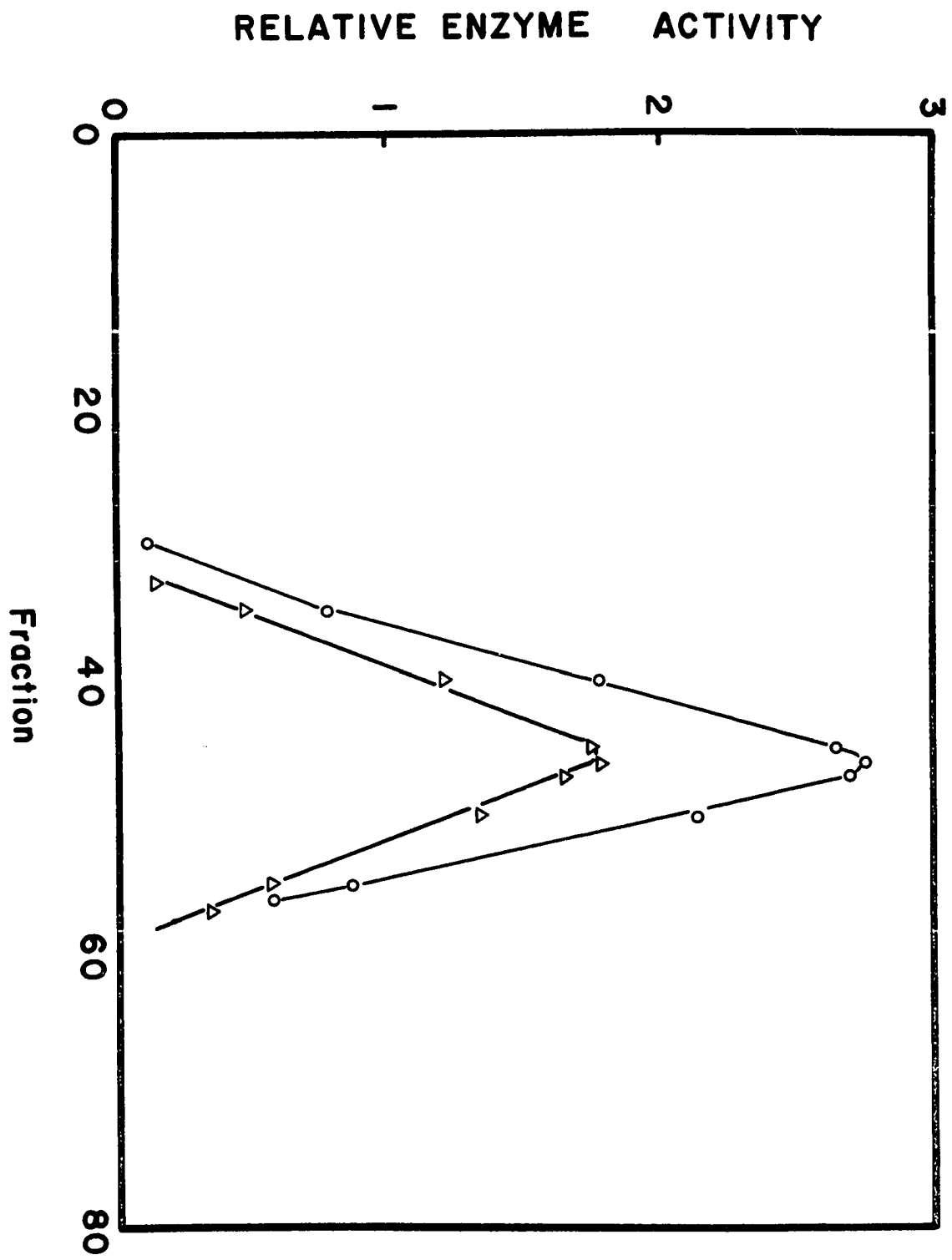
PURIFICATION OF PHENYLALANINE AND TYROSINE AMMONIA-LYASES FROM S. pararoseus

Step	Total enzyme units PAL[a]	Total enzyme units TAL[b]	Ratio units phe / tyr ammonia-lyase
1. Crude enzyme sonicate	12.4	2.95	4.20
2. Protamine sulfate (1 ml 5%/200 mg protein)	16.95	8.11	2.09
3. Ammonium sulfate fractionation			
22% saturation	10.70	2.10	5.10
26% saturation	1.48	0.30	4.97
4. Sodium citrate fractionation			
15.0% saturation	0.594	0.139	4.27
18.7% saturation	5.98	1.29	4.64
5. G-200 Sephadex chromatography			
Tube 35	0.077	0.012	6.40
40	0.179	0.030	5.96
45	0.265	0.043	6.19
46	0.276	0.044	6.21
47	0.270	0.041	6.61
50	0.214	0.034	6.36
55	0.088	0.014	6.08
Total combined above fractions	3.99	0.632	6.31

PAL[a] is phenylalanine ammonia-lyase

TAL[b] is tyrosine ammonia-lyase

Figure 4. Elution profile for phenylalanine and tyrosine ammonia-lyase in 50 mM Tris HCl buffer, pH 8.5, on G-200 Sephadex (3 x 100 cm) (o-o phenylalanine ammonia-lyase), (Δ-Δ tyrosine ammonia-lyase x 5).

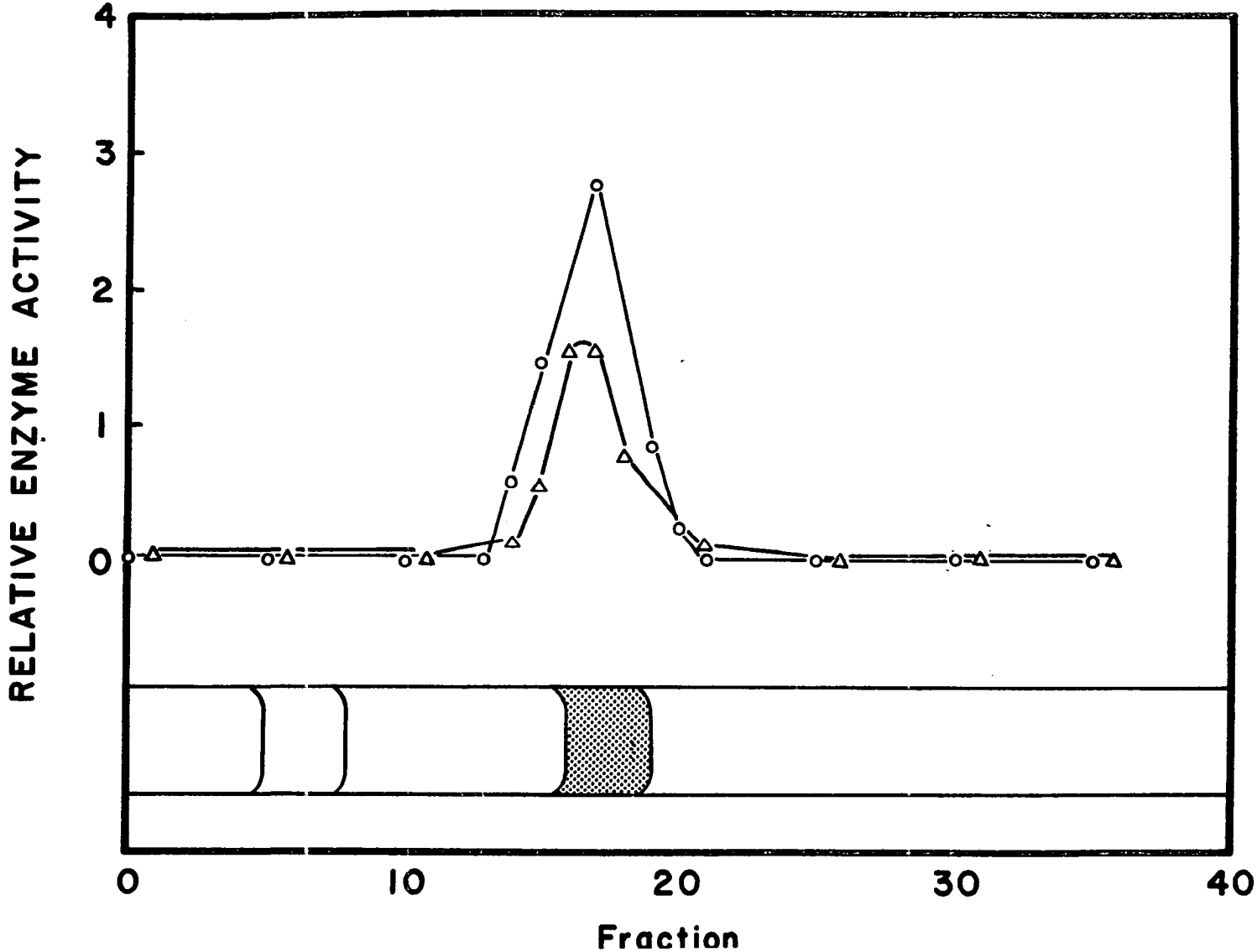

RELATIVE ENZYME ACTIVITY
0
1
2
3
0
20
40
60
80
Fraction

the purified enzymes was subjected to disc gel electrophoresis. Figure 5 shows the results obtained when duplicate gels were run, one gel being cut into 1 mm slices and eluted with 0.5 ml of 50 mM Tris HCl buffer, pH 8.5, while the other gel was stained with 1% Amido black. Enzyme assays with both substrates revealed only one activity peak for each and these peaks were again superimposable. The stained gel revealed one major protein band which corresponded in migration to the enzyme activities. Several media were substituted for the one described earlier, in an attempt to induce tyrosine ammonia-lyase. In two cases 1% L-tyrosine replaced 1% D,L-phenylalanine and in one of these, salts (48) replaced malt and yeast extract. When tyrosine and salts were used as the growth medium the resultant enzyme, purified as before, gave a ratio of phenylalanine to tyrosine ammonia-lyase units of 6.2. Under no conditions could a tyrosine specific ammonia-lyase be detected in this yeast.

