CHARACTERIZATION OF THE REACTIONS INVOLVED IN THE CONVERSION OF ORNITHINE TO 2-AMINO-4-KETOPENTANOIC ACID IN CLOSTRIDIUM STICKLAND II

Thesis for the Degree of Ph. D.
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RALPH SOMACK
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"CHARACTERIZATION OF THE REACTIONS INVOLVED IN THE CONVERSION OF ORNITHINE TO 2-AMINO-4-KETOPENTANOIC ACID IN *CLOSTRIDIUM STICKLANDII*"

presented by

Ralph L. Somack

has been accepted towards fulfillment of the requirements for

Ph. D. degree in Microbiology and Public Health

Major professor

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ABSTRACT

CHARACTERIZATION OF THE REACTIONS INVOLVED IN THE CONVERSION OF ORNITHINE TO 2-AMINO-4-KETOPENTANOIC ACID IN CLOSTRIDIUM STICKLANDII

By

Ralph Somack

The first intermediate in the oxidation of ornithine by Clostridium sticklandii, 2,4-diaminopentanoic acid, has been purified from cell extracts in gram quantities. The dibenzoyl derivative and the monopicrate and dihydrochloride salts were crystallized and characterized. These compounds were employed to verify the structure of the new amino acid by infrared, mass, and nuclear magnetic resonance spectrometry.

The NAD^+-$NADP^+$-dependent 2,4-diaminopentanoic acid C_4 dehydrogenase from Clostridium sticklandii has been purified to homogeneity by the criteria of disc gel electrophoresis and ultracentrifugation. The weight average molecular weight of the native enzyme as determined by high speed sedimentation equilibrium is 72,000. Sedimentation velocity indicated an $s_{20,w}$ of 4.47S. Sodium dodecyl sulfate disc gel electrophoresis and high speed sedimentation in 6 M guanidine·HCl established that the enzyme is composed of two subunits of identical size. The enzyme is

sensitive to thiol inhibitors and titration with 5,5'-dithiobis-2-nitrobenzoic acid and p-chloromercuribenzoate demonstrated the presence of six sylfhydryl groups per mole. Amino acid analysis indicated that the enzyme contains six half-cystine residues per mole.

The coenzyme B_{12}-dependent ornithine mutase from Clostridium sticklandii catalyzing the conversion of ornithine to 2,4-diaminopentanoic acid (2,4-DAP) has been purified to homogeneity. A radiochemical assay employing ^{14}C-labeled ornithin e and a rapid, coupled spectrophotometric assay employing 2,4-diaminopentanoic acid C_4 dehydrogenase are described. Analysis by gel electrophoresis, sucrose gradient centrifugation and SDS gel electrophoresis indicated that the mutase has a molecular weight of about 180,000 and consists of 2 subunits of identical size. The enzyme is specific for D-α-ornithine and is inhibited by L-α-ornithine, DL-α-lysine, and β-lysine. Kinetic and inhibitor studies showed that ornithine mutase and C_4 dehydrogenase are directly linked and that pyridoxal phosphate is a cofactor for ornithine mutase. The absorption spectrum of the mutase measured directly in analytical gels indicated that a substantial amount of native bound cobamide had been converted to inactive hydroxocobalamins. After incubation with B_{12} coenzyme and subsequent dialysis, the spectrum was more typical of bound B_{12} coenzyme. The ornithine mutase reaction is reversible and proceeds to approximately an equal extent in both directions. However, the product, 2,4-DAP, appeared to inhibit the reverse reaction at

concentrations greater than 0.7 mM when present alone. The enzyme contains labile sulfhydryl groups and is inhibited by oxygen. Experiments with H_2O-t indicated that the reaction proceeds by a mechanism which excludes exchange of hydrogen with the solvent.

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By

Ralph Somack

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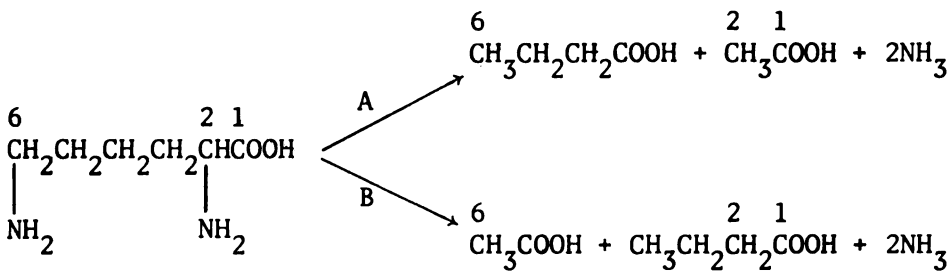
INTRODUCTION[1]

The initial steps in the fermentation of both ornithine and lysine in the putrefactive anaerobe, Clostridium sticklandii, involve a series of amino group migrations followed by oxidative deaminations which prepare the amino acids for subsequent thiolytic cleaveages. These cleavages result in the generation of acyl-CoA fragments from which energy may be derived in the form of ATP, and in the formation of short chain fatty acids which can be used for further biosynthetic processes.

DL-lysine is fermented by C. sticklandii and related organisms through a cleavage between carbon atoms 2 and 3 (Type A cleavage) or carbon atoms 4 and 5 (Type B cleavage) yielding acetate, butyrate and 2 equivalents of ammonia.



[1]A Literature Review which follows references the material presented in this Introduction.

The first reaction in the Type A cleavage is catalyzed by a PLP- and S-adenosylmethionine-dependent enzyme forming L-β-lysine from L-α-lysine. L-β-lysine is next converted to 3,5-diaminohexanoate by β-lysine mutase, an enzyme requiring a B_{12} coenzyme, ATP, a mercaptan, FAD, pyruvate, and a monovalent and divalent cation for full activity. A DPN-dependent dehydrogenase next catalyzes an oxidative deamination forming 3-keto-5-aminohexanoate, which undergoes a thiolytic cleavage yielding the fatty acids and ammonia.

The first and only transformation identified in the Type B cleavage involves the migration of the ε-amino group of D-α-lysine to carbon atom 5 forming 2,5-diaminohexanoate. The enzyme catalyzing this step, D-α-lysine mutase, is structurally identical to the β-lysine mutase and has similar cofactor requirements except that PLP replaces pyruvate and FAD is not stimulatory.

The initial reaction in the oxidation of ornithine to acetate, NH_3, alanine and CO_2 in C. sticklandii, proceeds through an initial B_{12} coenzyme-dependent migration of the δ-amino group of ornithine to carbon atom 4, forming 2,4-diaminopentanoic acid (2,4-DAP). This conversion is catalyzed by the enzyme ornithine mutase. A subsequent PLP-dependent epimerase catalyzing an inversion of the C_4-amino group of 2,4-DAP has been proposed to exist and to precede a TPN or DPN linked oxidative deamination forming 2-amino-4-ketopentanoic acid. The enzyme catalyzing this last step (2,4-diaminopentanoic acid C_4 dehydrogenase) has been partially purified and appears to have no further cofactor requirements.

In contrast to the thoroughly studied aminomutases in the lysine pathway, ornithine mutase has only been cursorily examined with respect to cofactor requirements using crude or partially purified extracts still containing 2,4-diaminopentanoic acid C_4 dehydrogenase. The purpose of the present study was to establish rigorously the structure of the product of the ornithine mutase reaction, 2,4-DAP, and to examine more thoroughly the properties of the enzyme catalyst. In the course of the investigation, methods were developed for obtaining gram quantities of 2,4-DAP and for separating ornithine mutase from C_4 dehydrogenase and purifying both enzymes to homogeneity. A sensitive and rapid spectrophotometric, coupled assay employing the pure C_4 dehydrogenase was devised for measuring mutase activity. It was demonstrated that PLP functions as a cofactor for ornithine mutase and not in a subsequent amino group inversion as previously suggested. In addition, the properties of both ornithine mutase and 2,4-diaminopentanoic acid C_4 dehydrogenase have been described.

This thesis is organized into four sections. The first is a literature review including a discussion of the metabolism of the putrefactive organisms of the genus Clostridium, the fermentation of ornithine and lysine in C. sticklandii, and a comparison of the B_{12}-dependent enzymes involved in these fermentations with related enzyme systems. The other three sections consist of a published manuscript concerning the structure of 2,4-diaminopentanoic acid, an accepted manuscript on the purification and properties of 2,4-diaminopentanoic acid C_4 dehydrogenase, and a manuscript prepared for publication on the purification and properties of ornithine mutase.

LITERATURE REVIEW

Part I
The Amino Acid Fermenting *Clostridia*

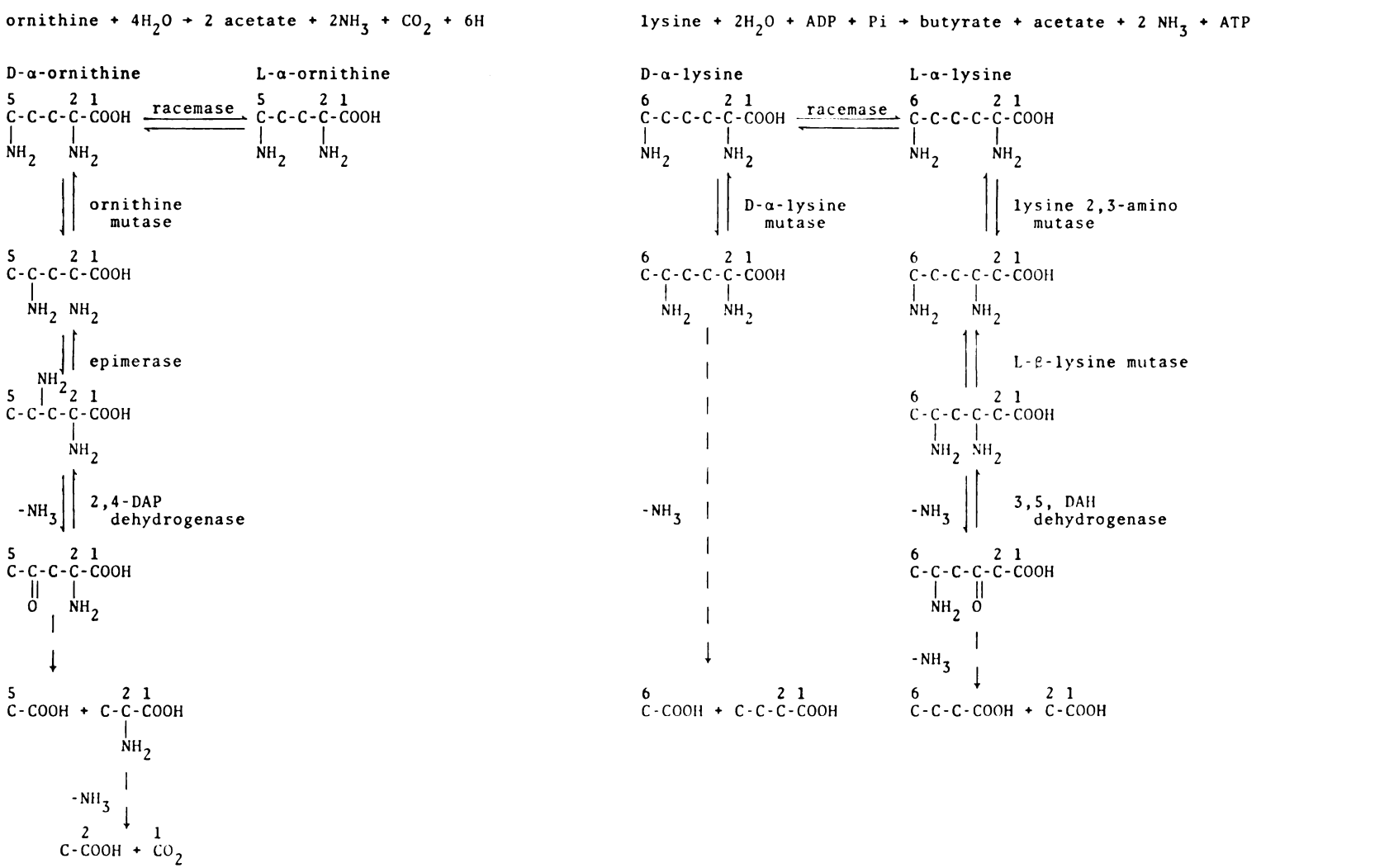
The non-saccharolytic, putrefactive organisms of the genus *Clostridium* are able to grow on media with amino acids as the only sources of energy. These anaerobes employ certain groups of amino acids as hydrogen (electron) donors and other groups as hydrogen (electron) acceptors in coupled oxidation-reductions known as the Stickland reaction (Stickland, 1934). The electron donor is oxidatively deaminated and thiolytically cleaved, eventually resulting in the formation of fatty acids, CO_2, NH_3, ATP and DPNH. The electron acceptor is reduced and sometimes deaminated at the expense of the reoxidation of DPNH. In *C. sporogenes*, amino acids which serve as efficient electron donors include alanine, valine and leucine. Amino acids which function as electron acceptors include proline, hydroxyproline, glycine and ornithine (Stickland, 1934; Woods, 1936).

Part II
The Fermentation of Ornithine and Lysine

Introduction. Since the early demonstration of the Stickland reaction in *C. sporogenes*, the details of these coupled oxidation-reduction fermentations have been investigated in a number of related

organisms. C. sticklandii oxidizes ornithine to acetate, NH_3 and CO_2 (Figure 1) in the presence of either proline or lysine (Stadtman, 1954; Stadtman and White, 1954). However, growth will not occur with any of these amino acids alone. Dyer and Costilow (1968) and Mitruka and Costolow (1967) showed that resting cells of C. sticklandii and C. botulinum ferment L-ornithine as a single substrate. In C. sticklandii part of the ornithine is oxidized to acetate, alanine and NH_3 and the remainder reduced to δ-aminovaleric acid and NH_3. However, if proline is present with ornithine all of the δ-aminovalerate is derived from the former amino acid. In contrast, δ-aminovalerate is the main product of ornithine fermentation by resting cells of C. botulinum. Costilow and Laycock (1971), using C. sporogenes extracts, demonstrated that this transformation proceeds by an initial conversion of ornithine to proline plus NH_3, catalyzed by the enzyme ornithine cyclase.

In the presence of ornithine, cells of C. sticklandii degrade lysine to a mole each of butyrate, acetate and NH_3 (Figure 1) (Stadtman, 1963). In addition, a mole of ATP is formed from ADP + P_i per mole of amino acid fermented. Since this conversion occurs without a net oxidation or reduction, lysine apparently does not serve directly as an electron donor or acceptor. However, the lysine pathway may function as a source of reducing power with ornithine present by generating acetyl-coenzyme A fragments, presumably through a thiolytic cleavage (Figure 1), which may reductively condense to form butyrate (Stadtman, 1954). Alternatively,



Ornithine fermentation pathway

Lysine fermentation pathways

FIGURE 1: Ornithine and lysine fermentation in Clostridium sticklandii.

an intermediate(s) in the breakdown of lysine, which can also be obtained from proline, may be required as a growth factor.

Fermentation of ornithine. The oxidation of ornithine by C. sticklandii to acetate, alanine and ammonia (Figure 1) involves a primary cleavage between carbon atoms 3 and 4, with alanine derived from carbon atoms 1 through 3, and acetate from carbon atoms 4 and 5 (Dyer and Costilow, 1968). Alanine can be further oxidized to acetate, CO_2 and NH_3 in the presence of lysine or an electron acceptor, proline (Stadtman, 1954; Stadtman and White, 1954; Dyer and Costilow, 1968). The first reaction in the pathway was discovered independently by Dyer and Costilow (1970) and Tsuda and Friedmann (1970) and involves the migration of the δ-amino group of ornithine to carbon atom 4, forming 2,4-diaminopentanoic acid (2,4-DAP). The enzyme catalyzing this reaction, ornithine-4,5-aminomutase, has been cursorily studied in crude extracts (Tsuda and Friedmann, 1970) and appears to be cobamide coenzyme dependent. Two further transformations proposed by Tsuda and Friedmann (1970) result in the formation of 2-amino-4-ketopentanoic acid. They proposed that the first of these involves the participation of a pyridoxal phosphate dependent epimerase which catalyzes the inversion of the C_4-amino group of 2,4-DAP. A pyridine nucleotide-linked dehydrogenase then oxidatively deaminates the C_4-amino group of 2,4-DAP forming 2-amino-4-ketopentanoic acid. The dehydrogenase was partially purified and was shown to be highly specific for

2,4-DAP, to utilize DPN^+ or TPN^+ equally well, and to have no further cofactor requirements (Tsuda and Friedmann, 1970). The equilibrium of the reaction is strongly in favor of oxidized pyridine nucleotide. The existence of a pyridoxal phosphate dependent epimerase between ornithine mutase and 2,4-diaminopentanoic acid C_4 dehydrogenase was only tentatively proposed from experiments with ammonium sulphate treated extracts which catalyzed the reduction of DPN^+ through the overall conversion of ornithine to 2-amino-4-ketopentanoic acid. It was suggested that a coenzyme B_{12}-dependent transformation inhibited by intrinsic factor preceded a pyridoxal phosphate dependent transformation which was inhibited by hydroxylamine.

Fermentation of lysine. The degradation of lysine by C. sticklandii and related organisms (Figure 1) occurs either through a cleavage between carbon atoms 2 and 3 (Type A cleavage) or carbon atoms 4 and 5 (Type B cleavage) yielding acetate, butyrate and 2 equivalents of ammonia (Stadtman, 1954, 1955). However, the formation of acetate in cell extracts has only been demonstrated by the former pathway (Stadtman, 1962). The initial steps in lysine fermentation by both pathways involve a series of amino group migrations, similar to the mutase reaction in the ornithine pathway. These prepare the amino acids for oxidative deaminations which can readily generate energy-rich acyl-coenzyme A intermediates through subsequent thiolytic cleavages.