Spectrum of Purified Enzyme

A spectrum of purified phenylalanine ammonia-lyase was obtained from 240 nm to 500 nm at an enzyme concentration of 4.75 mg/ml (specific activity 1.18) in 50 mM Tris HCl, pH 8.5. No significant spectral characteristics were observed other than an absorption peak at 280 nm characteristic of certain aromatic amino acids. Taylor and Jenkins (46) have shown that protein-bound pyridoxal phosphate has significant absorption in the ultraviolet region (300-400 nm). Phenylalanine ammonia-lyase exhibited no significant absorption in the 300-400 nm region making the presence of protein-bound pyridoxal phosphate unlikely.

Figure 5. Disc gel electrophoresis of 42.5 μg of purified phenylalanine ammonia-lyase, specific activity 0.934. Electrophoresis was at pH 8.9, 3 hr and 3 mamp/tube. One gel was sliced into 1mm pieces, eluted with buffer and assayed for ammonia-lyase activity. A duplicate gel was stained with 1% Amido black (at bottom) (0-0 phenylalanine ammonia-lyase) (Δ-Δ tyrosine ammonia-lyase x 5).


RELATIVE ENZYME ACTIVITY
4
3
2
1
0
0
10
20
30
40
Fraction

Molecular Weight Determination

The molecular weight of phenylalanine ammonia-lyase was estimated by both gel filtration with calibrated Sephadex G-200 columns and sedimentation on sucrose gradients using enzymes of known molecular weights as standards. When Sephadex elution volumes of the major activity peaks were plotted versus molecular weights by the method of Andrews (1) (Fig. 6), the molecular weight of phenylalanine ammonia-lyase was estimated to be 300,000, this being the average of results obtained from three such experiments. Three sucrose density gradient centrifugation experiments were performed where the rate of sedimentation of phenylalanine ammonia-lyase was compared to that of catalase, fumarase, or xanthine oxidase. Based on the relative enzyme sedimentation rates and applying the assumptions of Martin and Ames (32), [described in "Material and Methods" Chapter] the molecular weight of phenylalanine ammonia-lyase was estimated to be 275,000 with a sedimentation coefficient of 11.70S, these figures being the average of three such experiments. (Figure 7 shows the enzyme profiles for a typical centrifugal run.)

Subunits

Two experiments were performed to determine the number and size of subunits present in L-phenylalanine ammonia-lyase from S. pararoseus. In the first experiment the methods of Weber and Osborn (50) and Shapiro et al. (42) were followed. Briefly they consisted of:

1. Incubation of protein samples for 2-3 hr in 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol in 50 mM sodium phosphate buffer, pH 7.0.
2. Dialysis of protein samples in 500 ml of 10 mM $NaPO_4$ buffer, pH 7.0 containing 0.1% SDS and 0.1% mercaptoethanol.

Figure 6. Logarithmic plot by the method of Andrews (1) of Sephadex elution volumes against molecular weight for lactic dehydrogenase (LDH), fumarase (Fum), catalase (Cat), phenylalanine ammonia-lyase (PAL), glutamate dehydrogenase (GDH), and urease (U).

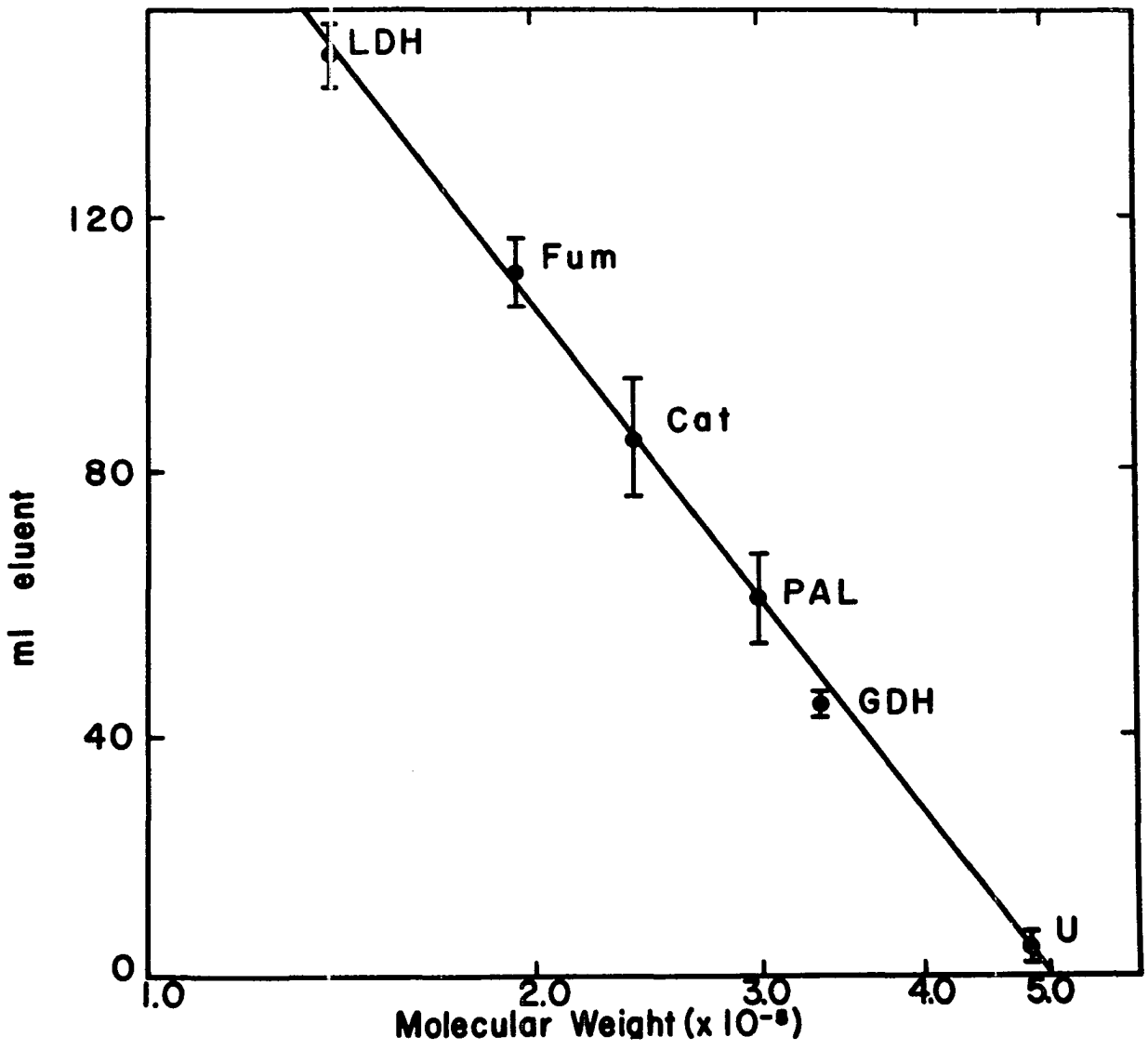
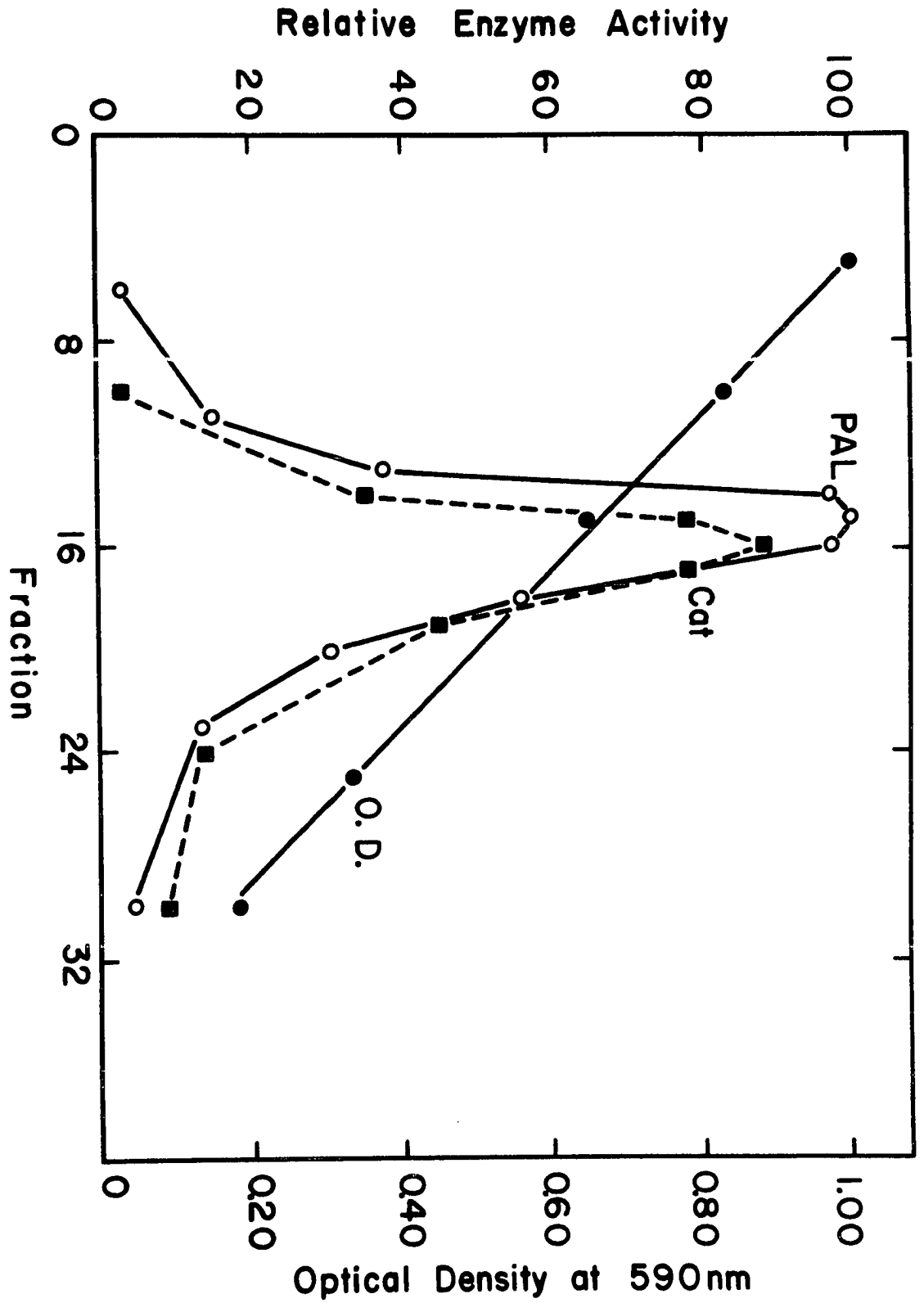

LDH
Fum
Cat
PAL
GDH
U
120
80
40
0
ml eluent
1.0
2.0
3.0
4.0
5.0
Molecular Weight (x 10⁻⁵)

Figure 7. Sucrose density gradient profile after fractionation showing L-phenylalanine ammonia-lyase activity (PAL,o), catalase activity (Cat,■), and optical density at 590 nm (measuring bromphenol blue, ●).


Relative Enzyme Activity
0
20
40
60
80
100
Fraction
0
8
16
24
32
PAL
Cat
O.D.
0
0.20
0.40
0.60
0.80
1.00
Optical Density at 590nm

3. Disc electrophoresis at 8 mamp/tube for 6 hr on 10% crosslinked polyacrylamide gels.

4. Gel staining in 1% Amido-Schwartz for 6 hr - destaining with 7.5% glacial acetic acid.

In this particular experiment four proteins were subjected to electrophoresis on separate gels. Table 6 shows the proteins utilized and the distance of migration for these SDS-protein complexes. Unlike the results of published procedures more than one band was observed for each protein after electrophoresis in SDS. It is not clear at this time why multiple bands were observed, although dimer formation or other multiple forms of enzyme were most probably present. Fig. 8 shows a semi-logarithmic plot of molecular weight versus the distance of migration for the SDS-subunits. The standards used and their nominal molecular weights were: carbonic anhydrase (30,000), bovine serum albumin (68,000), and bovine serum albumin dimer (136,000). The experimental data, when plotted as described above, produced a straight line which is consistent with published findings (42,50). Four SDS-protein bands were observed for phenylalanine ammonia-lyase (Table 6). When these distances were compared to the standard curve four molecular weight values were determined. They were: (A) 72,000-74,000, (B) 83,500, (C) 92,500, and (D) 142,000. The 72,000-74,000 molecular weight species (the most predominant form present) probably represents the monomeric species of this enzyme and the 142,000 species, a dimer. The 83,500 and 82,500 species are not identifiable. They could be artifacts or impurities. Thus, there may be more than one type of enzyme subunit, the most abundant has a molecular weight of 72,000 to 74,000. Four 72,000 to 74,000 subunits would give a molecular weight of 288,000 to

TABLE 6

DISC ELECTROPHORESIS OF PHENYLALANINE AMMONIA-LYASE AND STANDARDS IN SDS

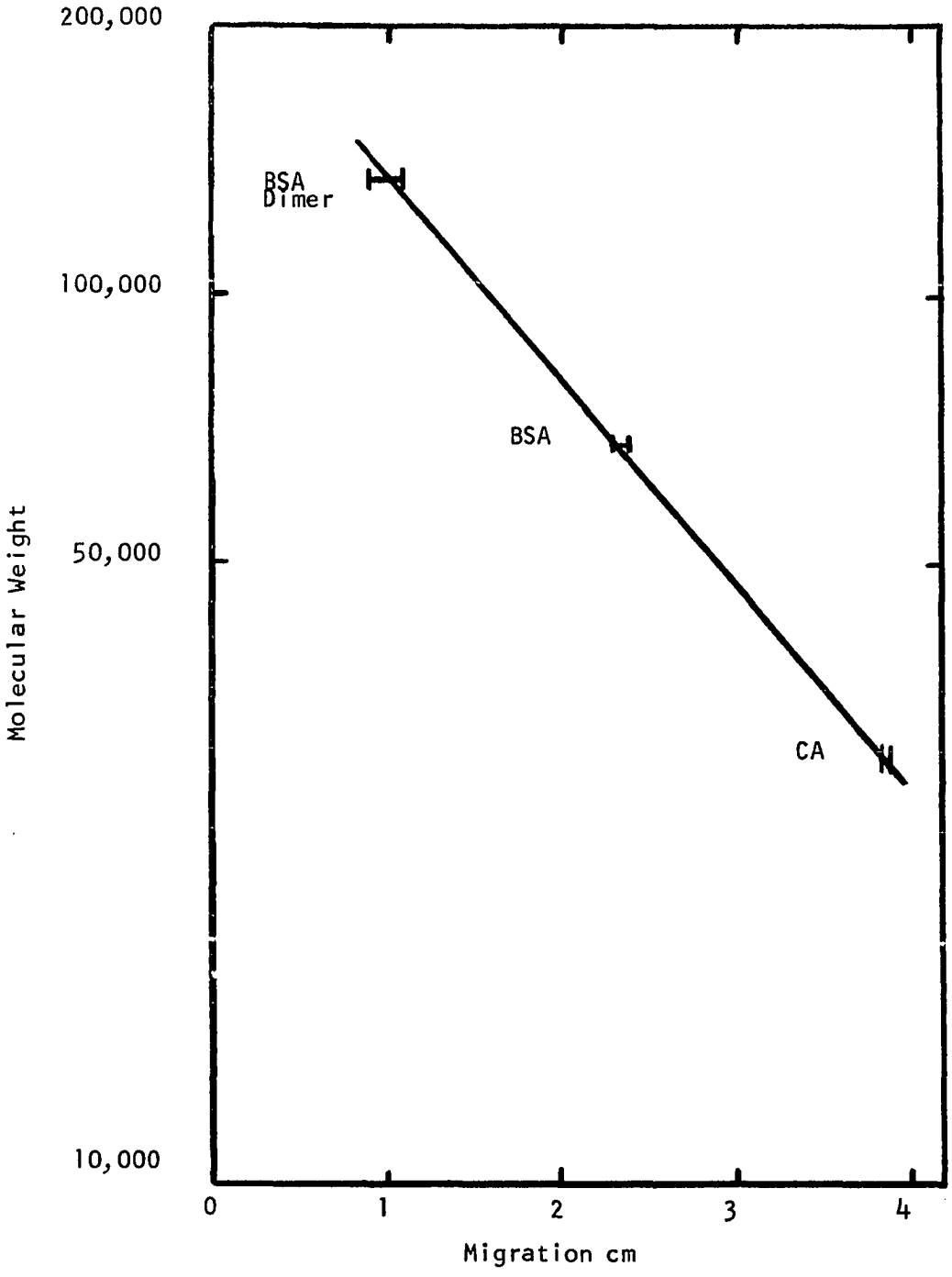
Protein	µg of Protein	distance of migration cm: major band	minor band
1. PAL[a]	200	2.15 - 2.20	1.92, 1.72, 0.89
2. BSA[b]	200	2.3 - 2.4	0.9 - 1.1
3. CA[c]	110	3.85 - 3.90	1.75 - 1.90

[a]PAL is phenylalanine ammonia-lyase

[b]BSA is bovine serum albumin

[c]CA is carbonic anhydrase

Figure 8. Migration of sodium dodecyl sulfate-proteins as a function of their molecular weight. A semi-logarithmic plot of molecular weight versus the distance of migration in cm for SDS-subunits was constructed from data obtained from three standards: carbonic anhydrase (MW = 30,000, CA); bovine serum albumin (MW = 68,000, BSA); and bovine serum albumin dimer (MW = 136,000, BSA Dimer) after polyacrylamide disc gel electrophoresis at 8 mamp/tube for 6 hr, after which the gels were stained in 1% Amido black for 6 hr and destained in 7.5% glacial acetic acid. The distance of migration from the top of the gel was determined for each stained protein band.