The first reaction in the type A cleavage, studied in Clostridium strain SB_4 extracts, is catalyzed by a pyridoxal phosphate and

S-adenosylmethionine-dependent mutase, forming L-β-lysine from L-α-lysine (Costilow et al., 1966; Chirpich et al., 1970). The enzyme contains bound pyridoxal phosphate and ferrous ion, is rapidly inactivated by oxygen, and requires the presence of mercaptans for full activity. The molecular weight of the protein is 285,000 and it contains 6 subunits, each with 3 sulfhydryl groups. One of the sulfhydryl groups is protected from titration with sulfhydryl reagents in the native state (Zappia and Barker, 1970). The next step is the type A cleavage involves the reversible conversion of L-β-lysine to 3,5-diaminohexanate and was demonstrated in Clostridium strain SB_4, C. sticklandii and Clostridium strain M-E extracts (Dekker and Barker, 1968; Tsai and Stadtman, 1968). The aminomutase catalyzing this reaction is resolved during purification from extracts of C. sticklandii into two distinct protein moieties: an acidic protein with a molecular weight of 150,000 containing tightly bound cobamide, and a labile sulfhydryl protein of molecular weight 60,000 (Stadtman and Renz, 1968). In addition to B_{12} coenzyme, the enzyme requires ATP, a mercaptan, FAD, pyruvate, a monovalent cation (K^+, Rb^+ or NH_4^+), and a divalent cation (Mg^{+2} or Mn^{+2}) for full activity. A DPN^+-dependent dehydrogenase next catalyzes an oxidative deamination forming 3-keto-5-aminohexanoic acid (Rimerman and Barker, 1968), which presumably undergoes a thiolytic cleavage yielding the fatty acid products.

The only transformation thus far identified in the type B cleavage (Figure 1) is the freely reversible migration of the ε-amino

group of D-α-lysine to carbon atom 5, forming 2,5-diaminohexanoate (Stadtman and Tsai, 1967). The aminomutase catalyzing this reaction, the D-α-lysine mutase, has been detected in Clostridium strain M-E and purified from C. sticklandii extracts (Morley and Stadtman, 1970). The enzyme is structurally identical to the L-β-lysine mutase; however, resolution of the complex is achieved after purification by acidification to pH 4.0, at which point the cobamide moiety is rendered insoluble. The sulfhydryl protein is stabilized in solution by the addition of mercaptans. The sulfhydryl proteins of both mutases appear to be interchangeable with the cobamide moieties directing the specificity for either D-α-lysine or L-β-lysine (Morley and Stadtman, 1970). The cofactor requirements of the D-α-lysine mutase are similar to those for L-β-lysine mutase except that the former enzyme is stimulated by pyridoxal phosphate instead of pyruvate, and FAD is stimulatory to the latter but not the former mutase. The cobamide moiety of the D-α-lysine mutase contains bound pyridoxal phosphate (Morley and Stadtman, 1972), and ATP is believed active as an allosteric effector (Morley and Stadtman, 1970).

Part III
Other Coenzyme B_{12}-dependent Mutases

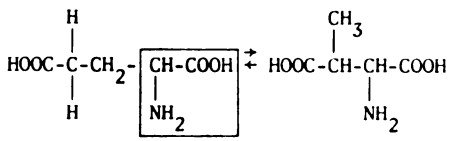
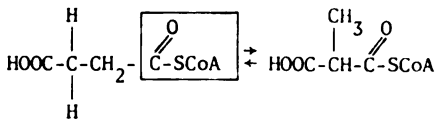
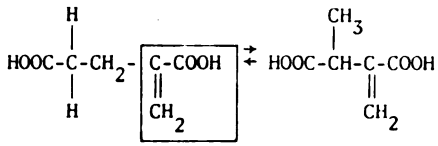
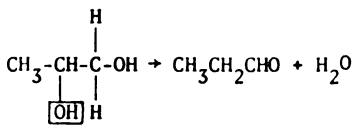
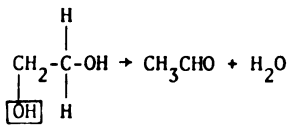
Introduction. The ornithine, D-α-lysine, and L-β-lysine mutases involved in the early transformations of ornithine and lysine fermentation belong to a class of enzymes which utilize B_{12} coenzymes as cofactors. The coenzyme forms of vitamin B_{12} are organo-cobalt derivatives of the vitamins that contain a 5'-deoxyadenosine moity

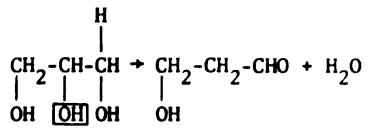
covalently bound to the cobalt atom in place of a CN^-, OH^- or H_2O ligand in the corresponding vitamin. The various B_{12} coenzymes differ with respect to the particular nucleotide base attached to the cobalt on the underside of the corrin ring (see Barker, 1966, 1967, and Hogenkamp, 1968, for comprehensive reviews of B_{12} biochemistry). Clostridium sticklandii normally synthesizes the B_{12} coenzyme containing adenine as the nucleotide base (pseudo-B_{12}) (Stadtman, 1960). Cobamide coenzyme (5,6-dimethylbenzimidazolyl-Co-5'-deoxyadenosylcobamide), as well as pseudo-B_{12}, satisfy the B_{12} coenzyme requirement of the mutases in the ornithine and lysine pathways (Tsuda and Friedmann, 1970; Stadtman and Renz, 1968; Morley and Stadtman, 1970). However, the tightly bound corrinoids native to these proteins have not yet been elucidated.

The remaining reactions catalyzed by the class of enzymes dependent on the coenzyme forms of vitamin B_{12} have recently been reviewed elsewhere (see below) and will be briefly mentioned. The transformation common to all these coenzyme B_{12}-dependent reactions involves the replacement of a group attached to one carbon atom of the molecule with a hydrogen from an adjacent carbon. Table 1 lists the individual reactions catalyzed by these enzymes and Table 2 summarizes a number of properties of these proteins.

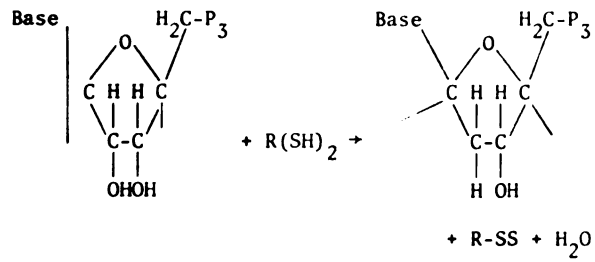
Carbon-nitrogen bond cleaving mutases (Stadtman, 1971, 1972). One other enzyme catalyzing this type of transformation in addition to the lysine and ornithine aminomutases has been described: ethanolamine

TABLE 1: B_{12} Coenzyme-dependent Reactions. In each reaction the migrating group that is replaced by a hydrogen is shown enclosed in a rectangle (from Stadtman, 1971).

Reaction Catalyzed	Enzyme	Distribution of Enzyme
Carbon-Carbon Bond Cleavage		
	Glutamate mutase	Bacteria
	Methylmalonyl-CoA mutase	Bacteria, mammals
	α-Methylene-glutarate mutase	Bacteria
Carbon-Oxygen Bond Cleavage		
	Diol dehydrase	Bacteria
	Diol dehydrase	Bacteria

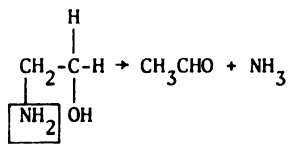


Glycerol dehydrase — Bacteria

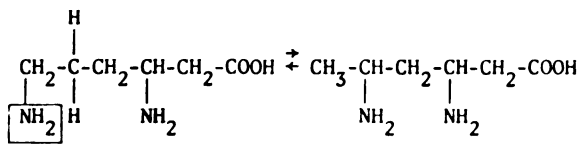


Ribonucleotide reductase — Bacteria

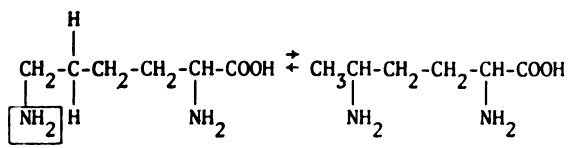
Carbon-Nitrogen Bond Cleavage



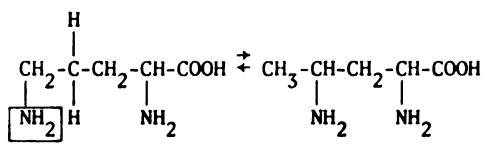
Ethanolamine deaminase — Bacteria



L-β-Lysine mutase — Bacteria



D-α-Lysine mutase — Bacteria



Ornithine mutase — Bacteria

TABLE 2: Properties of Coenzyme B_{12}-dependent Mutases.

Enzyme	Molecular Weight	Sub-units	Bound B_{12}		Groups	Reducing Agent Required	Cofactors
			Moles	Type			
β-lysine mutase	210,000	2	?	Strong	≥ 1	Yes	ATP, FAD, pyruvate Mg^{+2}, $K^{+}(Mn^{+})(NH_4^{+})$
D-α-lysine mutase	210,000	2	?	Strong	≥ 1	Yes	ATP, PLP, Mg^{+2}, $K^{+}(Mn^{+})$ (NH_4^{+})
Ornithine mutase	?	?	?	?	?	Yes	?
Ethanolamine deaminase	500,000	10	2	Weak	≥ 10	No	$K^{+}(NH_4^{+})(Rb^{+})$
Methyl malonyl-CoA mutase							
Bacterial	56,000	?	?	Weak	?	No	-
Animal	165,000	2	2	Strong	≥ 1	Yes	-
Glutamate mutase	145,000	2	7	Weak	5	Yes	-
α-methylene glutarate mutase	170,000	?	?	Weak	≥ 1	Yes	-
Diol dehydrase	240,000	?	1	Strong	≥ 1	No	$K^{+}(NH_4^{+})(Rb^{+})$
Glycerol dehydrase	188,000	2	1	Strong	≥ 1	No	$K^{+}(NH_4^{+})$
Ribonucleotide reductase	110,000	?	?	?	?	Yes	Mg^{+2}, K^{+}, ATP

deaminase. This enzyme is found in an unidentified Clostridium which utilizes choline and ethanolamine as substrates for growth. The migration and exchange of the amino group and hydrogen of ethanolamine results in the formation of an unstable intermediate which spontaneously decomposes to acetaldehyde and NH_3.

Carbon-carbon bond cleaving mutases (Barker, 1972). Methylmalonyl CoA mutase catalyzes the reversible formation of succinyl-CoA from methylmalonyl-CoA. This enzyme participates in the oxidation of propionate, methylmalonate and compounds converted to these acids during degradation by animal tissues and microorganisms, and also participates in the synthesis of propionate by the propionibacteria. The enzyme from both animal and bacterial sources contains cobamide coenzyme which is protected by the protein from photolysis, in contrast to the pseudo-B_{12} bound to ethanolamine deaminase, which is inactivated by visible light. With the bacterial mutase, coenzymes containing either a purine or benzimidazole nucleotide base can substitute for cobamide coenzyme. In contrast, the sheep liver enzyme is specific for benzimidazole-containing cobamides.

Glutamate mutase, found in C. tetanomorphum, Rhodospirillum rubrum and R. spheroides, catalyzes the initial step in the fermentation of glutamate in these organisms. The protein consists of two subunits, component E of molecular weight 128,000, and component S of molecular weight 17,000. Component E binds both substrate and B_{12}

coenzymes. Component S contains sulfhydryl groups which can form both inter- and intramolecular disulfides in the presence of O_2 resulting in inactivation. Component S apparently increases the affinity of component E for B_{12} coenzymes. The native-bound coenzyme is probably pseudo-B_{12}; however, other base substituted coenzymes are equally as effective with varying affinities for the E component.

α-methyleneglutarate mutase catalyzes the reversible conversion of α-methyleneglutarate to β-methylitaconate. The enzyme functions in the anaerobic degradation of nicotinic acid in C. barkeri. The B_{12} requirement is similar to that of glutamate mutase.

Carbon-oxygen bond cleaving mutases (Abeles, 1972). Dioldehydrase isolated from Aerobactor aerogenes catalyzes the conversion of 1,2-propanediol and ethylene glycol to the corresponding aldehydes. In addition to cobamide coenzymes, coenzymes in which the dimethylbenzimidazole moity is replaced with adenine, benzimidazole and chloroadenine are also active. Coenzyme B_{12} forms an irreversible and photolytically resistant complex with apoenzyme in the presence or absence of substrate. However, in the absence of substrate, the coenzyme, normally unreactive toward O_2, is converted to inactive hydroxocobalamin of O_2 is present. This fact, and the observed changes in the light and ESR spectra of enzyme-bound coenzyme in the presence of substrate, suggest that the interaction between apoenzyme and coenzyme leads to structural changes in the coenzyme.

Glycerol dehydrase has been isolated from Lactobacillus, Aerobacter aerogenes and a Clostridium sp. The enzyme catalyzes the conversion of glycerol to β-hydroxypropionaldehyde, although ethylene glycol and 1,2-propanediol also serve as substrates. The enzyme is purified as a stable but inactive hydroxocobalamin complex containing a large sulfhydryl protein and a smaller protein of molecular weight 22,000. The holoenzyme is activated by the addition of B_{12} coenzyme in the presence of Mg^{+2} and $SO_3^{=}$.

Ribonucleotide reductase from Lactobacillus leichmannii reduces the four nucleoside triphosphates to the corresponding 2'-deoxynucleotides. The immediate physiological reducing agent is a low molecular-weight dithiol protein which can be replaced by Escherichia coli thioredoxin or reduced lipoic acid and related dithiols. ATP apparently functions as an allosteric effector and Mg^{+2} regulates the rates at which the various substrates are reduced. The enzyme requires DBC coenzyme or an analogue containing another benzimidazole or purine nucleotide base.

Part IV
Vitamin B_{12}-dependent Reactions

In addition to the ten biochemical reactions mentioned above which utilize cobamide coenzymes as prosthetic groups, there are three other reactions known to require a vitamin form of B_{12}. These have been reviewed by Barker (1967) and Stadtman (1971), and will be briefly mentioned.

Methionine synthetase catalyzes the transfer of a methyl group from N^5-tetrahydrofolate to homocysteine, forming methionine. The enzyme is found in certain strains of E. coli, Streptococcus faecalis, several other bacteria, and in the livers of animals. Enzyme-bound vitamin B_{12} is the intermediate carrier of the methyl group in this reaction. S-adenosylmethionine is required as a cofactor but apparently serves to methylate a group on the enzyme rather than functioning in the methyl transfer reaction per se.

Methane synthetase, found in the methane bacteria, catalyzes the formation of methane derived from the methyl groups of various donor compounds. These include formate, serine, methanol, acetate, methyl-B_{12} and N^5-methyltetrahydrofolate. The corrinoid containing 5-hydroxybenzimidazole as the nucleotide base appears to be the native-bound B_{12} vitamin. The B_{12} vitamin is the intermediate carrier of the methyl group from donor molecules which is reductively cleaved in a final step to yield methane. Reducing equivalents are provided by molecular hydrogen and hydrogenase, or by an NADH generating system.

Acetate synthetase is found in Clostridium thermoaceticum and C. sticklandii. Methyl B_{12} functions as an intermediate methyl carrier in the formation of acetate from CO_2. The reduction of CO_2 to protein-bound methyl B_{12} proceeds through the intermediate formation of formate and N^5-methyltetrahydrofolate. The acetate synthetase-bound methyl B_{12} then undergoes methyl group carboxylation in the presence of CO_2, followed by reduction and cleavage of the carboxymethyl group to acetate at the expense of NADPH.

Part V
Mechanisms of B_{12}-dependent Reactions

It is apparent from the discussion in Part IV that enzyme systems dependent on a vitamin form of B_{12} are involved in methyl group transformations. The formation of methionine from homocysteine involves methyl transfer, methane formation involves methyl transfer and methyl group reduction, and acetate synthesis involves methyl transfer, carboxylation and carboxymethyl group reduction. The role of B_{12} in these systems as a methyl group carrier has been investigated by tracing radioactivity from ^{14}C-methyl donors to protein bound methyl B_{12} and then to reaction products (Barker, 1967).

The function of the corrinoid in the reactions catalyzed by the coenzyme forms of B_{12} is that of the intermediate carrier of the migrating hydrogen that exchanges for the group on a neighboring carbon atom. The hydrogen is transferred to the 5'-methylene group of the 5'-deoxyadenosyl moity of the coenzyme (which probably involves rupture of the cobalt-carbon bond) forming a 5'-methyl group (Miller and Richards, 1969; Eagar et al., 1972). One of the now equivalent hydrogens is subsequently transferred to the product. Tritium transfer from substrate to coenzyme and from coenzyme to product has thus far been demonstrated in the majority of the reactions listed in Table 1 (Stadtman, 1971). The kinetic isotope effects observed with tritium and deuterium labeled substrates and coenzymes in such experiments established that the rate limiting step in the mutase reactions is the breaking of the carbon-hydrogen

bond in the substrate (Abeles, 1971; Barker, 1972; Stadtman, 1972). The role of the cobamide in the glycerol dehydrase, ornithine mutase, and ribonucleotide reductase reactions has not yet been rigidly established. Attempts to detect hydrogen transfer from ^{3}H-5'-labeled coenzyme to deoxyribonucleotide products resulted in rapid exchange of tritium with H_2O (Beck et al., 1966). This was postulated to occur due to an enzyme catalyzed reversible exchange between coenzyme hydrogen and reduced lipoate, followed by a rapid exchange of thiol hydrogens with the solvent. No exchange has been reported to occur between the migrating hydrogen and H_2O in the other B_{12} coenzyme reactions studied (Abeles, 1971; Barker, 1972; Stadtman, 1971).

It is yet uncertain as to the species of hydrogen transferred in the mutase reactions. The appearance of unpaired electrons during the ethanolamine deaminase reaction suggests that the hydrogen migrates as a radical to the 5'-deoxyadenosyl moity of the coenzyme (Babior and Gould, 1969). A mechanism involving transient cleavage of the C-Co bond and formation of a deoxyadenosine intermediate derived from coenzyme would appear to be plausible. If such an intermediate exists, however, it must remain tightly bound to the enzyme since no exchange with free added 5'-deoxyadenosine has been detected under normal catalytic conditions (Stadtman, 1972). Such an intermediate has been detected during the diol dehydrase reaction in the presence of glycoaldehyde (Wagner et al., 1966) and during the ethanolamine deaminase reaction with ethylene glycol as substrate (Babior, 1970). Furthermore, 5'-deoxyinosine is liberated during the

abortive reaction of diol dehydrase in the presence of 1,2-propanediol and deoxyinosyl cobalamin (Jayme and Richards, 1971).

In contrast to the role of B_{12} coenzymes as the intermediate carrier of the migrating hydrogen, the mechanism of migration of the constituent which replaces the abstracted hydrogen in these reactions is open to speculation. The migration of these groups in the various mutase reactions appears to occur intramolecularly since neither free nor enzyme-bound intermediates have been detected. In addition, a variety of proposed alkyl intermediates for the reactions involving carbon-carbon bond cleavage and rearrangement fail to be incorporated into products (Barker, 1972). The β-lysine mutase reaction also occurs without exchange with ammonia nitrogen from the medium (Bray and Stadtman, 1968). Morley and Stadtman (1972) have proposed that pyridoxal phosphate is directly involved in the catalysis of the amino group migration in the D-α-lysine mutase reaction. The cobamide binding protein of the enzyme complex catalyzes a slow cobamide independent but pyridoxal phosphate- and Mg^{+2}-dependent exchange of the hydrogen at position 6 of D-lysine with H_2O. This suggests that pyridoxal phosphate may be the carrier of the migrating amino group through an intermediate involving Schiff-base formation between enzyme-bound pyridoxal phosphate and the ε-amino group of the substrate. Pyruvate, the carbonyl compound which stimulates the β-lysine mutase, may serve a similar function in the analagous amino group migration associated with this reaction (Stadtman and Renz, 1968).

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ARTICLE 1

PREPARATION AND CHARACTERIZATION OF 2,4-DIAMINOVALERIC ACID: AN INTERMEDIATE IN THE ANAEROBIC OXIDATION OF ORNITHINE

By

Ralph L. Somack, David H. Bing, and Ralph N. Costilow

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PREPARATION AND CHARACTERIZATION OF 2,4-DIAMINOVALERIC ACID: AN INTERMEDIATE IN THE ANAEROBIC OXIDATION OF ORNITHINE

RALPH L. SOMACK, DAVID H. BING, AND RALPH N. COSTILOW
Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

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ANALYTICAL BIOCHEMISTRY 41, 132–137 (1971)

Preparation and Characterization of 2,4-Diaminovaleric Acid: An Intermediate in the Anaerobic Oxidation of Ornithine[1]

RALPH L. SOMACK, DAVID H. BING, AND RALPH N. COSTILOW

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

Received September 25, 1970

The first intermediate in the anaerobic oxidation of ornithine (2,5-diaminovaleric acid) by *Clostridium sticklandii* is 2,4-diaminovaleric acid (2,4-DAV), which accumulates in reaction mixtures containing crude or dialyzed extracts from which cofactors are omitted (**1**). This compound is formed from ornithine by a cobamide coenzyme dependent reaction, and it is then oxidatively cleaved to form acetate, alanine, and ammonia. The intermediate was originally identified as a dibasic compound similar to ornithine by the identical electrophoretic mobilities with ornithine at five pH values. The positions of the amino groups were indicated by the results of oxidations with chloramine T, acid dichromate, and periodate.

Since 2,4-DAV is a new amino acid, we decided to undertake a more complete characterization. Methods are outlined for obtaining gram quantities of the compound from reaction mixtures containing extracts of *C. sticklandii*, and for preparing three derivatives. The derivatives are characterized and evidence of structure is presented.

MATERIALS AND METHODS

Media and Growth Techniques

C. sticklandii strain HF was grown in two-liter flasks in a medium consisting of 0.6% trypticase (BBL), 0.6% yeast extract, and 0.6% L-arginine·HCl in 0.04 *M* potassium phosphate buffer (pH 7.5) under natural gas. Cell extracts were prepared as previously described (**2**) and stored at −20°C. They were not dialyzed prior to use. The lysine in-

[1] Journal Article No. 5174, Michigan Agricultural Experiment Station.

corporated in previous media (1,3) was found to contribute little to cell growth, whereas the addition of trypticase significantly increased cell yields. Arginine, however, could not be replaced by trypticase without a great loss in cell and enzyme yield. Both the total and specific activities of extracts converting ornithine to 2,4-DAV paralleled cell yields under these conditions. Activity increased exponentially with cell growth and to a constant value at stationary phase.

Analytical Methods

Melting points were determined with a Hoover capillary melting point apparatus. The elemental analysis was performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan. The infrared spectrum was obtained with a Perkin-Elmer model 700 spectrometer, the nuclear magnetic resonance spectrum with a Varian NA-100 MHz spectrometer, and mass spectrometry with a LKB model 9000 combination gas chromatograph spectrometer. The optical rotation determination was made with a Carl Zeiss spectropolarimeter.

Isolation and Purification of 2,4-DAV

A reaction mixture of 300 ml containing 25 m*M* tris(hydroxymethyl)-aminomethane (Tris)-chloride (pH 7.5), 4 μM dimethylbenzimidazolylcobamide (DBC) coenzyme, 80 m*M* L-ornithine, 2.5 m*M* adenosine diphosphate, and crude cell extract (4 gm protein estimated by the method of Lowry *et al.* (4)) was incubated under argon in the dark on a magnetic stirrer at 37°C for 1.5 hr. The reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid (TCA). The protein was removed by centrifugation at 5° and 20,000*g* for 10 min, and the TCA in the supernatant fluid removed by extracting three times with an equal volume of ether. The supernatant solution was concentrated to a thick syrup under reduced pressure at 40° and taken up in 20 ml of water. Ornithine, 2,4-DAV, and alanine were separated from anions by adsorption to a Dowex 50W-X4 (200-400 mesh) H^+-form column (3 × 21 cm). After washing the column with five bed volumes of water, the amino acids were eluted with 1 *M* NH_4OH. Ninhydrin-positive fractions were pooled, concentrated to dryness, and brought to a 20 ml volume with chloroform/methanol/15% NH_4OH (40:40:10). This solution was placed on a 6 × 35 cm silicic acid column (SilicAR CC-4, 100-200 mesh, Mallinckrodt) which had been previously equilibrated with the above solvent. Fractions were monitored for ninhydrin-positive compounds, and the compounds identified by thin-layer chromatography using the chloroform/methanol/15% NH_4OH (36:46:20) solvent system (Table 1). Alanine appeared after about 600

TABLE 1
Chromatographic and Electrophoretic Properties of 2,4-Diaminovaleric acid-2HCl

Solvent	2,4-DAV	Unknown[a]
Chromatography[b] (R_f)		
Butyl alcohol/acetic acid/water (60:15:25)	0.28	0.18
Propanol/pyridine/water (1:1:1)	0.44	0.42
Methanol/water/pyridine (20:5:1)	0.34	0.46
Chloroform/methanol/15% NH_4OH (40:40:10)	0.13	0.14
Chloroform/methanol/15% NH_4OH (36:46:20)	0.66	—
Electrophoresis (cm from origin[c])		
0.2 *M* formic acid, pH 2.0, 42.5 V/cm, 45 min	25.40	23.40

[a] Values from Dyer and Costilow (1).

[b] All chromatograms were on Whatman 3MM paper except the chloroform/methanol/15% NH_4OH solvents, which were on thin-layer silica gel G (E. Merck AG) plates (40:40:10) and silicic acid TLC (ChromAR Sheets, Mallinckrodt) (36:46:20). All systems were ascending except the butanol/acetate/water, which was descending.

[c] The compounds migrated as cations.

ml of this solvent was added and was completely eluted by 2000 ml. At this point the solvent proportions were changed to 36:46:20 chloroform/methanol/15% NH_4OH and 2,4-DAV was eluted in the following 600 ml, after which ornithine appeared. The fractions containing 2,4-DAV were pooled, concentrated to dryness, and brought to a 15 ml volume with water. The oils accumulating from the solvents employed were removed by ether extraction. The product was adsorbed on a Dowex 50W-X4 (100-200 mesh) H^+-form column (1.3 × 15 cm), washed, eluted with 2 *N* NH_4OH, evaporated to dryness, and stored at −20°.

RESULTS: *Preparation of 2,4-DAV Derivatives and Evidence of Structure*

The purified product was taken up in 10 ml of water, to which was added 15 ml of 0.8% picric acid in methyl alcohol. The picrate crystallized on standing and was recrystallized twice from methanol/water (50:50), yielding 1.9 gm of monopicrate. The monopicrate was identified spectrophotometrically by the absorbance of a solution of known weight concentration employing a molar extinction coefficient (ϵ_{352}) measured for picric acid in water of 1.25×10^4 M^{-1} cm^{-1}. The monopicrate was found to have a melting point range of 124° to 137°C, melting with decomposition.

Crystalline 2,4-DAV dihydrochloride was prepared by adding 5 ml of 6 *N* HCl to 0.5 gm of the monopicrate. The picric acid was removed by extraction with ether, the aqueous solution evaporated to dryness, and the residue dissolved in 1 ml of absolute ethyl alcohol with a mini-

mum amount of water. Ether was added until a permanent turbidity developed and the suspension held at —20° overnight. The resinous deposit resulting was triturated with a small volume of absolute ethanol and ether, dissolved in water, and evaporated to dryness under reduced pressure at 40°. This deposit was then dissolved in absolute ethanol with a minimum amount of water. Crystallization was achieved by the addition of cold ether and the crystals were washed twice with ether. Centrifugation rather than filtration was used to collect the crystals since the ether-insoluble product formed a resinous deposit on paper which was difficult to remove. The yield was 135 mg of crystals which turned brown when stored at room temperature in closed vials. The dihydrochloride melts with decomposition at 172° to 179°.

Elemental analysis: $C_5H_{14}N_2O_2Cl_2$. Indicated: C 29.53, H 6.84, N 14.57, O 16.38, Cl 32.68. Calculated: C 29.28, H 6.88, N 13.66, O 15.60, Cl 34.57.

The reduced ninhydrin analysis (5) of a known weight concentration using ornithine as a standard indicated a molecular weight of 218. The infrared spectrum (Fig. 1) revealed a broad, strong absorption at $\nu =$

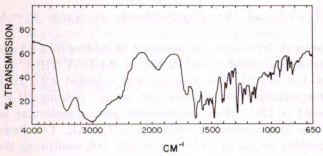

FIG. 1. Infrared absorption spectrum of 2,4-diaminovaleric acid dihydrochloride (KBr pellet).

2340–3300 cm^{-1} (> OH and > NH_3^+), a medium peak at $\nu =$ 1840-2150 cm^{-1} (> C—$NH_3^+Cl^-$), medium peaks at $\nu = 1635$ cm^{-1} and $\nu = 1480$ cm^{-1} (> NH_3^+), and a weak absorption peak at $\nu =$ 1700–1735 cm^{-1} (> C=O).

The nuclear magnetic resonance spectrum of the dihydrochloride (Fig. 2) demonstrated the single C-2 proton by the doublet of doublets at δ4.22 ppm, the C-3 methylene group by the sixteen line multiplet at approximately δ2.33 ppm, the lone C-4 proton by the two overlapping quartets at δ3.8 ppm, and the C-5 methyl by the doublet at δ1.53 ppm. Irradiation of the proton at δ3.8 ppm collapsed the methyl group to a singlet, confirming the position of the amino group at C-4. The optical

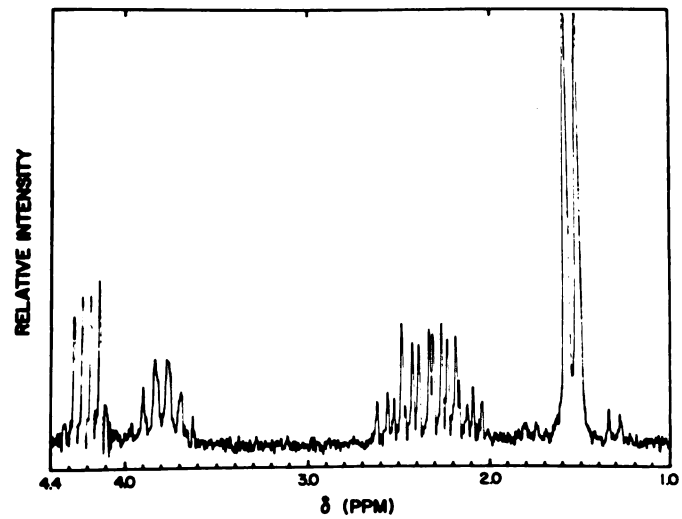

FIG. 2. 100 MHz nuclear magnetic resonance spectrum of 2,4-diaminovaleric acid dihydrochloride (D_2O solution, TMS as external reference).

rotation $[(\alpha)^{23}_{578}]$ of the dihydrochloride in water ($c = 5\%$) was $-17.39°$.

The dibenzoyl derivative was prepared by adding 0.12 ml of dibenzoyl chloride to a cold solution of 32 mg of 2,4-DAV·2HCl in an equal volume of 1 *N* NaOH. The solution was acidified with HCl and the product recrystallized twice from hot water, yielding 21.4 mg. The melting point is 120°. Mass spectrometry gave a molecular ion of 340, equivalent to an empirical formula of $C_{19}H_{20}N_2O_4$. The fragmentation pattern revealed an ion of molecular weight 148, confirming the position of the benzoyl amide on C-4.

Chromatography of a solution of the dihydrochloride supplied the R_f values in five solvent systems and the electrophoretic mobility (Table 1). The values obtained by Dyer and Costilow (1) are included for comparison. For unknown reasons, our results with the butyl alcohol/acetic acid/water and methanol/water/pyridine systems differ considerably. Complete separation was achieved only with the chloroform/methanol/15% NH_4OH (36:46:20) solvent on thin-layer ChromAR sheets.

These results fully confirm that the intermediate described by Dyer and Costilow (1) is 2,4-diaminovaleric acid, and provide the necessary characteristics for simple presumptive detection (TLC), and for identification by derivative formation and analysis by infrared and nuclear magnetic resonance spectroscopy.

SUMMARY

2,4-Diaminovaleric acid was purified in gram quantities, three derivatives were crystallized and characterized, and the structure was verified by infrared and nuclear magnetic resonance spectroscopy.

ACKNOWLEDGMENTS

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The mass spectrum and its interpretation were kindly performed by Professor C. C. Sweeley and his associates at the Department of Biochemistry, Michigan State University. We are indebted to Professor H. Hart, of the Department of Chemistry, Michigan State University, for arranging the use of the Varian HA-100 MHz spectrometer and to G. Love and E. Roach for their expertise in performing and interpreting the nuclear magnetic resonance spectrum. We thank Professor J. C. Speck for performing the optical rotation analysis. The DBC coenzyme was generously supplied by H. A. Barker, Department of Biochemistry, University of California, Berkeley.

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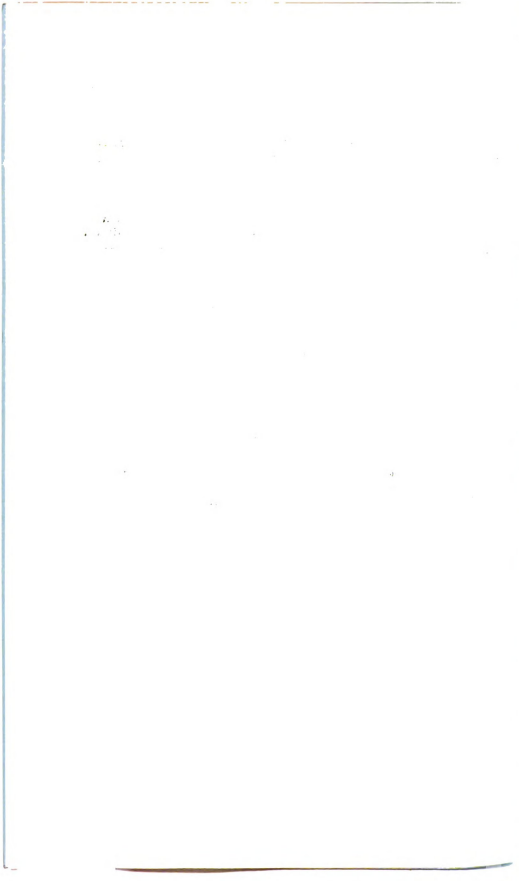

ARTICLE 2

2,4-DIAMINOPENTANOIC ACID C_4 DEHYDROGENASE: PURIFICATION AND PROPERTIES OF THE PROTEIN*

By

Ralph Somack and Ralph N. Costilow

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RALPH SOMACK[†] AND RALPH N. COSTILOW[§]

From the Department of Microbiology and Public Health,

Michigan State University, East Lansing, Michigan 48823

SUMMARY

The NAD^+-$NADP^+$ dependent 2,4-diaminopentanoic acid C_4 dehydrogenase from Clostridium sticklandii has been purified to homogeneity by the criteria of disc gel electrophoresis and ultracentrifugation. The weight average molecular weight of the native enzyme as determined by high speed sedimentation equilibrium is 72,000. Sedimentation velocity indicated an $s_{20,w}$ of 4.47S. Sodium dodecyl sulfate disc gel electrophoresis and high speed sedimentation in 6 M guanidine•HCl established that the enzyme is composed of two subunits of identical size. The enzyme is sensitive to thiol inhibitors and titration with 5,5'-dithiobis-2-nitrobenzoic acid and p-chloromercuribenzoate demonstrated the presence of six sulfhydryl groups per mole. Amino acid analysis indicated that the enzyme contains six half-cystine residues per mole.