200,000
100,000
50,000
10,000
Molecular Weight
BSA
Dimer
BSA
CA
0
1
2
3
4
Migration cm

296,000, which is consistent with the native enzyme's molecular weight.

The second type of experiment employed to determine the molecular weight of L-phenylalanine ammonia-lyase subunits was sucrose density gradient centrifugation. Gradients were prepared as they were for the molecular weight determination of native enzyme and also with the addition of 0.1% SDS and 0.1% mercaptoethanol. A quantity of enzyme was inactivated with ^{14}C-NaCN. An aliquot of ^{14}C-enzyme was treated as mentioned before for application to disc electrophoresis gels. Two 5-20% linear sucrose gradients were prepared, the first containing native enzyme plus an aliquot of inactivated ^{14}C-enzyme. The second gradient contained 0.1% SDS and 0.1% mercaptoethanol plus ^{14}C-enzyme treated with SDS. The gradients were centrifuged in a SW50L rotor with a Beckman-Spinco L-265-B ultracentrifuge for 15.5 hr at 5^{o} and 90,972 x g. After centrifugation the gradients were fractionated and analyzed for radioactivity and enzyme activity. In the gradient containing no SDS or mercaptoethanol a single symmetrical enzyme activity peak was observed which was superimposable upon the peak of radioactivity. The gradient containing 0.1% SDS and 0.1% mercaptoethanol contained no L-phenylalanine ammonia-lyase activity due to dissociation of the enzyme into subunits, but did contain a single symmetrical radioactivity peak. Using the assumptions of Martin and Ames (32) the molecular weight of the species in SDS was estimated using L-phenylalanine ammonia-lyase at 275,000 as the standard. The ^{14}C-SDS species was found to have an estimated molecular weight of 70,000. Four of these subunits would give a molecular weight of approximately 280,000.

The second technique employed for the determination of subunit

molecular weight has several advantages over the polyacrylamide disc gel electrophoresis method. First, the only subunits detected by the sucrose density gradient technique would be those containing the active site of phenylalanine ammonia-lyase. This is true because ^{14}C-NaCN reacts exclusively with the active site located dehydroalanine moiety (described in detail in this Chapter under Effects of Carbonyl Inactivating Reagents). Second, impurities reduced to monomeric form by SDS would not enter into the results obtained as they would not contain a radioactive label. The obvious question posed by this experiment is whether or not all subunits of phenylalanine ammonia-lyase contain an active site, since only those containing an active site would contain radioactivity. It seems most likely that phenylalanine ammonia-lyase from S. pararoseus is not an allosteric enzyme due to the fact that the enzyme contains only one size of subunit and has a Hill coefficient of 1.0 which is indicative of an enzyme having non-interacting active sites.

Substrate Specificity of L-Phenylalanine Ammonia-Lyase

Phenylalanine and tyrosine are deaminated by L-phenylalanine ammonia-lyase. Table 7 shows a comparison of the Rhodotorula glutinus enzyme and the Sporobolomyces pararoseus enzyme as regards their Michaelis constants (K_m) for these two substrates. The Vmax for L-phenylalanine and L-tyrosine are 1.17 and 0.33 μmoles/min/unit respectively.

Inhibition Studies

In an attempt to define the substrate binding characteristics of phenylalanine ammonia-lyase, several substrate analogs were tested an inhibitors of L-phenylalanine deamination and their inhibitory constants were graphically estimated (Table 8). Of the compounds tested

TABLE 7

SUBSTRATE SPECIFICITY OF PHENYLALANINE AMMONIA-LYASE FROM *Rh. glutinus* AND *S. pararoseus*

Substrate	*Rh. glutinus*[a] Km (μM)	*S. pararoseus* Km (μM)
1. L-phenyl-alanine	250	300
2. L-tyrosine	150	85

[a] Hodgins (26).

TABLE 8

INHIBITORY CHARACTERISTICS OF VARIOUS SUBSTRATE ANALOGS

Inhibitory	*Rh. glutinus*[a] Ki (mM)	*S. pararoseus* Ki (mM)	Type of Inhibition
2-OH-Cinnamic acid	0.027	0.0046	Competitive
Cinnamic acid	0.026	0.018	Competitive
L-β-Phenyllactic acid	1.02	0.953	Competitive
Glycine	----	2.12	Competitive
Benzyl Alcohol	----	25.5	Competitive
Phenylpropiolic acid	----	0.56	Non-Competitive
Phenol	----	2.79	Non-Competitive
Glycolic acid	----	242.0	Non-Competitive
Alanine	----	∞	None
Phenethylamine	----	∞	None
Leucine	----	∞	None
Isoleucine	----	∞	None
Valine	----	∞	None

[a] Hodgins (26).

five were found to be effective competitive inhibitors, three exhibited non-competitive patterns and five had no effect. Also shown on this table are three competitive inhibitors of the Rhodotorula glutinus enzyme with their respective inhibitory values. In comparison, the inhibitors have the same relative effectiveness with either enzyme but, of the three inhibitors cited, all appear to have more affinity for the Sporobolomyces pararoseus enzyme than for that from Rhodotorula glutinus. Two additional competitive inhibitors, benzyl alcohol and glycine, demonstrate the wide specificity of binding exhibited by phenylalanine ammonia-lyase. Benzyl alcohol presumably mimics the aromatic side chain of phenylalanine and glycine the aliphatic portion of the substrate molecule. Alanine, leucine, isoleucine, valine and phenethylamine at all concentrations tested (5-40 mM) did not inhibit the enzyme.

Interacting Active Sites

For enzymes obeying Michaelis kinetics the saturation of any active site does not affect the saturation of other active sites on the same molecule. On the other hand, regulatory enzymes show a changing strength of interaction between active sites as the saturation of the enzyme is varied. A simplified procedure has been devised to qualitatively determine whether or not one is dealing with a regulatory or non-regulatory enzyme. It employs the Hill equation which is derived from the Michaelis-Menten equation (equation 1) by a rearrangement and converting to the logarithmic form (equation 2):

$$K = [S]^n \frac{Vmax-1}{V} \qquad (1)$$

$$\log \frac{V}{Vmax - V} = n \log [S] - \log Km \qquad (2)$$

where n is the number of substrate binding sites and is a measure of interaction of catalytic sites, V is the observed initial velocity of the enzyme reaction, Km is the concentration of substrate giving half maximal velocity, S is the substrate concentration, and Vmax is the velocity obtained when all active sites are saturated. When log [V/(Vmax - V)] is plotted versus log [S] a slope of 1.0 is obtained for non-regulatory enzymes; whereas for regulatory enzymes the slope is greater than 1.0. When a Hill Plot (51) was constructed from kinetic data a slope of 0.962 was obtained for the L-phenylalanine ammonia-lyase enzyme. This suggests that there is probably no cooperativity present and the active sites do not interact.

Effects of Carbonyl Inactivating Reagents

It has been previously shown that phenylalanine ammonia-lyase can be inactivated by borohydride, bisulfite, and cyanide (20,24). Experiments similar to those of Hodgins (26) were performed to determine the site of attack of cyanide on this phenylalanine ammonia-lyase. Approximately 1.7 moles of bound ^{14}C-NaCN were required to totally inactivate one mole of the enzyme. Prior incubation of the enzyme with the substrate analog DL-2-hydroxyphenylalanine prevented loss of activity due to NaCN treatment.