The first two transformations in the fermentation of ornithine to acetate, alanine and ammonia in Clostridium sticklandii have previously been identified (1,2). Ornithine is initially converted to 2,4-diaminopentanoic acid in a coenzyme B_{12} and possibly pyridoxal phosphate dependent reaction catalyzed by ornithine mutase (1,2). The 2,4-diaminopentanoate is then oxidatively deaminated forming 2-amino-4-ketopentanoic acid (2). The enzyme catalyzing this latter reaction, a C_4 dehydrogenase, has been partially purified and shown to utilize NAD^+ or $NADP^+$ as cofactors (2). This report describes a procedure for purifying the C_4 dehydrogenase to homogeneity. In addition, determinations were made of the molecular weight of the native enzyme and subunits, the amino acid composition, and the sulfhydryl group content.

METHODS

General--All reagents were obtained from commercial sources except for coenzyme B_{12}[1] which was provided by Dr. H. A. Barker, University of California, Berkeley, and 2,4-diaminopentanoate which was generated as described previously (3). The procedures for growing, harvesting, storing and extracting cells of Clostridium sticklandii were as reported elsewhere (1). Protein concentrations were measured by the method of Lowry et al. (4).

Enzyme Assays--The activity of the C_4 dehydrogenase was measured by the rate of reduction of $NADP^+$ using 2,4-diaminopentanoate as substrate or by a coupled reaction using D-ornithine as substrate in the presence of an excess of ornithine mutase. The former procedure was as described by Tsuda and Friedmann (2) except that (a) $NADP^+$ was used instead of NAD^+ to eliminate problems with NADH oxidase in extracts prior to hydroxylapatite chromatography, and (b) no dithiothreitol was added since it was found not to stimulate the reaction. The reaction mixtures for the coupled assay contained: 10 mM Tris buffer, pH 8.5; 5 μM B_{12} coenzyme; 20.2 μM pyridoxal phosphate; 3.0 mM $NADP^+$; 5 mM D-ornithine; 1 mM dithiothreitol; excess levels of C_4 dehydrogenase-free mutase obtained from the hydroxylapatide purification step; and the C_4 dehydrogenase in a total volume

of 0.5 ml. Enzyme was added after gassing the cuvettes with argon, the mixture equilibrated to 25° and the reaction initiated by addition of D-ornithine. The absorbance change at 340 nm in all reactions was monitored using a Gilford model 2000 spectrophotometer. One unit of enzyme is defined as that amount which catalyzes the formation of 1 μmole of NADPH per minute under assay conditions.

Disc Gel Electrophoresis--Preparative disc gel electrophoresis utilized a Canalco "Prep-Disc" apparatus with the PD-2/150 lower column insert. The Ornstein and Davis (5) gel system was used with the gel modifications employed by Gilpin and Sadoff (6). The resolve gel was 25 mm long and the stacking gel was 10 mm long. Both gels contained 10% glycerol (v/v). The cathode and anode reservoir buffers consisted of 0.3 g Tris, 1.44 g glycine, 100 ml glycerol and 0.154 g dithiothreitol per liter, pH 8.2-8.4. The elution buffer contained 3.12 ml concentrated HCl. 28.4 g Tris, 100 ml glycerol and 0.154 g dithiothreitol per liter, pH 8.8-8.9. Gels were polymerized at 5° and the electrophoresis unit cooled during operation by circulating ice water.

Analytical disc gel electrophoresis in anionic (pH 7.3) and cationic (pH 4.3) buffer systems were performed at room temperature with a Buchler apparatus. The gels were stained with amido Schwartz and destained in 7% acetic acid. A modification of the sodium dodecyl sulphate method of Weber and Osborn (7) was used for subunit analysis. The markers used and assigned molecular weights were: glutamate dehydrogenase, 53,000 (8); yeast alcohol dehydrogenase,

37,000 (9); chymotrypsinogen A, 25,700 (10); ovalbumin, 43,000 (11); and D-amino acid oxidase, 37,000 (12).

Ultracentrifugation Studies--Dialyzed enzyme was used for studies of sedimentation velocity at 59,896 rpm at 7.3° and for high speed equilibrium experiments. A Spinco model E ultracentrifuge with appropriate equipment was used. The equilibrium experiments were conducted as outlined by Yphantis (13).

Amino Acid and Sulfhydryl Group Analysis--After dialysis against distilled water, samples containing 475 μg of protein were hydrolyzed in 6 N HCl under vacuum at 110° for 24 and 72 hours. The hydrolysates were analyzed in an ultrasensitive amino acid analyzer by the procedure of Robertson et al. (14). Tryptophan was determined by the method of Beaven and Holiday (15) and threonine and serine estimated by extrapolation to zero time hydrolysis. Sulfhydryl group content was measured by titration with DTNB and p-chloromercuribenzoate as outlined by Finlay and Adams (16).

RESULTS

Purification--A summary of the entire purification is presented in Table 1. The procedures used through the hydroxylapatite column (Step IV) were similar to those used by Tsuda and Friedmann (2) for partial purification of this enzyme.[2] However, there were some differences in elution patterns from DEAE cellulose and hydroxylapatite since we routinely used buffers at pH 7.5 while Tsuda and Friedmann used a pH of 7.0. At the higher pH, the C_4 dehydrogenase was eluted from DEAE cellulose with 0.175 M potassium phosphate buffer, and from hydroxylapatite with 0.025 M phosphate buffer. The dehydrogenase was free of ornithine mutase at this point, and the mutase could be eluted from this column by increasing the buffer strength to 0.05 M.

The C_4 dehydrogenase was far from pure at this point (see inset in Fig. 1). Final purification was achieved by using preparative polyacrylamide gel electrophoresis. A 2-ml volume of the concentrated eluate from the hydroxylapatite step was made 8% (w/v) with respect to sucrose and 40 µl of a 0.01% solution of bromphenol blue was added as an anionic marker. The sample was applied to the preparative column which was run at a constant current of 10 milliamps. Elution was begun immediately at the rate of 1 ml per min to insure the removal of possible electrophoretically generated contaminants. Fig. 1 shows the electrophoretic elution profile. A brown band was

eluted soon after the dye which was collected in 17 2-ml fractions. The 280 nm absorption profile of these fractions traced the C_4 dehydrogenase activity. The most active fractions (No. 26-34) were concentrated and then dialyzed for 12 hours against standard 0.1 M potassium phosphate buffer, pH 8.5. The concentrate was stored under liquid N_2.

The specific activity of the enzyme was essentially doubled, and only a small amount of enzyme was lost during this step. Not only did the enzyme appear homogeneous after electrophoresis in both anionic (pH 9.3) and cationic (pH 4.5) systems (see inset in Fig. 1), but a single peak with no sign of heterogeneity was observed in a high speed sedimentation velocity experiment. This was conducted using enzyme at a concentration of 3.9 mg per ml in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 N KCl. The $S_{20,w}$ value calculated from this experiment was 4.47S.

Molecular Weight of Enzyme and Subunits--Three high speed sedimentation equilibrium experiments were conducted with the purified enzyme in 0.05 M potassium phosphate buffer containing 0.1 M KCl. One run was at pH 7.0 and sedimented for 24 hours at 25,910 rpm and 10.4^{o} and the other two at pH 7.9 for 24 hours at 25,965 rpm and 13.9^{o}. The plots of the ln fringe displacement against the radius squared were linear in all cases for fringe displacements over 100 $\mu m.^2$ The weight average molecular weights calculated from these three experiments were 68,260 (pH 7.0 run), 73,450 and 74,340. The average value was 72,070.

Duplicate determinations of subunit structure and molecular weight by sodium dodecyl sulphate gel electrophoresis were run on different occasions. A single protein band was observed in all gels containing the C_4 dehydrogenase and the molecular weight estimated from the standards employed was 40,000 ± 10%. This suggests that the enzyme has a native molecular weight of 80,000 ± 10% and consists of two polypeptide chains of equal size.

The molecular weight of the subunit was also estimated by a high speed sedimentation equilibrium experiment with 0.6 mg/ml of enzyme in 6 M guanidine hydrochloride containing 0.12 M mercaptoethanol and 0.05 M potassium phosphate buffer, pH 7.7. The plot of the ln fringe displacement versus the radius squared was linear and indicated a subunit molecular weight of 35,380.

Amino Acid Analysis--The amino acid analysis of the C_4 dehydrogenase failed to indicate anything particularly unique about the enzyme. The averages of four analyses of 72-hour and two analyses of 24-hour hydrolyzed samples indicated the following amino acid residues per subunit of enzyme: 28 Asp, 12 Thr, 13 Ser, 32 Glu, 19 Pro, 32 Gly, 21 Ala, 27 Val, 3 1/2 Cys, 16 Met, 26 Ile, 17 Leu, 5 Tyr, 8 Phe, 17 Lys, 5 His, 9 Arg, and 22 Try.[2] Calculations based on these data indicated a molecular weight of 35,000 per subunit and a partial specific volume of 0.73 cc g^{-1}.

Sulfhydryl Group Content--The C_4 dehydrogenase was strongly inhibited by p-chloromercuribenzoate (100% by 0.2 mM), iodoacetate

(98% by 2 mM) and N-ethylmaleimide (98% by 2 mN).[2] Titration of 0.294 mg (4.12 mμmoles) of pure enzyme with DTNB yielded a final absorbance change of 0.309 O.D. unit after 4.5 hours indicating 5.6 sulfhydryl groups per mole of enzyme (72,000 g). Using p-chloromercuribenzoate for titration of 2.06 mμmoles of enzyme, the final corrected absorbance change was 0.109 O.D. unit which is equivalent to 7.0 sulfhydryl groups per mole. These results and the amino acid analysis indicate that the C_4 dehydrogenase contains 6 sulfhydryl groups (3 per subunit) and no disulfide bonds.

Absorption Spectrum--The absorption spectra of the enzyme in Tris buffer at pH 8.8 and in phosphate buffer at pH 8.0 (both buffers with 10% glycerol and 0.5 mM dithiothreitol) were identical and typical of many proteins. There was a single absorption peak in the ultraviolet range at 276.5 nm and none in the visible range. The 280/260 absorbance ratio was 1.33.

DISCUSSION

For the first time, a dehydrogenase catalyzing a NAD^+-linked oxidative deamination of a dibasic α-amino acid other than the α-position has been purified to homogeneity. The existence of such an enzyme in oxidative degradation of ornithine was first suggested by Dyer and Costilow (18) who demonstrated that ornithine was cleaved to form alanine from carbons 1-3 and acetate from 4 and 5. Later they showed that 2,4-diaminopentanoate was an intermediate in this degradation (1). Tsuda and Friedmann (2) subsequently confirmed the formation of this intermediate and demonstrated the activity of a C_4 dehydrogenase in partially purified extracts. The specific activity of the dehydrogenase preparation studied by Tsuda and Friedmann was only 1/4 of that of our final preparation. Our data indicate that the partially purified preparation used by Tsuda and Friedmann probably contained many contaminating proteins. The results of the present study provide unequivocal proof for the existence of a single protein which catalyzes the oxidation of 2,4-diaminopentanoic acid at the 4-position with the reduction of either NAD^+ or $NADP^+$.

Similar dehydrogenases are probably involved in the lysine fermentation. Rimerman and Barker (19) demonstrated the activity of 3,5-diaminohexanoate C_3 dehydrogenase. The partially purified C_4 dehydrogenase preparation of Tsuda and Friedmann (2) was not active on

this substrate. It appears likely that there is also a C_5 dehydrogenase for 2,5-diaminohexanoate, a product of D-α-lysine mutase (20). Intact cells of C. sticklandii have been shown to cleave lysine at both the C_3 and C_5 (21) positions but the C_5 cleavage has not been observed in cell extracts and the activity of such a dehydrogenase has not been reported. Therefore, it appears unlikely that the dehydrogenase described herein would be active on 2,5-diaminohexanoate.

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FOOTNOTES

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† U.S. Public Health Predoctoral Trainee, Public Health Training Grant GM-01911 of the National Institute of General Medical Sciences. This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Biochemistry, University of California, Berkeley, California 94720.

§ To whom correspondence should be addressed.

[1] The appreviations used are: coenzyme B_{12}, dimethylbenzimidazolyl cobamide coenzyme; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid.

[2] Detailed purification procedures and results of equilibrium sedimentation experiments, amino acid analysis and inhibition studies are available as JBC Document Number , in the form of one microfiche or 5 pages. Orders for supplementary material should specify the title, author(s) and reference to this paper and the JBC Document number, the form desired (microfiche or photocopy) and the number of copies desired. Orders should be addressed to The

Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland, 20014, and must be accompanied by remittance to the order of the Journal in the amount of $2.50 for microfiche or for photocopy, with a minimum charge of $2.50.

TABLE 1: Purification of C_4 Dehydrogenase. Conditions of purification and definition of units are described in the text.

Step	Total Enzyme Activity (units)	Specific Activity (units/mg)	Fold Purification (-fold)	Recovery (%)
I. Crude extract	58,000	5.2	1	100
II. Streptomycin sulfate + ammonium sulfate (40-70%), dialyzed	39,750	8.05	1.5	68.5
III. DEAE-cellulose column	19,075	40.15	7.6	32.9
IV. Hydroxylapatite column	8,075	152.0	28.8	13.9
V. Preparative disc gel electrophoresis	7,125	272.5	51.5	12.3

FIGURE 1: Preparative disc gel electrophoretic elution profile of C_4 dehydrogenase. Details of the purification step appear in the text. Enzyme was assayed using D-ornithine as substrate (see "Methods") with excess mutase from step IV of the purification procedure and 50 µl of each fraction. o, absorbance at 280 nm; o, C_4 dehydrogenase (ΔA_{340} per min). Inset: Analytical disc gel electrophoresis of the input extract from the hydroxylapatite column (left) and of the concentrate from the preparative column illustrated in this figure (center and right). Electrophoresis was performed as described under "Methods" using 50 µg protein of the input extract and 20 µg and 50 µg of the purified enzyme in the anionic and cationic systems, respectively. The direction of migration was from top to bottom in each case. The wires at the bottom indicate the positions of the dye markers.

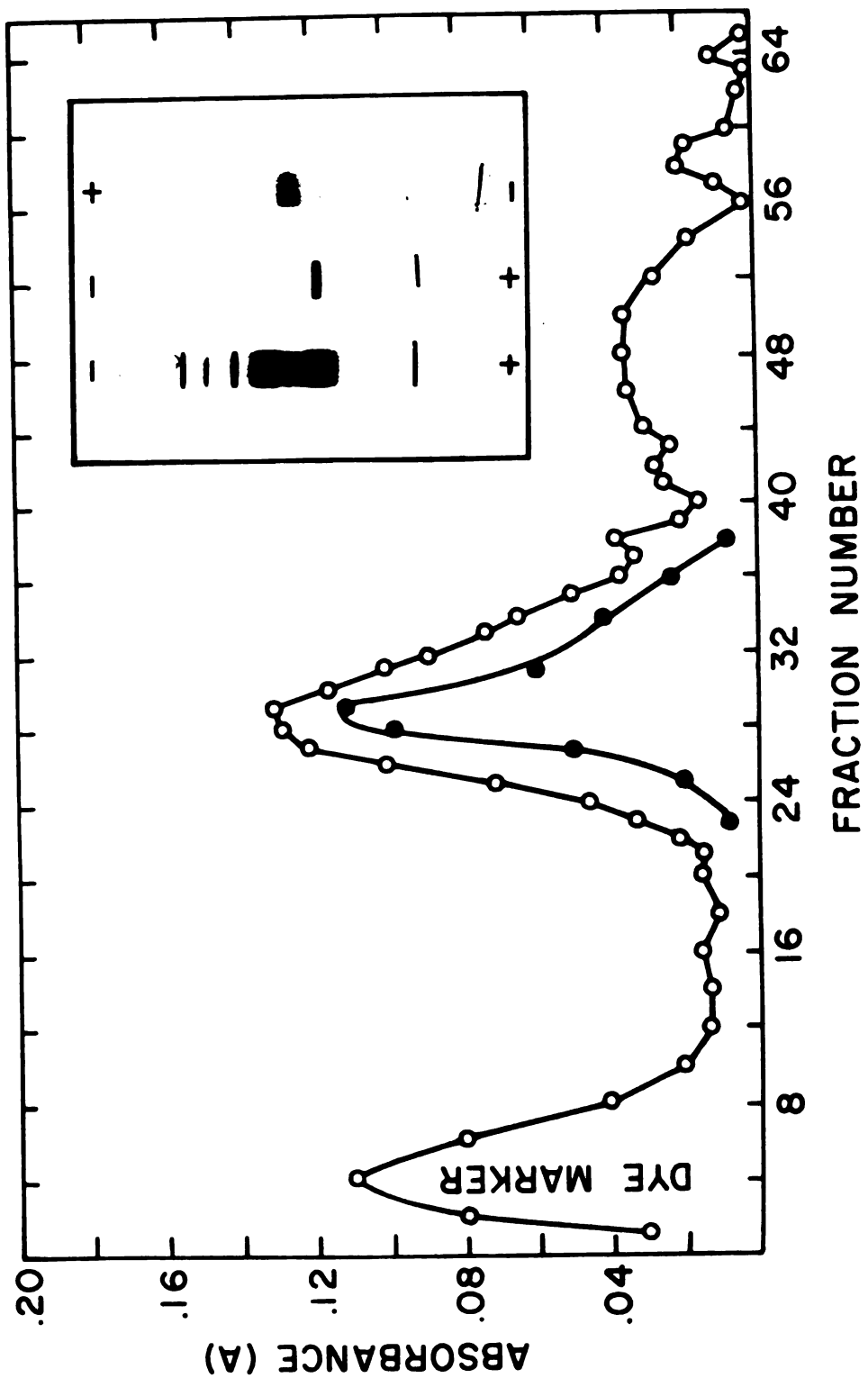
ABSORBANCE (A)
.20
.16
.12
.08
.04
DYE MARKER
8
16
24
32
40
48
56
64
FRACTION NUMBER
+
-
-
+
-
+

FIGURE 1.

Supplementary data:

a. Details of partial purification procedure.

b. High speed equilibrium centrifugation of the C_4 dehydrogenase.

c. Amino acid analysis.

d. Inhibition by sulfhydryl inhibitors.

Authors:

Ralph Somack and Ralph N. Costilow

Title:

2,4-Diaminopentanoic Acid C_4 Dehydrogenase

PURIFICATION AND PROPERTIES OF THE PROTEIN

SUPPLEMENTARY RESULTS

All purification steps were conducted at 0-4°. Where standard buffer is indicated, the buffer also contained 10% glycerol (v/v) and 0.5 mM dithiothreitol. A summary of the entire purification is presented in Table 1.

Streptomycin Sulfate Treatment--To the crude extract, obtained as described under "Methods," an equal volume of 5% streptomycin sulfate in 0.1 M potassium phosphate buffer, pH 7.5, was added slowly with stirring. The mixture was stirred overnight and the precipitate removed by centrifugation.

Ammonium Sulfate Fractionation--The streptomycin sulfate treated extract was diluted with buffer to approximately 10 mg protein per ml and solid ammonium sulfate added to 40% saturation. After stirring for 30 min, the precipitate was removed by centrifugation. The supernatant solution was brought to 70% saturation with ammonium sulfate, stirred for 30 min and then centrifuged. The pellet was resuspended and dialyzed overnight against standard 0.15 M Tris buffer, pH 7.5.

DEAE-cellulose Column Chromatography--A volume of dialyzed extract containing 1000 mg of protein was placed on a DEAE-cellulose column (1.7 x 25 cm) which had been equilibrated with standard 0.15 M Tris buffer. The column was washed with the same buffer, followed by 0.175 M standard Tris buffer, which removed most of the protein. The C_4 dehydrogenase was then eluted by increasing the buffer strength to 0.2 M. Fractions containing activity were concentrated by ultrafiltration and dialyzed overnight against standard 5 mM potassium phosphate buffer, pH 7.5.

Hydroxylapatite Column Chromatography--An aliquot of dialyzed extract from the previous step containing 200 mg of protein was applied to a hydroxylapatite column (4 x 12 cm) which had been equilibrated with the buffer used for dialysis in the previous step. After thorough washing, the C_4 dehydrogenase was eluted with standard 0.025 M potassium phosphate buffer. Ornithine mutase may be eluted by increasing the buffer strength to 0.05 M. The fractions containing activity were concentrated by ultrafiltration to a protein concentration of approximately 10 mg per ml.

TABLE 1: Amino Acid Analysis of C_4 Dehydrogenase. The data were obtained from the average of four analyses from the 24-hour and two analyses from the 72-hour hydrolyzed samples, as described under "Methods."

Amino Acid	Residues per 17 Leucine Residues		Residues per Mole/2
	24 Hours	72 Hours	
Asp	28.70	26.85	28
Thr[a]	11.60	10.50	12
Ser[a]	11.93	9.37	13
Glu	33.25	30.55	32
Pro	18.90	-	19
Gly	32.45	31.10	32
Ala	21.10	21.50	21
Val	26.40	27.10	27
1/2 Cys	3.08	2.61	3
Met	17.25	15.93	16
Ile	25.70	26.10	26
Leu	17.00	17.00	17
Tyr	4.72	4.88	5
Phe	7.50	8.13	8
Lys	17.35	16.70	17
His	4.46	4.84	5
Arg	8.95	8.13	9
Try[b]	-	-	22

[a]Extrapolated to zero hour hydrolysis.

[b]Estimated from ultraviolet absorption in 0.1 N NaOH, assuming five tyrosine residues per mole/2 (16).

TABLE 2: Effect of Sulfhydryl Inhibitors. Assays were performed with 2,4-diaminopentanoate as substrate as described under "Methods" and enzyme with a specific activity of 43.7 units/mg. The inhibitors were added at the indicated concentrations before addition of enzymes.

Additions	Concentration (mM)	Activity (μmoles NADPH/ min x 10^3)	Inhibition
None	-	25.70	-
p-CMB	2	0	100
	0.2	0	100
	0.02	3.05	88.1
Iodoacetate	2	0.48	98.1
	0.2	20.6	20.0
Ethylmaleimide	2	0.48	98.1
	0.2	22.7	11.6

FIGURE 1: High speed equilibrium centrifugation of C_4 dehydrogenase. The initial protein concentration was 0.4 mg per ml and was centrifuged at 25,965 rpm and 13.9^{o} for 24 hours. Details of the experiment are presented in the text. Δy, fringe displacement.

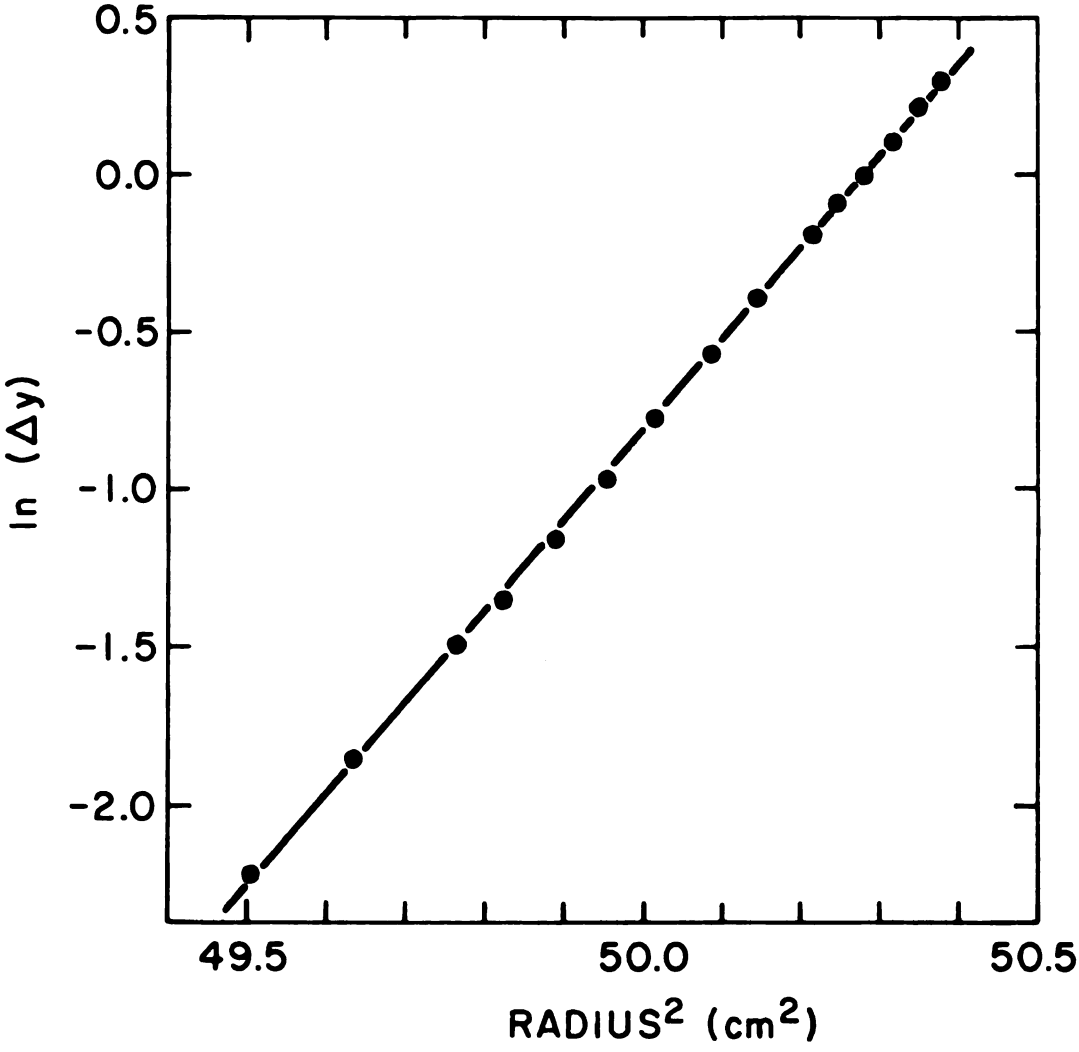

FIGURE 1.

ARTICLE 3

PURIFICATION AND PROPERTIES OF A PYRIDOXAL PHOSPHATE AND B_{12}-COENZYME-DEPENDENT D-α-ORNITHINE-4,5-AMINOMUTASE

By

Ralph Somack and Ralph N. Costilow

Submitted To:

Biochemistry

PURIFICATION AND PROPERTIES OF A PYRIDOXAL PHOSPHATE AND B_{12}-COENZYME-DEPENDENT D-α-ORNITHINE-4,5-AMINOMUTASE+

Ralph Somack† and Ralph N. Costilow*

Running Title: D-α-ORNITHINE-4,5-AMINOMUTASE

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FOOTNOTES

† Present address: Department of Biochemistry, University of California, Berkeley, California 94720.

[1] Abbreviations used are: 2,4-DAP, 2,4-diaminopentanoic acid; coenzyme B_{12} (DBC coenzyme), dimethylbenzimidazolyl cobamide coenzyme; PLP, pyridoxal phosphate; DTT, dithiothreitol.

ABSTRACT

The coenzyme B_{12}-dependent ornithine mutase from Clostridium sticklandii catalyzing the conversion of ornithine to 2,4-diaminopentanoic acid (2,4-DAP) has been purified to homogeneity. A radiochemical assay employing ^{14}C-labeled ornithine and a rapid, coupled spectrophotometric assay employing 2,4-diaminopentanoic acid C_4 dehydrogenase are described. Analysis by gel electrophoresis, sucrose gradient centrifugation and SDS gel electrophoresis indicated that the mutase has a molecular weight of about 180,000 and consists of 2 subunits of identical size. The enzyme is specific for D-α-ornithine and is inhibited by L-α-ornithine, DL-α-lysine, and β-lysine. Kinetic and inhibitor studies showed that ornithine mutase and C_4 dehydrogenase are directly linked and that pyridoxal phosphate is a cofactor for ornithine mutase. The absorption spectrum of the mutase measured directly in analytical gels indicated that a substantial amount of native bound cobamide had been converted to inactive hydroxocobalamins. After incubation with DBC coenzyme and subsequent dialysis, the spectrum was more typical of bound DBC coenzyme. The ornithine mutase reaction is reversible and proceeds to approximately an equal extent in both directions. However, the product, 2,4-DAP, appeared to inhibit the reverse reaction at concentrations greater than 0.7 mM when present alone. The enzyme contains labile sulfhydryl groups and is inhibited

by oxygen. Experiments with H_2O-t indicated that the reaction proceeds by a mechanism which excludes exchange of hydrogen with the solvent.

The initial steps in the fermentation of ornithine and lysine in Clostridium sticklandii involve a series of amino group migrations followed by oxidative deaminations which prepare the amino acids for subsequent thiolytic cleavages and conversions to the fatty acid products. The oxidation of ornithine to acetate, carbon dioxide and ammonia proceeds by an initial migration of the δ-amino group to carbon atom 4 forming 2,4-DAP[1] (Dyer and Costilow, 1968, 1970; Tsuda and Friedmann, 1970; Somack et al., 1971). The accumulation of this compound is stimulated by DBC coenzyme. A subsequent PLP-dependent reaction involving a C_4 amino group inversion was next proposed to precede a TPN^+ or DPN^+ linked oxidative deamination forming 2-amino-4-ketopentanoic acid (Tsuda and Friedmann, 1970). The enzyme catalyzing this last step (2,4-diaminopentanoic acid C_4 dehydrogenase) appears to have no further cofactor requirements and has been purified to homogeneity (Somack and Costilow, 1972).

DL-Lysine is fermented by intact cells of C. sticklandii and related organisms through a cleavage between carbon atoms 2 and 3 (Type A cleavage) or carbon atoms 4 and 5 (Type B cleavage) yielding acetate, butyrate and 2 equivalents of ammonia (Stadtman, 1954, 1955). However, the formation of acetate in cell extracts has only been demonstrated by the former pathway (Stadtman, 1962). The first reaction in the type A cleavage is catalyzed by a PLP- and S-adenosylmethionine-dependent mutase, forming L-β-lysine from L-α-lysine (Costilow et al.,

1966; Chirpich et al., 1970; Zappia and Barker, 1970). L-β-Lysine is next converted to 3,5-diaminohexanoate by an aminomutase consisting of an acidic cobamide protein and a smaller sulfhydryl protein (Dekker and Barker, 1968; Stadtman and Renz, 1968). In addition to a B_{12} coenzyme, the enzyme requires ATP, a mercapten, FAD, pyruvate, and a monovalent and divalent cation for full activity. A DPN^+-dependent dehydrogenase next catalyzes an oxidative deamination forming 3-keto-5-aminohexanoate (Rimerman and Barker, 1968), which presumably undergoes thiolytic cleavage yielding the fatty acids and ammonia.

The first reaction leading to the type B cleavage is believed to be an initial migration of the ε-amino group of D-α-lysine forming 2,5-diaminohexanoate (Stadtman and Tsai, 1967). The aminomutase catalyzing this reaction is structurally identical to the β-lysine mutase with similar cofactor requirements except that PLP replaces pyruvate (Morley and Stadtman, 1970).

Since the initial demonstration of ornithine mutase activity in crude extracts (Dyer and Costilow, 1970), the enzyme has only been cursorily examined with respect to cofactor requirements using partially purified extracts (Tsuda and Friedmann, 1970). The present paper describes a method for separating ornithine mutase from the 2,4-diaminopentanoic acid C_4 dehydrogenase and purifying the mutase to homogeneity. The molecular weight and subunit composition suggests a physical structure quite different from the cobamide-dependent aminomutases in the lysine pathway. In addition, evidence is presented

which indicates that PLP is required in the mutase reaction per se and not in a subsequent amino group inversion as previously suggested (Tsuda and Friedmann, 1970).

MATERIALS AND METHODS

Materials. Acrylamide and N,N'-methylbisacrylamide were purchased from Canal Industrial Corporation. Coenzyme B_{12} and [^{14}C]β-L-lysine were kindly provided by Dr. H. A. Barker, Department of Biochemistry, University of California, Berkeley. Non-radioactive β-L-lysine and 2,4-DAP were generated with C. sticklandii crude extracts and purified by the methods of Costolow et al. (1966) and Somack et al. (1971) respectively. The concentrations of these diaminoacids were estimated by the reduced ninhydrin assay of Moore and Stein (1954). L-Lysine-6-^{14}C and DL-lysine-1-^{14}C were obtained from Calbiochem. DL-Ornithine-5-^{14}C and H_2O-t were products of New England Nuclear Corporation. All other reagents were obtained from commercial sources.

Methods. Culture and cultural methods for growing, harvesting, storing and extracting cells of C. sticklandii (ATCC 12662) have been reported previously (Somack et al., 1971).

Preparative and Analytical Disc Gel Electrophoresis were performed with a Buchler apparatus employing 6.0 mm (I.D.) diameter glass tubing. The method of Ornstein and Davis (1964) was followed with the gel modifications described by Gilpin and Sadoff (1971). Both stacking gels (5 cm) and resolve gels (0.5 cm) contained 10% glycerol

(v/v). The buffer used in both the cathode and anode reservoirs contained 0.3 g Tris, 1.44 g glycine and 0.154 g DTT per liter, pH 8.2-8.4. Analytical gels were stained with amido Schwartz, destained by diffusion and stored in 7% acetic acid.

Subunit analyses were performed by a modification of the SDS electrophoresis technique of Weber and Osborn (1969) using 5% polyacrylamide gels. The following proteins of known molecular weight were employed as markers: glutamate dehydrogenase (53,000), bovine serum albumin (68,000), chymotrypsinogen A (25,700), ovalbumin (45,000), and D-amino acid oxidate (37,000). The markers were added at a level of 5 µg/gel.

Molecular Weight Determinations. The molecular weight was estimated by the electrophoretic method of Hedrick and Smith (1968) and by sucrose density gradient centrifugation (Martin and Ames, 1961). The following standards were used in the electrophoretic determination: human transferrin (74,000), beef liver catalase (24,000), alcohol dehydrogenase (314,000), and horse ferritin (monomer) (450,000). The standards were added to gels at a level of 50 µg/gel and the mutase at 150 µg/gel. The relative migration of the mutase was established from the position of the pink band in unstained gels. For the sucrose gradient determination, 150 µg of mutase and 6 µg of lactic dehydrogenase were layered on a 5-20% linear gradient (4.55 ml) in cellulose nitrate tubes. The gradients were centrifuged in a SW39L rotor in a Spinco model L preparative centrifuge at 48,000 rpm for 6.5 hr at 5^{o}.

Six-drop fractions were collected and mutase activity measured by the spectrophotometric coupled assay (below).