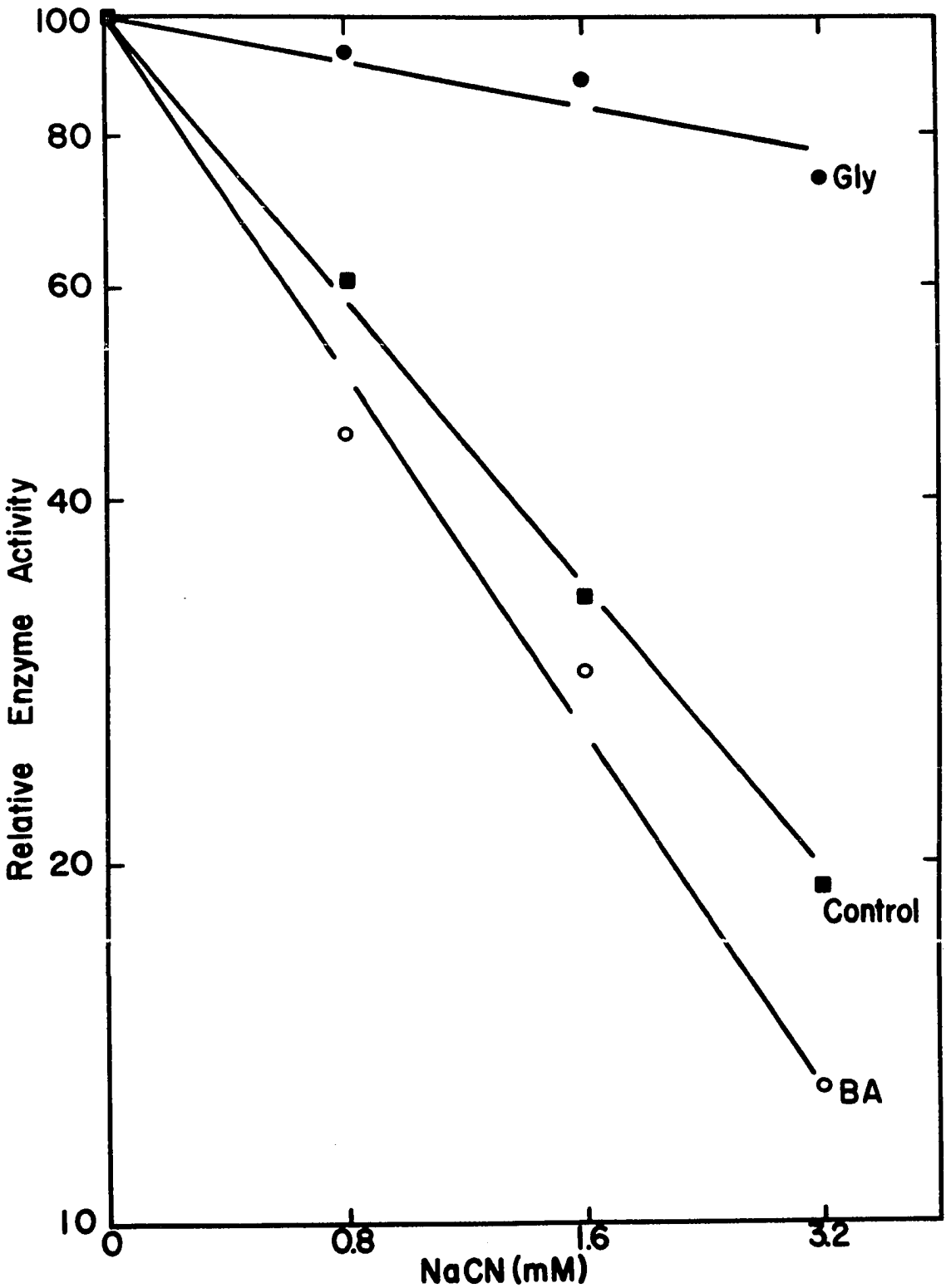
A quantity of ^{14}CN-inactivated enzyme was prepared. The incubation mixture (1.8 ml) containing 1.5 mg protein (1.84 units of enzyme), 0.565 mM ^{14}C-NaCN (52.6 mCi per mmole), and 50 mM Tris HCl, pH 8.5 was prepared. After 45 min at 25°, the mixture was extensively

dialyzed. The enzyme had lost 90% of its catalytic activity and had incorporated 1.62 moles of ^{14}C-cyanide per mole of enzyme.

The ^{14}C-CN-enzyme was subjected to acid hydrolysis (6 M HCl at 110^{o} for 18 hr, in vacuo) and the acid was removed by evaporation under partial vacuum. Aliquots of the hydrolysate were subjected to high voltage paper electrophoresis at pH 1.9 and 8.9 with appropriate standards. Scanning of the high voltage electrophoresis strips for radioactivity indicated that 70-90% of the radioactivity present comigrated with L-aspartic acid. This finding is consistent with earlier reports (18,26) indicative of cyanide addition to an essential dehydroalanine moiety at the active site of phenylalanine ammonia-lyase.

In an attempt to determine the relative location of the dehydroalanine moiety with respect to substrate binding, three competitive inhibitors (cinnamic acid, benzyl alcohol, and glycine) were evaluated for their ability to protect the enzyme against inactivation by cyanide. Benzyl alcohol and glycine presumably mimic the aromatic and aliphatic portions of the substrate molecule L-phenylalanine. Fig. 9 shows the results obtained when various concentrations of cyanide ranging from 0.0 to 3.2 mM were incubated at 30^{o} for 15 min with and without 5.1 mM (2.4 x Ki) glycine or 61.25 mM (2.4 x Ki) benzyl alcohol. Inactivation by cyanide alone represents the control which is a linear logarithmic inactivation under these conditions. Glycine protected the enzyme whereas benzyl alcohol appears to have increased the rate of inactivation slightly. Cinnamic acid was also capable of protecting against inactivation by cyanide, at 100 μM (5 x Ki) cinnamic acid protection was 95% complete.

Figure 9. Protection from NaCN inactivation by substrate analogs. Benzyl alcohol (61.25 mM, BA, o) or glycine (5.1 mM, Gly,●) both at 2.4 x Ki were incubated with enzyme and various concentrations of NaCN at 30° for 15 min and assayed for activity. The NaCN control (■) lacked substrate analogs.


100
80
60
40
20
10
Relative Enzyme Activity
0
0.8
1.6
3.2
NaCN (mM)
Gly
Control
BA

Effects of Amino Attacking Reagents

Phenylalanine ammonia-lyase was inactivated by trinitrobenzene sulfonic acid (TNBS), a reagent capable of attacking amino groups (18). TNBS was studied as to its ability to inactivate phenylalanine ammonia-lyase in the presence of benzyl alcohol and cinnamic acid, two competitive inhibitors. It was not possible to use glycine for this evaluation because it contains an α-amino group which reacted with TNBS. Fig. 10 shows the results obtained when TNBS and enzyme were incubated both with and without either 100 μM (5 x Ki) cinnamic acid or 61.25 mM (2.4 x Ki) benzyl alcohol. Cinnamic acid afforded 66% protection of activity at 60 min whereas benzyl alcohol had no effect.

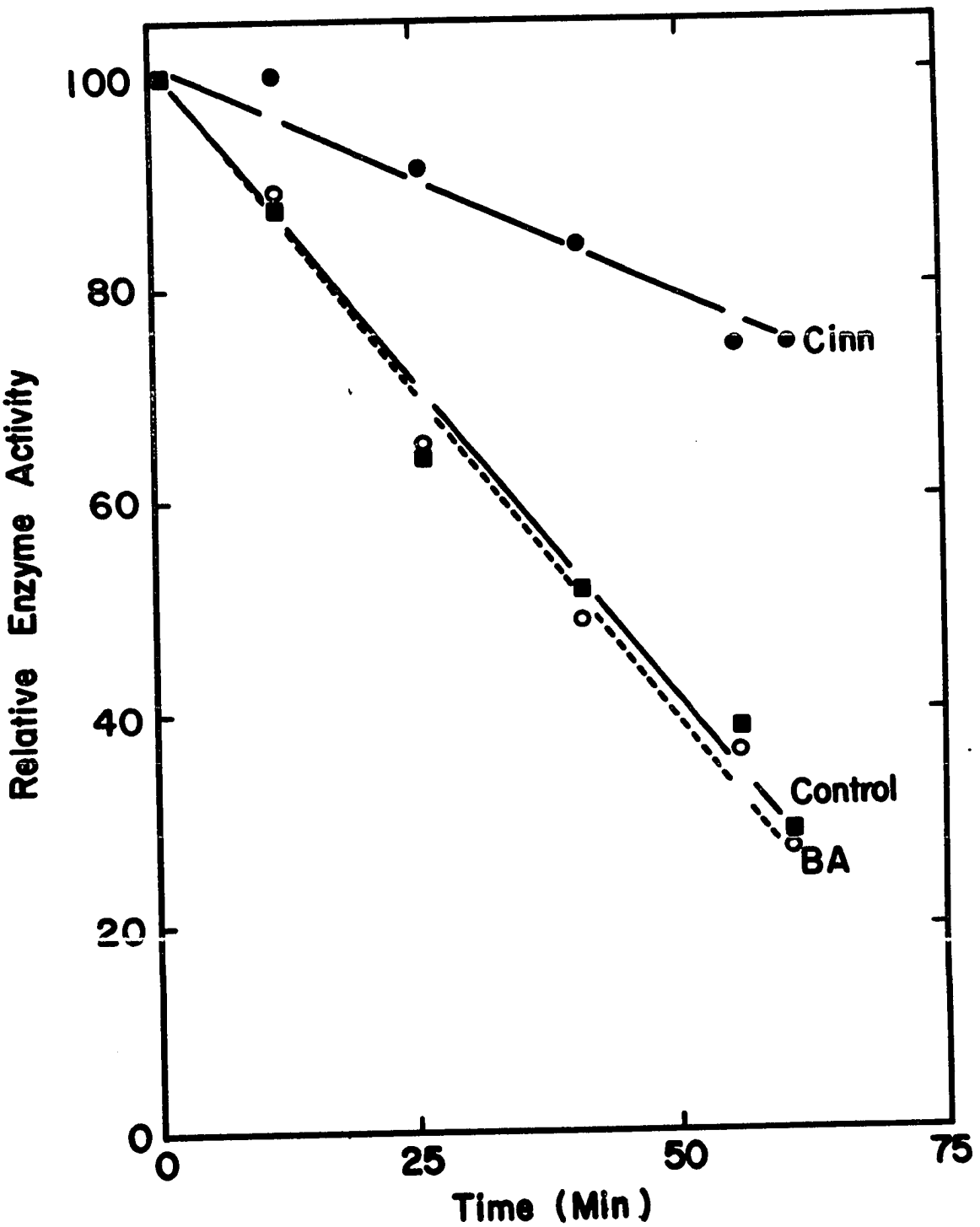
Effects of Sulfhydryl Attacking Reagents

Total inactivation of phenylalanine ammonia-lyase was attainable with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). No level of competitive inhibitor tested [2.12 mM (1 x Ki) glycine; 360 μM (20 x Ki) cinnamic acid, or 900 μM (200 x Ki) DL-2-hydroxyphenylalanine] was capable of affording complete protection from DTNB inactivation even at the minimum level of DTNB necessary for enzyme inactivation. DL-2-hydroxyphenylalanine at 200 x Ki gave 25% protection which was the highest observed.

Enzyme-Ammonia Intermediate in Phenylalanine Ammonia-Lyase

It has previously been postulated that the reaction catalyzed by histidine ammonia-lyase and potato tuber phenylalanine ammonia-lyase proceeds via an enzyme-ammonia intermediate (18,40). Evidence is presented indicating that the yeast phenylalanine ammonia-lyase catalyzed reaction may also involve an enzyme-ammonia intermediate. A postulated catalytic scheme for the deamination of L-phenylalanine by phenylalanine

Figure 10. Protection from TNBS inactivation by substrate analogs. Cinnamic acid (100 μM, 5 x Ki, ●) and benzyl alcohol (61.25 mM, 2.4 x Ki, o) plus 125 μM TNBS and enzyme were incubated at 30° in 0.10 M potassium phosphate, pH 8.0, for 0 to 60 min in a total volume of 0.20 ml. The TNBS control (■) lacked any substrate analogs. The reaction was stopped by a dilution of 1:14 followed by an assay for enzyme activity.


100
80
60
40
20
0
0
25
50
75
Relative Enzyme Activity
Time (Min)
Cinn
Control
BA

ammonia-lyase is:

$$(1) \quad E + P \rightleftharpoons [EP \rightleftharpoons ENC]$$

$$(2) \quad [EP \rightleftharpoons ENC] \rightleftharpoons EN + C$$

$$(3) \quad EN \rightleftharpoons E + N$$

where E is enzyme, P is phenylalanine, C is cinnamic acid and N is ammonia.

If this scheme is correct then any increase in the level of phenylalanine up to a saturating level for the enzyme, would result in an increase in the amount of the enzyme-ammonia complex. It is with these assumptions that the following experiment was designed. Three levels of phenylalanine 1/2, 1 and 2 x Km (0.45, 0.90 and 1.8 mM) were incubated with 0.10 potassium phosphate buffer, pH 6.8 and 98 μM cinnamic acid (0.09 mCi/mM) and 0.05 units of enzyme in a volume of 2.0 ml for 0, 15, 30, 45 or 60 min at 30°. At the times indicated 0.40 ml aliquots of the incubation were removed and placed in an 80° bath for 10 min, (sufficient time to denature the enzyme). Optical density readings were made at 290 nm to assess the amount of cinnamic acid formed. In all cases less than 10% of the original phenylalanine was converted to cinnamic acid. High voltage electrophoresis (pH 1.9 for 3 hr and 2,500 volts) separated the ^{14}C-phenylalanine formed from the ^{14}C-cinnamic acid. The ^{14}C-phenylalanine was eluted from the paper assayed for radioactivity and a quantitative phenylalanine determination performed. Fig. 11 shows the ^{14}C-phenylalanine formed as a function of time and phenylalanine concentration. Fig. 12 shows the relationship of ^{14}C-phenylalanine production to cinnamic acid formation. We have no explanation at this time why the data in Fig. 12 does not extrapolate through the origin.

Figure 11. Formation of ^{14}C-phenylalanine from ^{14}C-cinnamic acid at three phenylalanine concentrations: 0.45 mM (1/2 x Km, ●); 0.90 mM (1 x Km, o); and 1.8 mM (2 x Km, ■). The above value for Km (0.90 mM) was obtained at pH 6.8 in phosphate buffer. Incubations were at 30° for 0 to 60 min with 50 m units of enzyme, 98 µM cinnamic acid (90 µCi/mMole), and phenylalanine in 0.10 M potassium phosphate buffer, pH 6.8 and a volume of 2.0 ml.

Deviations from zero were calculated for the three zero time points, which averaged about 0.1 nMoles ^{14}C-phenylalanine. This deviation was also expressed in the subsequent time points to estimate the limits of deviation in experimental results.