Enzyme Assays. Two procedures were used to assay ornithine mutase: a radiochemical assay employing D-ornithine-1-^{14}C as substrate and a rapid and sensitive coupled spectrophotometric assay using 2,4-diaminopentanoic acid and C_4 dehydrogenase. The C_4 dehydrogenase was separated from the mutase during the hydroxylapatite chromatography step described later in this paper, and was further purified to homogeneity by preparative disc gel electrophoresis (Somack and Costilow, 1972). The radiochemical assay was carried out anaerobically and protected from direct light in culture tubes as previously described (Costilow and Laycock, 1968). The reaction mixture contained: 20 mM D-ornithine containing 25-50 mCi DL-ornithine-5-^{14}C; 0.1 M Tris-Cl, pH 9.0; 10 mM DTT; 20 µM DBC coenzyme; 81 µM PLP; and from 0.015 to 0.15 units of enzyme in a total volume of 50 µl. After adding enzyme, the tubes were gassed with argon for one min, stoppered, and incubated for 5 min at 37°. The reaction was initiated by the addition of substrate and terminated after 5 min with 25 µl of 0.5 M formic acid. Precipitated protein was centrifuged out, 20 µl spotted on Chromar 500 thin layer silicic acid sheets (Mallinkrodt), and the chromatograms developed by ascending chromatography with choloroform:methanol:15% NH_4OH (36:46:20). Ornithine and 2,4-DAP were located by spraying with 0.01% ninhydrin in acetone. The spots were excised and the radioactivity measured using a toluene-based scintillation fluid (Costilow

and Laycock, 1968). There is considerable variation between batches of the thin layer silicic acid sheets which has resulted in deviations reported by several investigators (personal communication from manufacturer). We have noticed with some batches appreciable spreading of radioactivity not restricted to the usual spots comprising the amino acids. This effect is pronounced by increasing the specific activity of the ornithine used. Therefore, it is necessary to chromatograph a sample of unreacted ornithine-^{14}C to make appropriate corrections. The spectrophotometric assay was performed in the manner described previously (Somack and Costilow, 1972) by measuring the rate of TPN^+ or DPN^+ reduction at 27^o using D-ornithine as substrate for the mutase and coupling the reaction with the C_4 dehydrogenase (Tsuda and Friedmann, 1970; Somack and Costilow, 1972). One-cm light path cuvettes contained: 50 mM sodium pyrophosphate buffer, pH 8.6; 4 mM TPN^+; 1 μM DBC coenzyme; 40.4 μM PLP; 20 mM D-ornithine; 0.001-0.002 units of ornithine mutase; and approximately 1 unit of C_4 dehydrogenase in a volume of 0.25 ml. One unit of enzyme is that amount which catalyzes the formation of 1 μmole of 2,4-DAP per min in the radiochemical assay or 1 μmole of reduced pyridine nucleotide per min in the coupled assay under standard assay conditions. Specific activity is the number of units per mg of protein. When both assays were performed simultaneously at 30^o with 10X the level of mutase in the radiochemical assay than was present in the coupled assay, the specific activity calculated from the coupled system was 60% of that from the radiochemical system.

β-lysine mutase, D-α-lysine mutase and L-lysine mutase were assayed radiochemically by the methods described by Chirpich et al. (1970), Morley and Stadtman (1970), and Stadtman and Renz (1968), respectively. Lactic acid dehydrogenase was assayed spectrophotometrically with pyruvate and DPNH as substrates.

Analytical Methods. Ornithine and 2,4-DAP in reactions containing H_2O-t were adsorbed on small Dowex 50W-X8, H^+ form columns and washed with copious amounts of H_2O. The amino acids were eluted with 3 N NH_4OH and dried under vacuum. They were taken up in small volumes of H_2O and separated on thin layer silicic acid sheets. The amino acid spots were excised, solubilized in 10X hydroxide of hyamine, and assayed for tritium by scintillation spectrometry after adding a toluene-based fluid (Costilow and Laycock, 1968).

Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. All optical absorbance measurements were performed with a Gilford model 2000 spectrophotometer. Polyacrylamide gel scans were conducted with the same instrument employing a Gilford linear transport attachment. Spectra of solutions were determined with a Coleman, model 124, double beam spectrophotometer. All radioactive measurements employed a Packard Tri-Carb, model 3320, scintillation spectrometer.

RESULTS

Purification of Ornithine Mutase. A summary of the purification is presented in Table 1. All operations were carried out at 4^{o} in dim light. In all chromatography steps, buffers contained 10% glycerol (v/v) and 5×10^{-4} M DTT unless otherwise stated.

INITIAL STEPS. Ornithine mutase was partially purified by the identical procedure developed for the purification of the next enzyme in the pathway, the 2,4-diaminopentanoic acid C_4 dehydrogenase (Somack and Costilow, 1972). The method involves an initial streptomycin sulphate treatment of crude extract, followed by ammonium sulphate fractionation and then by DEAE-cellulose and hydroxylapatite chromatography. The C_4 dehydrogenase is eluted from the hydroxylapatite column with 0.025 M potassium phosphate buffer, pH 7.5. The column is next washed with 0.035 M buffer and ornithine mutase is eluted by increasing the buffer strength to 0.05 M. The C_4 dehydrogenase may be further purified to homogeneity by preparative disc gel electrophoresis (Somack and Costilow, 1972).

DISC GEL ELECTROPHORESIS. The 0.05 M potassium phosphate buffer eluate from hydroxylapatite containing ornithine mutase activity was concentrated by ultrafiltration to 4-5 mg of protein per ml. From 50 to 75 µl containing 250 to 500 µg of protein was then layered

on individual analytical gels which were prepared and electrophoresed in dim light as described in the "Methods" section. The electrophoresis unit was maintained as close to 0^{o} with a circulating ice water bath. With the Buchler apparatus, 12 gels containing as much as 6 mg of total protein could be electrophoresed in a single run. During the run, two protein bands were clearly visible, a pink band containing mutase activity and a faster migrating yellow band. Electrophoresis was terminated when the yellow bands reached the ends of the gels. The pink bands were carefully excised and macerated with a glass Dounce homogenizer in 3.2 ml of a mixture containing: 0.1 M Tris buffer, pH 8.5; 13 μM DBC coenzyme; 2×10^{-3} M DTT and 10% glycerol (v/v). The addition of DBC coenzyme to the elution buffer significantly increased recovery. The macerate was gently stirred at 0^{o} under argon for 6 hr, the gel pieces removed by centrifugation, and the supernatant solution concentrated to 1 ml by ultrafiltration. Routinely, we recovered about 2/3 of the total mutase activity that was placed on the gels, and observed about a 3X increase in the specific activity after this step. Typically, about 13% of the mutase activity observed in the crude extracts was recovered, and the specific activity of the purified enzyme was about 70X that of the crude preparations (Table 1).

The mutase appeared homogeneous by the criterion of disc gel electrophoresis. Single discrete bands developed on both 7% and 5% gels electrophoresed at pH 9.3. Figure 1 shows a scan of a 7% gel containing the pure mutase superimposed on a scan of a 7% gel

electrophoresed with enzyme from step 4 (hydroxylapatite) of purification. The faster migrating large peak in the latter gel is the yellow band mentioned above. The pure mutase was found to be free of D-α-lysine mutase, L-α-ornithine mutase and β-lysine mutase activity. (Enzyme, 8.5 μg per reaction, with a specific activity of 1.8 units per mg, was used in these assays.)

Stability. The highly purified mutase lost 35% of its activity in one month when stored in 50 μl aliquots at -20°. Enzyme from step 4 of purification lost 30% of its activity after two days at 4°. By including 10% and 20% glycerol (v/v), the loss at 4° was reduced to 20% and 9%, respectively. The addition of 20 μM DBC coenzyme also reduced the loss at 4° to 9%. The inclusion of D- or L-ornithine or PLP in the buffer had no effect on stability. The protection afforded by glycerol and DBC coenzyme was also observed during attempts to purify the mutase on Sepharose-6-B columns. A 4-fold increase in recovery was achieved by the addition of 10% glycerol and 2 μM DBC coenzyme to the buffer.

Molecular Weight and Subunits. The molecular weight of the mutase from step 4 of purification was estimated by the gel electrophoresis technique of Hedrick and Smith (1968) and by sucrose density gradient centrifugation. The sucrose gradient (Figure 2) utilizing lactic acid dehydrogenase as a reference standard indicated a $s_{20,w}$ value of 8.7S which corresponds to a molecular weight in the order of 170,000. The molecular weight estimated by the Hedrick-Smith technique indicated a value of 175,000-180,000.

The number and molecular weight of subunits was investigated by SDS gel electrophoresis. In two experiments using as reference standards either ovalbumin, D-amino acid oxidase, and chymotrypsinogen A or glutamate dehydrogenase, ovalbumin and bovine serum albumin, one major band with a molecular weight of 95,000-98,000 and a minor component with a molecular weight of approximately 83,000 was observed. A scan of the mutase gel (Figure 3) shows that the minor component represented only approximately 2% of the total protein and therefore probably was a breakdown product or minor contaminant rather than a subunit of the enzyme. These data and the results obtained with the intact enzyme indicate that ornithine mutase has a molecular weight of about 180,000 and consists of 2 polypeptide chains of equal size.

Temperature, pH Optimum, and Protein Concentration. The optimum temperature for the mutase reaction under standard radiochemical assay conditions was approximately 37^{o}, with one-half maximal activities occurring at 23^{o} and 49^{o} (Figure 4). Other experiments demonstrated that the mutase is rapidly inactivated at temperatures exceeding 45^{o}.

The optimum pH was found to be 9 with one-half maximal activities occurring at approximately 7.4 and 9.7. The pH curve (Figure 5) indicates that potassium phosphate buffer is inhibitory. We also observed that the addition of potassium ion to reactions in Tris buffer decreased activity.

With the radiochemical assay, a plot of activity versus protein concentration was linear in the range of 10 μg to 90 μg of protein per reaction and extrapolated through the origin. Enzyme from step 4 of purification with a specific activity of 1.2 units per mg was used in this experiment.

Substrate Specificity and Analogues. The enzyme from DEAE-cellulose columns utilized either D- or L-ornithine as substrate. However, following hydroxylapatite treatment (Step 4), no activity was detectable with L-ornithine as substrate when tested with either assay. Therefore, D-ornithine is the true substrate for the ornithine mutase reaction and it is likely that an ornithine racemase was removed by step 4 of purification.

The apparent Km for D-ornithine obtained by the radiochemical assay was 6.7×10^{-3} M (Figure 6). The Km observed using the coupled assay was $4.4 \cdot 10^{-4}$ M. This large difference in observed Km values may have resulted from the great differences in the protein concentrations utilized in the two assay systems and/or the differences in temperature and buffers used. It was not possible to determine the Km by both procedures under the same conditions because of differences in the sensitivity of the assays and the optimum conditions for the C_4 dehydrogenase activity. Table 2 summarizes the effect of a number of amino acids and compounds structurally related to ornithine when equimolar amounts were added with D-ornithine. It is of interest to note that the presence of L-ornithine and the substrates of the

D-α-lysine mutase, L-α-lysine mutase and the β-lysine mutase significantly decreased activity. Tsuda and Friedmann (1970) reported that L-α-lysine did not act as an inhibitor when tested using a partially purified extract.

Pyridoxal Phosphate Requirement. The highly purified mutase from step 5 of purification was almost completely dependent on PLP. The deletion of this cofactor from the radiochemical assay mixture (8.5 μg of pure mutase, specific activity of 1.8 units per mg) resulted in an 85% loss of activity, and the addition of either isoniazid or hydroxylamine at a concentration of 20 mM completely inhibited the reaction. These results provide evidence for enzyme bound PLP or a PLP analog. PLP was previously shown to stimulate the conversion of ornithine to 2-amino-4-ketopentanoic acid in ammonium sulphate fractionated extracts containing both ornithine mutase and C_4 dehydrogenase (Tsuda and Friedmann, 1970). However, it was proposed that PLP functioned as a cofactor in a reaction following ornithine mutase which catalyzed the inversion of the C_4-amino group of 2,4-DAP prior to the oxidative deamination by the C_4 dehydrogenase. The Km for PLP reported by Tsuda and Friedmann using a partially purified extract containing both mutase and C_4 dehydrogenase was 1.3×10^{-6} M. The apparent Km which we obtained with pure ornithine mutase employing the coupled assay and pure C_4 dehydrogenase was 3.6×10^{-7} M (Figure 7). The value obtained using the radiochemical assay was 1.8×10^{-6} M. Again, the differences in assay conditions may have resulted in the

differences in the apparent Km observed. Further evidence that the two enzymes are directly linked is provided in Figure 8. It can be seen that the product generated by pure ornithine mutase served as a substrate for the pure C_4 dehydrogenase. The initial rate of TPN^+ reduction was directly correlated with the amount of 2,4-DAP produced by the mutase. There was no reduction of TPN^+ in the absence of either enzyme with D-ornithine as substrate.

Absorption Spectra of the Enzyme. The marked stimulation of mutase activity in crude or partially purified extracts by DBC coenzyme, and the inhibition resulting from the addition of intrinsic factor previously established the participation of a B_{12} coenzyme (Dyer and Costilow, 1970; Tsuda and Friedmann, 1970). In the present study, very little activity was detected in the absence of DBC coenzyme with enzyme preparations following the hydroxylapatite step of purification. The addition of DBC coenzyme to the extraction buffer to stabilize the mutase during the final step of purification (step 5) precluded the spectral investigation of the nature of the native bound corrinoid. Therefore, a spectrum was derived directly from the pink band constituting ornithine mutase activity in gels electrophoresed with enzyme from step 4 of purification. This was accomplished with a linear transport gel scanner attached to the Gilford spectrophotometer as described in detail in Figure 9. The spectrum in the UV region shows an absorbance maximum at 280 nm and a 280/260 ratio of 1.34. The spectrum in the visible region is typical of a corrinoid compound

with a peak at 355-360 nm and a smaller absorption at 520-540 nm. The former peak indicates that a substantial amount of bound coenzyme is in the hydroxocobalamin form. The spectrum of pure enzyme from step 5 of purification, dialyzed to remove the DBC coenzyme added to stabilize the enzyme, is shown in the same figure. The broad absorption band in the visible region with shoulders in the neighborhood of 355 nm and 520-530 nm and the high absorbance in the 260 nm region is indicative of the DBC coenzyme. Even after exposure at 4^{o} to a 100 watt tungston filament lamp at 12 cm for 4 hr, the spectrum was essentially unchanged.

Extent of Conversion of Ornithine to 2,4-DAP. The conversion of ornithine to 2,4-DAP approached 45% using either enzyme from step 4 of purification or pure mutase from step 5. Equilibrium was usually attained within 40 min employing D-ornithine-^{14}C at a concentration of 20 mM. Attempts to measure the extent of the reverse reaction using similar levels of 2,4-DAP-^{14}C resulted in little, if any, accumulation of ornithine. Performing assays with much lower concentrations of product to test for possible inhibition would have required excessively high specific activity radioactive 2,4-DAP. Therefore, the extent of the reverse reaction using low levels of 2,4-DAP was estimated by employing the C_4 dehydrogenase reaction modified as a substrate assay (see legend of Figure 10). Levels of 2,4-DAP in excess of approximately 0.7 mM led to progressively less utilization. The extent of conversion in both directions was measured in the manner indicated

above employing 20 mM D-ornithine or 0.7 mM 2,4-DAP. The results presented in Figure 10 demonstrate that the reaction is readily reversible and proceeds to approximately the same extent in both directions.

Essential Sulfhydryl Groups and Effect of Oxygen. The activity of the mutase is markedly decreased in the presence of 0.2 mM p-chloromercuribenzoate or N-ethylmaleimide and moderately affected by iodoacetate at a level of 2 mM (Table 3). This suggests that ornithine mutase contains sulfhydryl groups essential for activity, a feature common to the majority of coenzyme B_{12}-dependent mutases catalyzing carbon chain rearrangements and those catalyzing amino group migrations. The failure of arsenite to cause inhibition suggests that the essential sulfhydryl groups are not vicinal.

The effect of DTT and oxygen are presented in Table 4. Similar to the three mutases in the lysine pathway, ornithine mutase exhibited little activity under aerobic conditions either in the presence or absence of a mercaptan. However, in contrast to the former enzymes, about 75% of maximal activity was recovered in the absence of DTT if oxygen was depleted by gassing with argon. DTT completely replaced the anaerobic requirement achieved with argon. Presumably, the mercaptan functions to keep essential, labile sulfhydryl groups reduced and protected from oxygen.

Hydrogen Exchange. A standard reaction employing pure ornithine mutase (8.5 μg, specific activity of 2 units per mg) was

carried out in the presence of a level of H_2O-t providing a total of 5×10^8 cpm or 9×10^6 cpm per µatom of hydrogen. The reaction mixture was incubated for one hr and the diamino acids separated and analyzed for the incorporation of tritium as described under "Methods." There was no incorporation of tritium into non-exchangeable positions of either 2,4-DAP or ornithine. An identical reaction employing D-ornithine-^{14}C as substrate indicated the formation of 0.12 µmoles of 2,4-DAP. If exchange had occurred to the extent of one µatom of hydrogen per µmole of substrate converted and there was no isotope selection, approximately 3.2×10^5 cpm would have been detected in the 2,4-DAP analyzed.