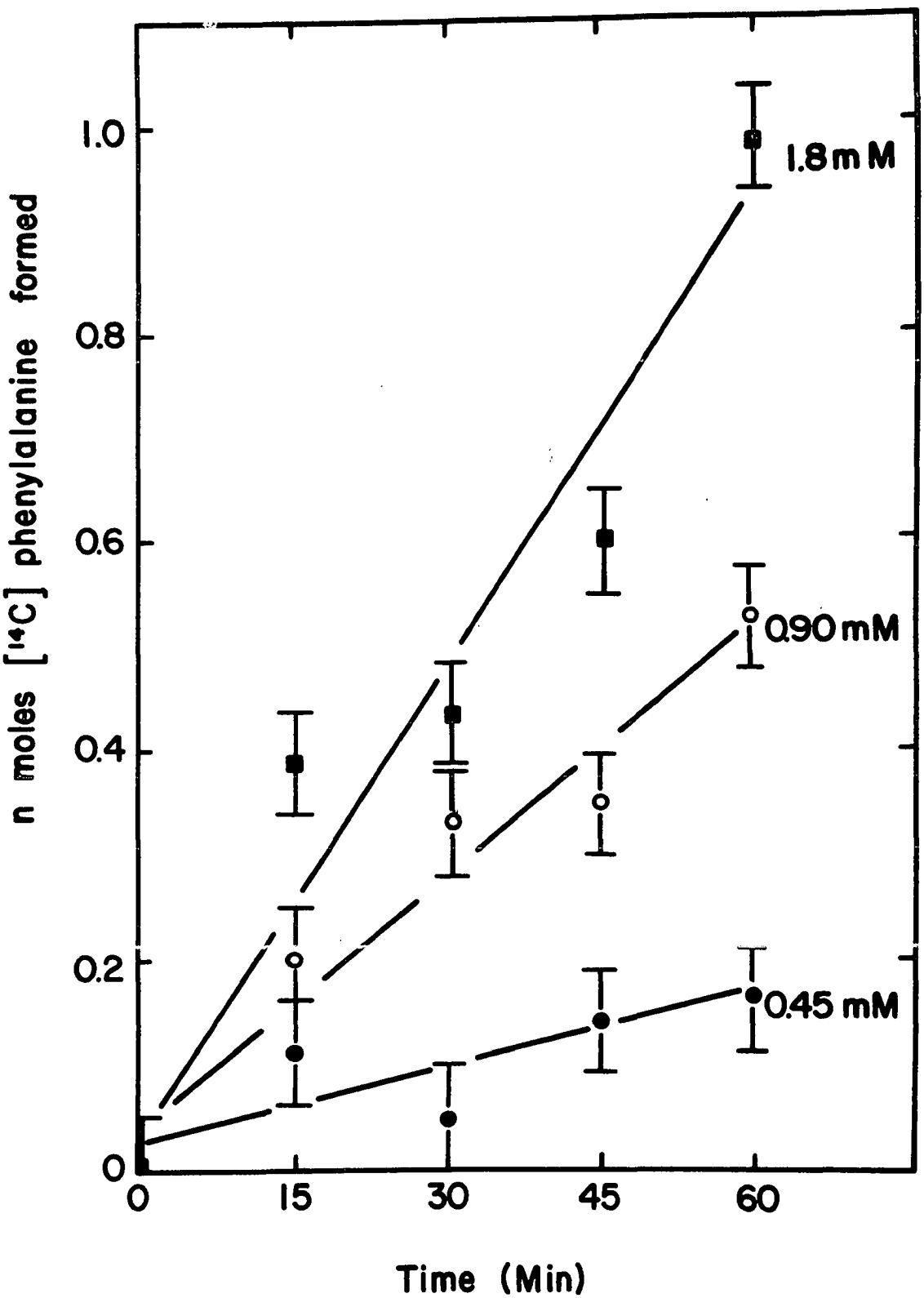
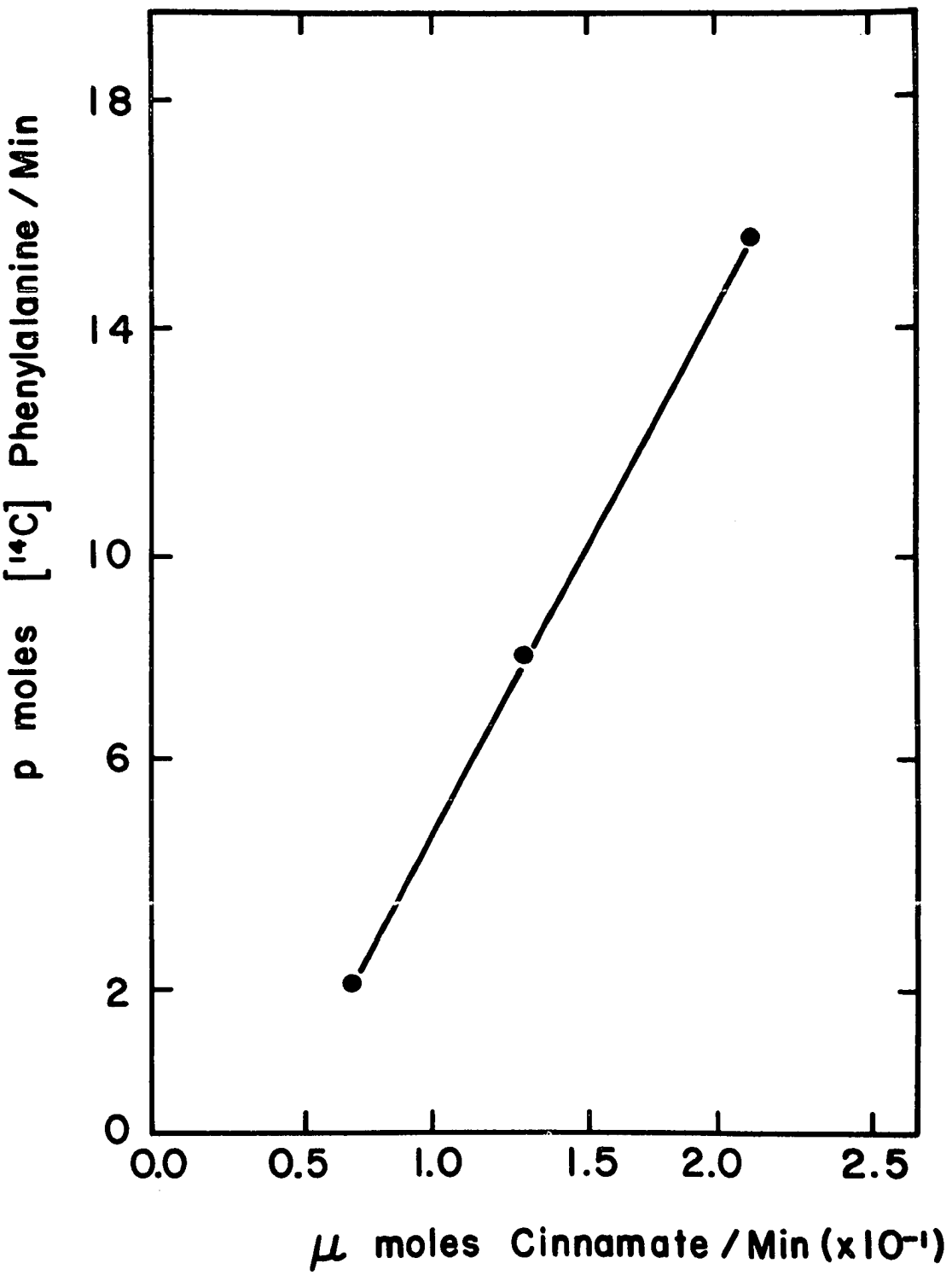

n moles [14C] phenylalanine formed
1.0
0.8
0.6
0.4
0.2
0
0
15
30
45
60
Time (Min)
1.8 mM
0.90 mM
0.45 mM

Figure 12. The rate of ^{14}C-phenylalanine production as a function of the rate of cinnamic acid formation. The rate of ^{14}C-phenylalanine formation was computed from the slopes of the lines in Fig. 11. Cinnamic acid concentrations were determined after 0, 15, 30, 45 and 60 min of incubation and the rate of formation assessed.


p moles [14C] Phenylalanine / Min
18
14
10
6
2
0
0.0
0.5
1.0
1.5
2.0
2.5
μ moles Cinnamate / Min (x10-1)

CHAPTER IV

DISCUSSION

Phenylalanine ammonia-lyase was purified 450 fold using the described procedure which resulted in a specific enzyme activity of 1.3 units/mg protein. After purification the enzyme showed one major protein band on disc gel electrophoresis, a minor slower moving band and trace impurities. When a large amount of enzyme (100 μg) was used for electrophoresis, deaminating activity was also found to correspond to the light slower moving band seen in Figure 3. This lighter band represented only a small percentage of the total phenylalanine ammonia-lyase activity. Due to the consistent appearance of this band and the fact that it apparently has some phenylalanine ammonia-lyase activity it is possible that it is an aggregate form of the enzyme as seem with potato tuber phenylalanine ammonia-lyase (19).

Phenylalanine and tyrosine ammonia-lyase activities in S. pararoseus were not separable by any of the steps in the purification procedure. They gave superimposable activity peaks during Sephadex chromatography and polyacrylamide disc gel electrophoresis. Because of the inseparability of these activities it is very likely that a single enzyme from S. pararoseus has bisubstrate activity for tyrosine and phenylalanine. Whether phenylalanine or tyrosine was used during yeast culture, essentially the same deamination activities were observed.

There appears to be no independent phenylalanine or tyrosine ammonia-lyase in this yeast when cultured under our conditions.

Camm and Towers (8) postulated on the basis of experiments with a crude enzyme preparation that the enzymes responsible for the deamination of tyrosine and phenylalanine in S. roseus were different proteins. The data indicate a contrasting situation with S. pararoseus in that our purest enzyme deaminates both substrates. It is possible, however, but unlikely that a minor species catalyzing solely tyrosine deamination could have been deactivated during the purification procedure.

Purified enzyme exhibited a molecular weight of 300,000 as estimated by gel filtration with several standards, and 275,000 by sucrose density gradient centrifugation.

Disc gel electrophoresis of phenylalanine ammonia-lyase in 0.1% SDS and 0.1% mercaptoethanol indicated that purified phenylalanine ammonia-lyase contains subunits having a monomer molecular weight between 72,000 and 74,000. Similarly, experiments using ^{14}C-inactivated enzyme on 5 to 20% sucrose gradients containing 0.1% mercaptoethanol and 0.1% SDS resulted in a single monomer of 70,000. Results from the disc gel electrophoresis experiments suggest that there may be more than one size of subunit, whereas those from sucrose density gradient centrifugation suggest only a single size of subunit. If it is assumed that aggregation in the disc electrophoresis is responsible for the multiple bands found for both standards and phenylalanine ammonia-lyase and that the monomer molecular weight is 72,000 to 74,000, four monomers would give a native enzyme molecular weight of 286,000 to 296,000. Comparatively, four subunits of molecular weight 70,000 would result in a native enzyme

molecular weight of 280,000. Both of these figures are consistent with the estimated native enzyme molecular weight of 275,000 to 300,000. Hence phenylalanine ammonia-lyase probably contains four subunits of equal molecular weight this being from 70,000 to 74,000. Similarly Havir and Hanson (21) have reported that phenylalanine ammonia-lyase from maize (MW = 306,000) and potato tuber (MW = 330,000) contains 4 identical subunits of molecular weight of 82,800 and 83,900 respectively. Due to the similarities between these enzymes and the yeast phenylalanine ammonia-lyase enzyme it is reasonable to tentatively conclude that the analogous results obtained for the yeast enzyme are accurate.

The discrepancy between the postulated number of subunits for phenylalanine ammonia-lyase (i.e., 4) and the amount of ^{14}C-NaCN necessary to totally inactivate the enzyme (i.e., 1.7 moles/mole of enzyme) suggests that either all the active sites are not catalytically active or that there are not four catalytic subunits. The Hill plot interactive coefficient of 0.962 obtained for the enzyme suggests a non-regulatory enzyme with non-interactive active sites and thus, most probably one type of subunit. It is possible that there exists an *in vivo* mechanism for activation or inactivation of the enzyme via an alteration of dehydroalanine to alanine or serine. If this were the case, the number of subunits would remain constant, whereas the catalytic activity might vary. *In vitro* inactivation of the enzyme also occurs without changes in the electrophoresis patterns on polyacrylamide disc gels; thus, this inactivation may also be active site directed. It is the belief of the author that the enzyme probably contains four subunits of equal size any number of which may either be catalytically active or inactive.