DISCUSSION

The conversion of ornithine to 2,4-DAP catalyzed by ornithine mutase has been previously studied only in crude (Dyer and Costilow, 1970) or partially purified extracts (Tsuda and Friedmann, 1970) of C. sticklandii. In the present paper, a method is described for separating ornithine mutase from 2,4-diaminopentanoic acid C_4 dehydrogenase and obtaining the mutase as a pure protein. The C_4 dehydrogenase may be further purified to homogeneity by an additional preparative disc gel electrophoresis step (Somack and Costilow, 1972) and employed in a sensitive spectrophotometric assay to measure mutase activity. The addition of DBC coenzyme to the buffer used to extract the mutase from individual analytical disc gel slices significantly increases the yield in the final purification step. This could explain the failure of numerous initial attempts at final purification using preparative gel electrophoresis columns where the B_{12} compound was not employed in elution buffers. As much as 2 mg of pure enzyme may be obtained in a single run by the present procedure using the Buchler analytical system with large (6 mm I.D.) diameter glass tubing.

The ornithine mutase appeared to be homogeneous by the criterion of disc gel electrophoresis in 5% and 7% anionic gels. On SDS gels, however, a small amount of material constituting 2% or less of the total protein migrated slightly ahead of the dissociated mutase

and probably represented a breakdown product of the mutase or a minor contaminant. The 70-fold purification achieved indicates that the mutase accounts for about 1.5% of the total soluble protein in crude extracts.

Molecular weight estimations using sucrose gradient centrifugation and gel electrophoresis combined with SDS gel electrophoresis indicate that the enzyme has a molecular weight of approximately 180,000 and is composed of 2 subunits of identical size. The ornithine mutase appears to be strikingly different from the two enzymes in the lysine pathway catalyzing analogous amino group migrations. The β-lysine mutase (Stadtman and Renz, 1968) and the D-α-lysine mutase (Morley and Stadtman, 1970) are physically indistinguishable and exist as complexes of two distinct protein moieties, an acidic protein with a molecular weight of 150,000 containing tightly bound cobamide, and a labile sulfhydryl protein of molecular weight 60,000. The two proteins of the β-lysine mutase are resolved during purification but resolution of the D-α-lysine mutase has been achieved only after acidification to pH 4.0 where the cobamide protein is rendered insoluble. When crude dialyzed extracts containing ornithine mutase were treated with acid at pH 5.0, the activity recovered (50%) was found only in the resulting precipitate.

D-α-Ornithine has been identified as the true substrate for the ornithine mutase reaction since L-ornithine fails to serve as substrate for the purified enzyme. The L-isomer is inhibitory. It is likely that an ornithine racemase was removed by purification step 4

since either D- or L-ornithine is used as substrate by more crude enzyme preparations.

The evidence presented strongly argues against the proposal by Tsuda and Friedmann (1970) that a PLP-dependent epimerase catalyzing the inversion of the C_4 amino group of 2,4-DAP follows the ornithine mutase reaction in the conversion of ornithine to 2-amino-4-keto-pentanoic acid. The data in Figure 8 show that only two pure proteins, ornithine mutase and C_4 dehydrogenase, are required for this conversion. Furthermore, the almost complete dependence of the mutase on PLP with a very low apparent Km (2×10^{-6} M to 4×10^{-7} M) and the complete inhibition of activity by isoniazid and hydroxylamine, indicate that PLP functions as a cofactor for ornithine mutase. The possibility of contamination of the highly purified mutase by a C_4 epimerase is further discounted by the complete lack of hydrogen-exchange noted during the mutase reaction. Highly purified preparations of D-α-lysine mutase are contaminated by a PLP-dependent lysine racemase (Morley and Stadtman, 1972) which catalyzes the incorporation of hydrogen from water into the α-position of both isomers of lysine.

The ornithine mutase has a number of features in common with the lysine aminomutases. Thus, ornithine mutase, D-α-lysine mutase and L-α-lysine mutase are PLP dependent whereas pyruvate may serve the function of PLP in the β-lysine mutase reaction. Morley and Stadtman (1972) have demonstrated that the cobamide moiety of the D-α-lysine mutase complex contains bound PLP and in the presence of Mg^{+2} catalyzes a PLP dependent exchange of a C_6 methylene hydrogen

of D-lysine with the solvent. This suggests that PLP may be the carrier involved in these amino group migrations through enzyme directed PLP-substrate amino group Schiff base intermediates.

Like the D-α-lysine and β-lysine mutases, purified ornithine mutase contains tightly bound cobamide or degradation products thereof and activity is stimulated by the addition of DBC coenzyme. The native bound coenzyme is nevertheless readily exchangeable with DBC coenzyme which subsequently is resistant to photolysis. Since free DBC coenzyme is readily inactivated by light, bound coenzyme is presumably protected in such a way as to prevent the photolytic reaction. This result is reminiscent of the binding of a benzimidazole-containing cobamide coenzyme to sheep methyl-malonyl coenzyme A mutase (Cannata et al., 1965). The indication that a substantial amount of native bound cobamide appears in the hydroxocobalamin form after purification (Figure 9) suggests that either a photolytically susceptible bound cobamide other than DBC coenzyme is the native prosthetic group (C. sticklandii synthesizes adenyl-cobamide coenzyme, Stadtman, 1960), or a significant amount of hydroxocobalamin from photolytic decomposition in extracts is subsequently bound to the enzyme.

Presumably, the cobamide functions as the carrier of the ornithine C_4 hydrogen which exchanges for the amino group on C_5. This hydrogen carrier function has been demonstrated in the analagous L-β-lysine mutase (Retey et al., 1969), the D-α-lysine mutase (Morley and Stadtman, 1971), and a number of other B_{12}-coenzyme dependent

reactions (Abeles, 1971; Barker, 1972). In all cases, the hydrogen migration occurs without exchange with solvent hydrogen. This is apparently also true for the ornithine mutase reaction, since tritium uptake could not be detected when the reaction was carried out in the presence of H_2O-t. However, the possibility of isotope selection has not been eliminated.

Ornithine mutase contains sulfhydryl groups which must be kept reduced either by the exclusion of oxygen or the addition of a mercaptan. This is a common feature of the lysine aminomutases which exhibit little activity in the absence of added mercaptans (Stadtman and Renz, 1968; Morley and Stadtman, 1970; Chirpich et al., 1970).

The D-α-lysine mutase is inhibited by a number of diamino-acids including L-β-lysine, 3,5-diaminohexanoate, and L-ornithine (Morley and Stadtman, 1970). It is interesting to note that the substrates of the lysine aminomutases, D-α-lysine, L-α-lysine and β-lysine also inhibit ornithine mutase.

The precise equilibrium positions of the D-α-lysine and β-lysine mutase reactions are unknown. Both proceed in the forward direction to the extent of approximately 45-50% and in the reverse direction to about 25-30% (Stadtman, 1972). The formation of addition products between pyruvate and 3,5-diaminohexanoate probably inhibit the β-lysine mutase reaction in the reverse direction (Stadtman and Renz, 1968). With respect to the ornithine mutase reaction, the inhibition observed in the reverse direction with 2,4-DAP concentrations in excess of 0.7 mM is undoubtedly a different phenomenon

unless adduct formation with PLP prevents the cofactor from participation in the reaction.

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TABLE 1: Purification of Ornithine Mutase.[a]

Step	Total Enzyme Activity (units)	Specific Activity (units/mg)	Fold Purification (-fold)	Recovery (%)
I. Crude extract	61.5	0.061	1	100
II. Streptomycin sulphate + ammonium sulphate (40-70%), dialyzed	44	0.077	1.25	80
III. DEAE-cellulose column	25	0.37	4.8	40
IV. Hydroxylapatite column	11.8	1.51	24.5	19.3
V. Disc gel electrophoresis	7.8	4.38	71	12.8

[a]Conditions of purification are outlined in the text. Activity was measured by the radiochemical assay as described under "Methods."

TABLE 2: Effect of Compounds Structurally Related to Ornithine on Mutase Activity.[a]

Amino Acid Added	Activity (cpm in standard assay)	Inhibition (%)
None	1100	-
L-α-Ornithine	464	57
L-α-Lysine	799	26
DL-α-Lysine	413	62
β-Lysine	477	56
N-α-Acetyl-L-ornithine	1100	0
Putrescine	1140	0
δ-Amino-n-valerate	1000	9
Cadaverine	1200	0

[a]The standard radiochemical assay was employed with 8.5 μg of pure enzyme. All compounds were present at a concentration of 20 mM.

TABLE 3: Effect of Sulfhydryl Inhibitors on Mutase Activity.[a]

Additions	Concentration (mM)	Activity (units X 10^2)	Inhibition (%)
None	-	32.2	-
p-CMB	0.2	3.9	88
	0.02	22.5	30
Ethylmaleimide	0.2	11.0	66
	0.02	31.0	4
Iodoacetate	2.0	20.3	37
	0.2	25.7	20
Arsenite	2.0	33.5	0

[a]Radiochemical assays were performed as described under "Methods." Reactions contained 15 µg of protein from step 4 of purification which was dialyzed against 100 mM Tris-Cl (pH 8.0) containing 10% glycerol (v/v) to remove DTT. The inhibitors were added at the indicated concentrations before the addition of enzyme.

TABLE 4: Effect of Dithiothreitol and Oxygen on Mutase Activity.[a]

Addition[b]		Treatment[c]	Activity ($_2$) (units x 10^2)	% of Control (1)
(1)	DTT	Argon	21.1	-
(2)	-	Argon	16.2	77
(3)	DTT	-	21.2	101
(4)	-	-	4.0	19
(5)	DTT	O_2	9.4	45
(6)	-	O_2	3.0	14
(7)	DTT	O_2 → argon	19.9	94
(8)	-	O_2 → argon	9.2	44

[a]The enzyme preparation free of DTT used for the experiments reported in Table 3 and the standard radiochemical assay were employed. [b]When present, the mercaptan was added to reactions prior to enzyme at a final concentration of 10 mM. [c]Gasing with oxygen (1 min) and/or argon (2 min) was initiated after the addition of enzyme.

FIGURE 1: Disc gel electrophoresis of pure ornithine mutase. Analytical disc gel electrophoresis was performed and gels scanned in electrophoresis buffer at 650 nm as described under "Methods." Protein from step 4 of purification (44 µg) was electrophoresed on one gel (----) and 14 µg of pure mutase on the other (——).

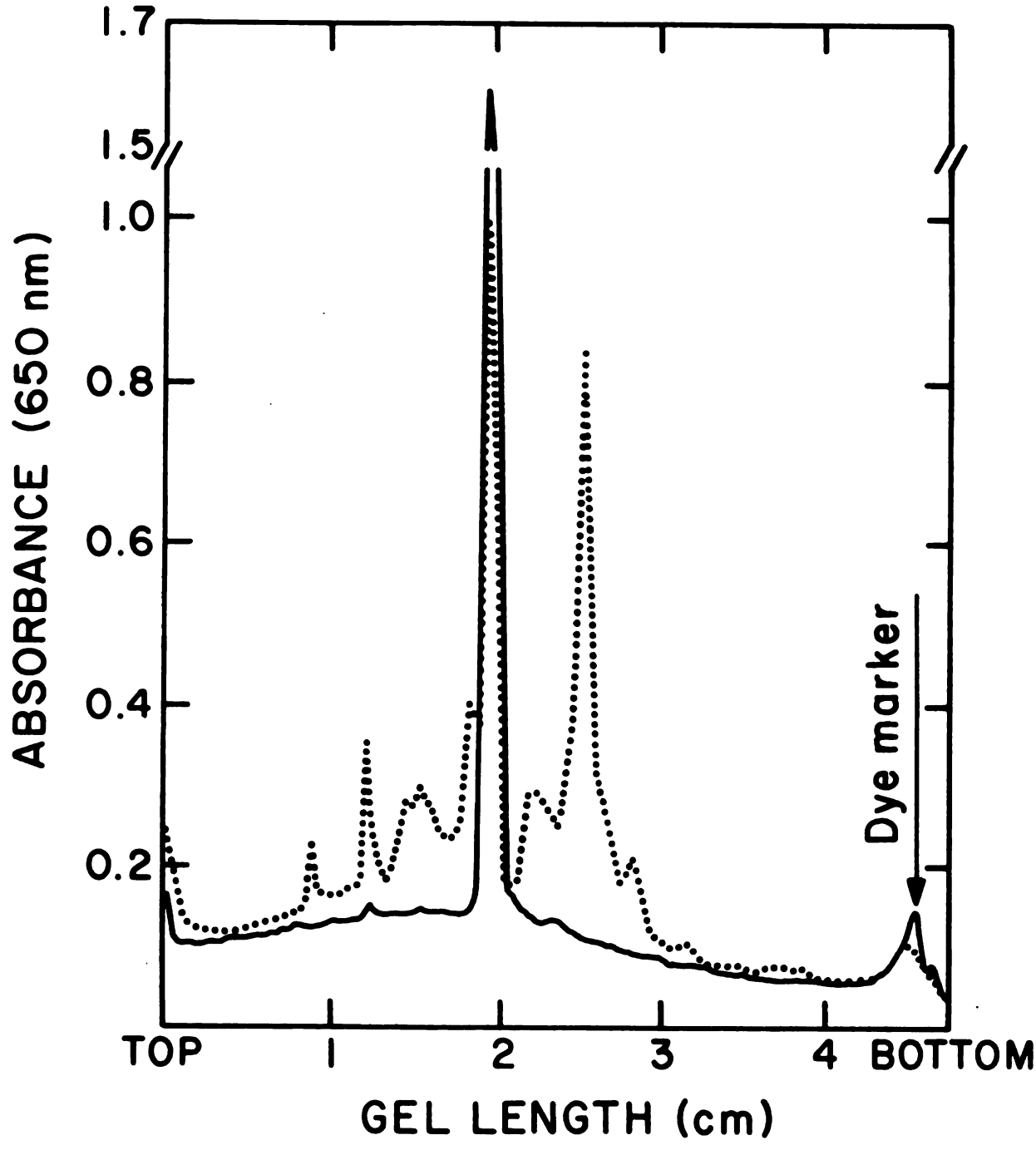
1.7
1.5
1.0
0.8
0.6
0.4
0.2
ABSORBANCE (650 nm)
Dye marker
TOP
1
2
3
4
BOTTOM
GEL LENGTH (cm)

FIGURE 1.

FIGURE 2: Sucrose density gradient centrifugation of ornithine mutase. A 5-20% linear sucrose gradient (4.55 ml) was layered with 500 µg of protein from step 4 of purification and 6 µg of lactic acid dehydrogenase in a volume of 0.15 ml containing 10^{-3} M DTT and 0.1 M Tris, pH 8.5 (see "Methods" for experimental details and enzyme assays). (●) ornithine mutase; (o), LDH ($-\Delta A_{340}$ per min).

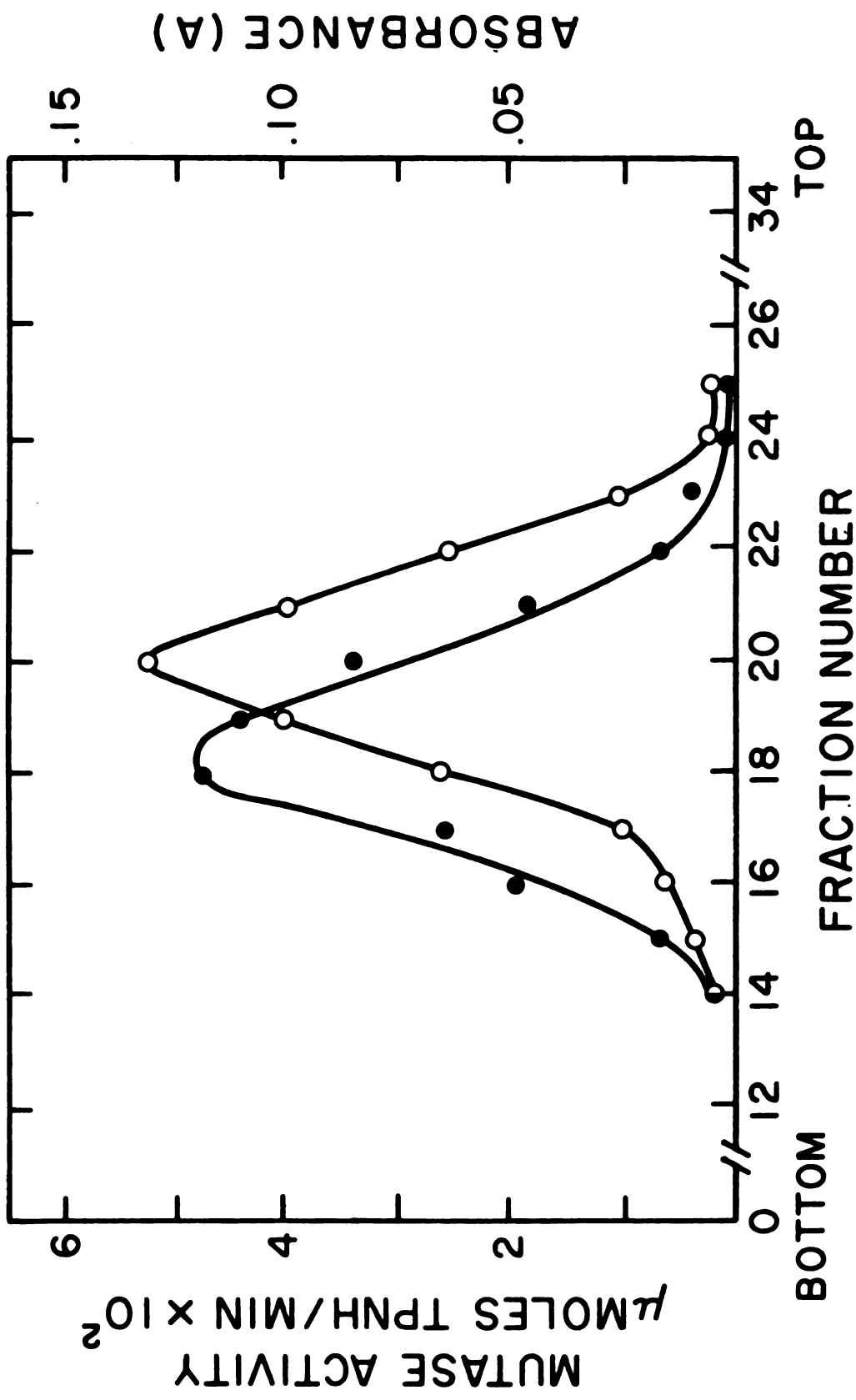
ABSORBANCE (A)
.15
.10
.05
MUTASE ACTIVITY
μMOLES TPNH/MIN × 10²
6
4
2
0
BOTTOM
12
14
16
18
20
22
24
26
34
TOP
FRACTION NUMBER

FIGURE 2.