The observation that phenylalanine ammonia-lyase from S. pararoseus does not have significant absorption between 300 nm and 400 nm makes the presence of pyridoxal phosphate seem unlikely.

As with the Rhodotorula glutinus enzyme the inactivation of phenylalanine ammonia-lyase from Sporobolomyces pararoseus with radioactive cyanide results in the formation after acid hydrolysis of ^{14}C-aspartic acid which is indicative of a catalytically essential dehydroalanine moiety. Cinnamic acid and DL-2-hydroxyphenylalanine provided protection from cyanide inactivation, consistent with the location of dehydroalanine at the active site of the enzyme. Borohydride or bisulfite, two other carbonyl attacking reagents, were also found to be capable of inactivating the enzyme.

Phenylalanine ammonia-lyase was also totally inactivated by reagents other than those cited above. TNBS, a reagent which primarily reacts with amino groups, and DTNB, a reagent which reacts with sulfhydryl groups, are both potent inactivators of the enzyme. In the case of both the carbonyl and amino attacking reagents, inactivation was prevented by cinnamic acid. However, no compound tested could afford complete protection from DTNB although partial protection was possible with high concentrations of DL-2-hydroxyphenylalanine. Experiments using cyanide, TNBS or DTNB, as inactivating reagents and benzyl alcohol, glycine, cinnamic acid or DL-2-hydroxyphenylalanine as protecting agents have revealed information as to the possible location of several essential functional groups at the active site. Two of the competitive inhibitors, benzyl alcohol and glycine, resemble respectively the aromatic and aliphatic portions of the substrate molecule L-phenylalanine; and hence,

provide information concerning the functional groups which they can physically mask. For instance, protection from cyanide inactivation by glycine but not by benzyl alcohol indicates that the catalytically essential dehydroalanine moiety may be physically masked by glycine and thus located adjacent to the aliphatic portion of the bound substrate molecule. The slight acceleration of inactivation produced by benzyl alcohol is not understood at this time. A possibility is that the binding of benzyl alcohol alters the conformational arrangement of the enzyme making the dehydroalanine more sensitive to cyanide attack. Cinnamic acid, in a similar experiment, was found to prevent cyanide inactivation. Results similar to those obtained with Na CN were observed for TNBS in the presence of benzyl alcohol and cinnamic acid; again leading to the tentative conclusion that the catalytically essential amino group is physically masked by the binding of the aliphatic portion of the substrate molecule. However, it is also conceivable that glycine and cinnamic acid do not physically mask these essential groups, but instead bring about a conformation change in the enzyme which affords protection from cyanide or TNBS. Because no substrate analog could completely protect against inactivation by DTNB, it is doubtful that the essential sulfhydryl group or groups are located such that they are masked by substrate analog binding.

Results analogous to those of Givot, et al. (16) with histidine ammonia-lyase have been obtained concerning the inhibition of phenylalanine deamination of glycine and alanine. Glycine was a competitive inhibitor (Ki = 2.12 mM) while alanine failed to inhibit the reaction at any concentrations tested (< 40 mM). It has previously been proposed

for histidine ammonia-lyase that the conversion of substrate to product involves the transformation of an $\underline{SP^3}$ to an $\underline{SP^2}$ carbon atom and that the enzyme facilitates this reaction by distorting the β-carbon of substrate toward the $\underline{SP^2}$ geometry. Energy for this distortion would be provided by enzyme-substrate interaction involving three binding sites, namely for the carboxyl, amino and aromatic residues. Alanine, having a β-carbon, must be distorted to bind, but does not have the aromatic residue necessary for the contribution of sufficient binding energy and therefore fails to interact successfully with the enzyme. Glycine, on the other hand, has no β-carbon and therefore can bind to the enzyme without distortion (16,28). In addition, phenylalanine deamination is not inhibited by isoleucine, leucine or valine, reaffirming the data obtained with alanine and furthering the above hypothesis with phenylalanine ammonia-lyase. Analogous observations were made utilizing compounds similar to the aromatic end of the substrate molecule. Benzyl alcohol (Ki = 25.5 mM) competitively inhibited phenylalanine deamination while phenethylamine ($\lesssim$ 40 mM) failed to have any effect. These observations thus strengthen the hypotheses of Givot, et al. (16) concerning the nature of enzyme substrate interactions for histidine ammonia-lyase and extend them to include phenylalanine ammonia-lyase.

Peterkofsky (40) has postulated the existence of an enzyme-ammonia intermediate for histidine ammonia-lyase based upon the incorporation of tritium and urocanate into histidine during the deamination process. Similarly, Havir and Hanson (20) presented evidence to support the enzyme-ammonia intermediate hypothesis in potato tuber phenylalanine ammonia-lyase by performing exchange experiments. Results from our

cinnamate exchange experiments using a constant concentration of ^{14}C-cinnamic acid and three levels of L-phenylalanine (1/2, 1, and 2 x Km) have confirmed the hypotheses that phenylalanine ammonia-lyase from Sporobolomyces pararoseus also containes an enzyme-ammonia intermediate. Based on the assumption that the level of the enzyme-ammonia intermediate increases as the enzyme is saturated, experiments were performed to determine if the rate of radioactive phenylalanine formation from ^{14}C-cinnamic acid was dependent on the relative saturation of the enzyme with unlabeled phenylalanine. Our results indicate an increase in the rate of ^{14}C-phenylalanine formation proportional to the rate of substrate deamination. The breakdown of an enzyme-ammonia intermediate however does not appear to be a rate limiting step in the reaction sequence as cinnamic acid inhibition is strictly competitive thus suggesting only one predominate form of enzyme (26). Also the difference in Vmax for tyrosine and phenylalanine suggest that the breadkown of a common enzyme-ammonia intermediate is not a rate-limiting step in the reaction sequence. Thus, although there does appear to be an enzyme-ammonia intermediate in the phenylalanine ammonia-lyase catalyzed reaction which is dependent on the extent of enzyme saturation with substrate, this intermediate is not a relatively stable form of enzyme.

As yet the exact role of the catalytically essential dehydroalanine, amino, and sulfhydryl groups are not known. As suggested earlier for potato tuber and Rhodotorula glutinus phenylalanine ammonia-lyase (18,26), and for histidine ammonia-lyase (16) the β-carbon of dehydroalanine could be linked to the amino group of substrate thus

increasing the leaving ability of this α-amino group. The resultant complex formed after cinnamate dissociation would be the enzyme-ammonia form of enzyme, which would either expel ammonia or form L-phenylalanine after cinnamic acid addition. The masking of the dehydroalanine by glycine but not benzyl alcohol is also consistent with this hypothesis.

The suggestion that the essential amino group may facilitate the binding of substrate (27) appears to be a likely function of this group. It is possible that the binding of glycine and cinnamic acid to enzyme may be in part through a charge-charge interaction of their carboxyl groups and the essential amino group at the active site of phenylalanine ammonia-lyase. Furthermore, an intriguing possibility is that the essential amino group is the α-amino group of dehydroalanine. If this were the case then the possibility of either hydrogen bond formation or a charge-charge interaction could exist between the carboxyl of substrate and the amino group of dehydroalanine. This interaction, in addition to that of the substrate α-amino group and the β-carbon of dehydroalanine, may result in a cyclic intermediate which could act as a stabilizing force for a transition state by restricting bond rotation thus making catalysis more probable. As yet, the role of the essential sulfhydryl group or groups has not been established, possibly they aid in the abstraction of a proton or protons from the substrate molecule, as previously hypothesized for the Rhodotorula enzyme (27).

CHAPTER V

SUMMARY

L-Phenylalanine ammonia-lyase from Sporobolomyces pararoseus was purified more than 450-fold. Polyacrylamide disc gel electrophoresis of this purified enzyme gave a single major protein band. Tyrosine ammonia-lyase activity was monitored during the purification of phenylalanine ammonia-lyase. Deaminating activities specific for phenylalanine and tyrosine were not resolved during the purification process. It was concluded that phenylalanine ammonia-lyase contains bisubstrate activity.

The purified enzyme has a molecular weight between 275,000 and 300,000 as judged by sucrose density gradient centrifugation and G-200 Sephadex chromatography, respectively. Disc gel electrophoresis and sucrose density gradient centrifugation of standards and purified phenylalanine ammonia-lyase in sodium dodecyl sulfate and mercaptoethanol indicated that the enzyme contains four subunits of equal molecular weight between 70,000-74,000. The enzyme is inactivated by carbonyl, amino, and sulfhydryl attacking reagents. Dehydroalanine has been shown to be present at the active site and essential for catalysis. Protection against inactivation by substrate analogs has revealed the possible active site localization of the essential dehydroalanine and amino group with respect to the substrate molecule.

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