FIGURE 3: SDS gel electrophoresis of pure ornithine mutase. Ten µg of pure mutase were applied to the gel which was electrophoresed at 20^{o} as described under "Methods."

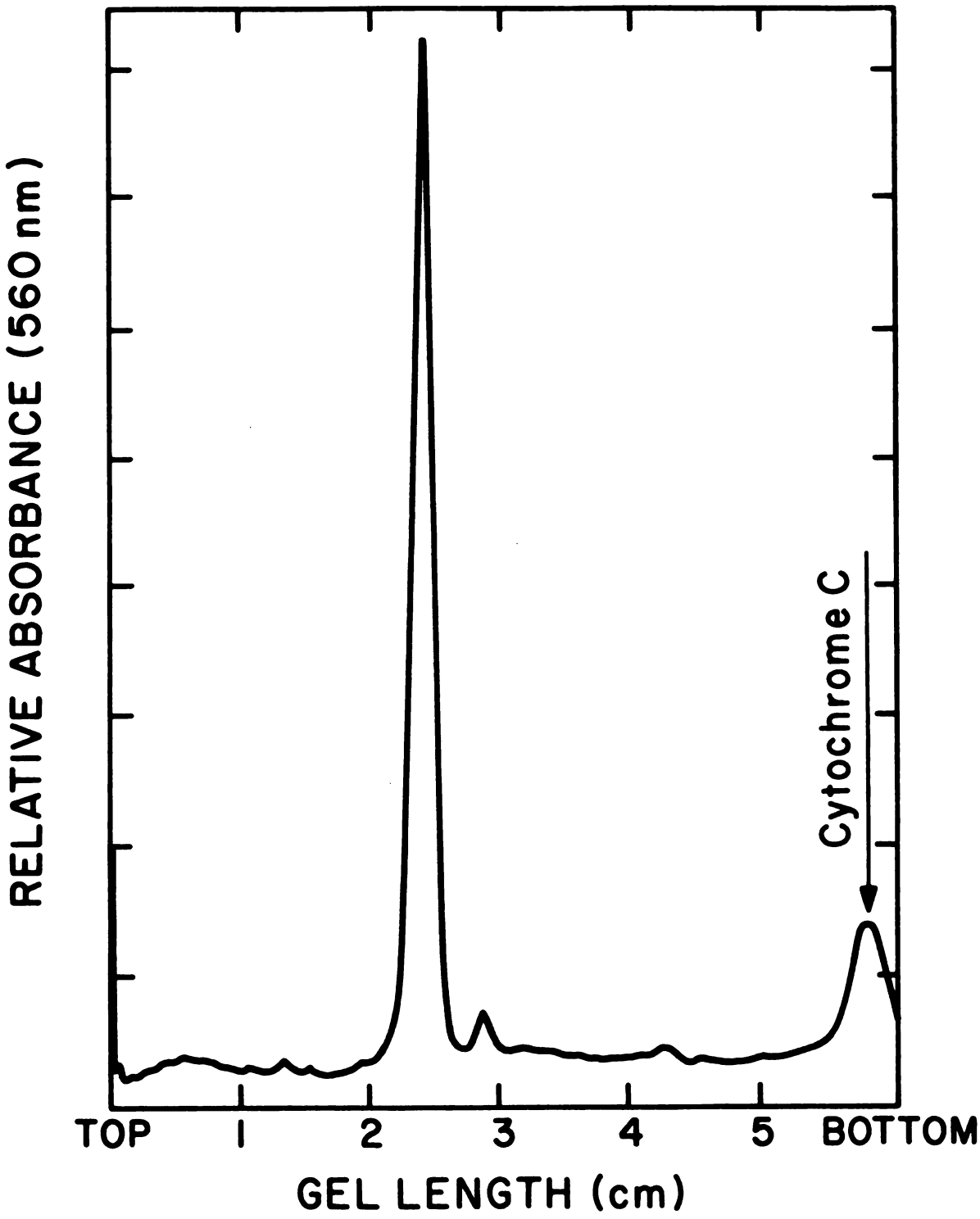
RELATIVE ABSORBANCE (560 nm)
Cytochrome C
TOP
1
2
3
4
5
BOTTOM
GEL LENGTH (cm)

FIGURE 3.

FIGURE 4: Temperature optimum of ornithine mutase. Radioactive assays were conducted in the standard manner except for the variation in temperature. Reactions contained 22 μg of mutase from step 4 of purification (1.0 unit per mg).

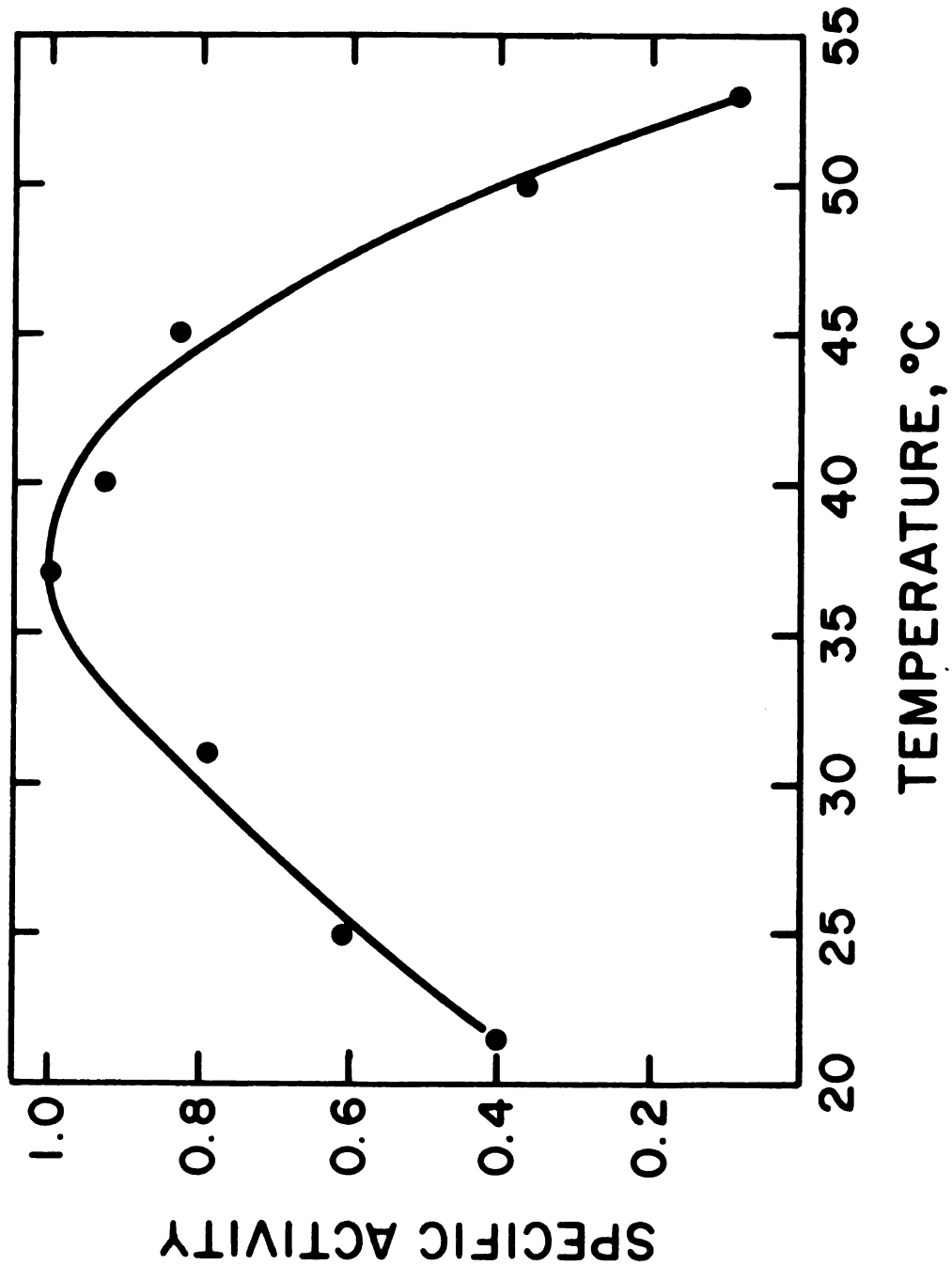
SPECIFIC ACTIVITY
1.0
0.8
0.6
0.4
0.2
20
25
30
35
40
45
50
55
TEMPERATURE, °C

FIGURE 4.

FIGURE 5: pH optimum of ornithine mutase. Standard radiochemical assays were conducted with 22 μg of protein from step 4 of purification (1.4 units per mg). The final pH was measured from scaled up, control reaction mixtures.

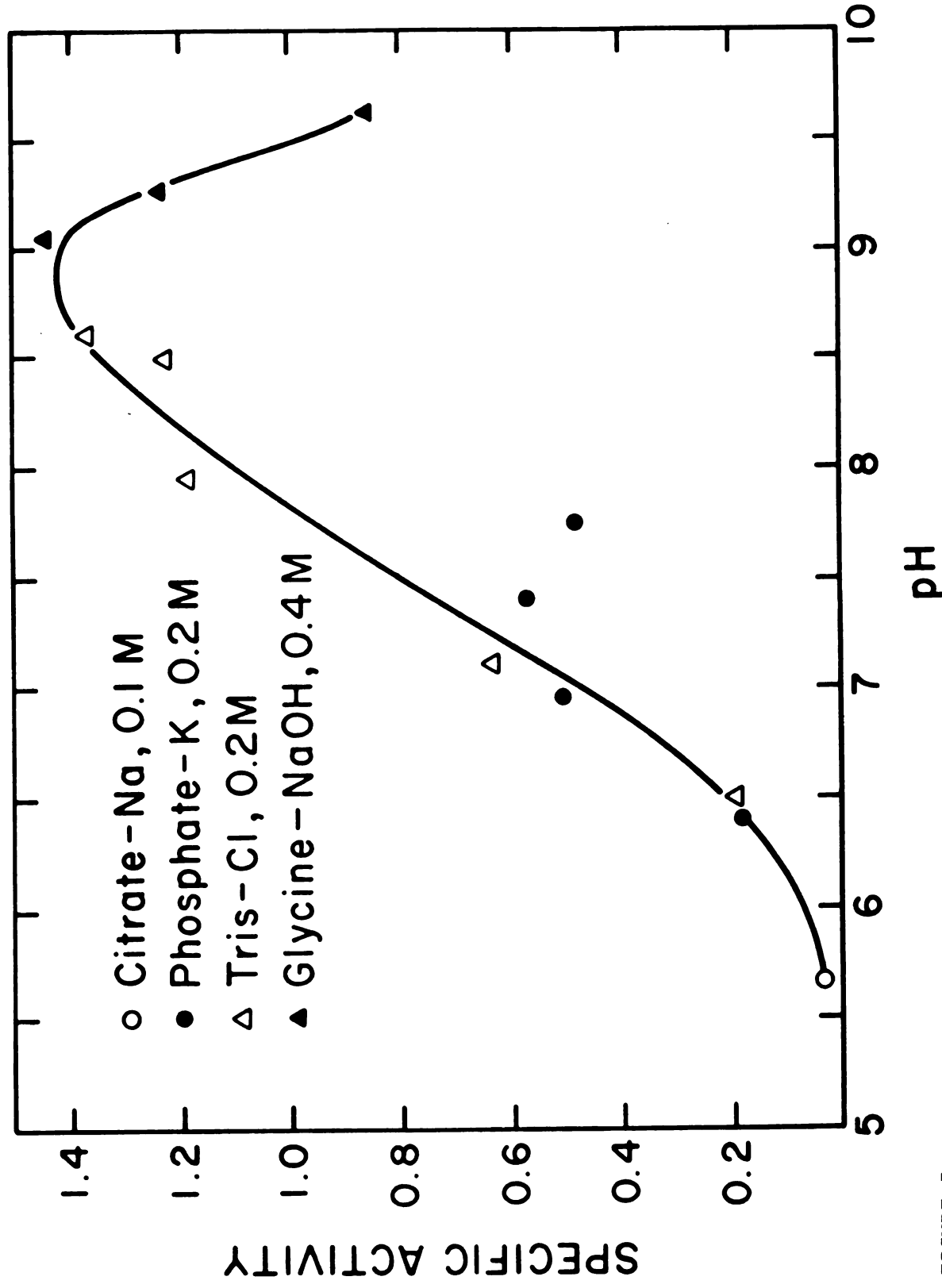
SPECIFIC ACTIVITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5
6
7
8
9
10
pH
o Citrate-Na, 0.1 M
● Phosphate-K, 0.2 M
△ Tris-Cl, 0.2M
▲ Glycine-NaOH, 0.4 M

FIGURE 5.

FIGURE 6: Lineweaver-Burk plot for D-ornithine with pure ornithine mutase. The standard radiochemical assay was employed except for the variation in D-ornithine level. Each reaction contained 8.5 μg of pure ornithine mutase with a specific activity of 2.8 units per mg.

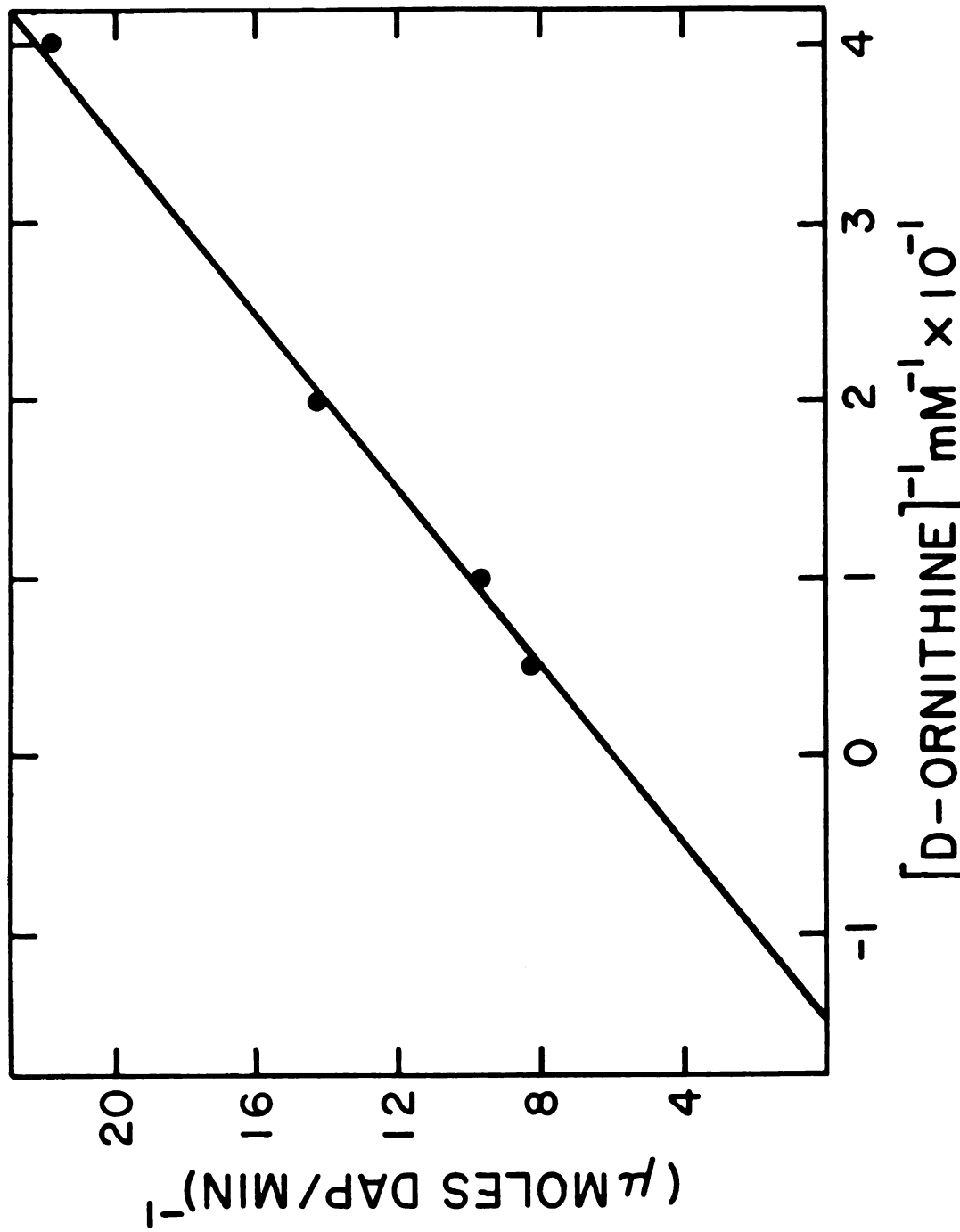

FIGURE 6.

FIGURE 7: Lineweaver-Burk plot for pyridoxal phosphate with pure ornithine mutase. The standard coupled assay was employed except for the variation in PLP level. Reactions contained 0.85 μg of pure ornithine mutase (specific activity, 3.8 units per mg).

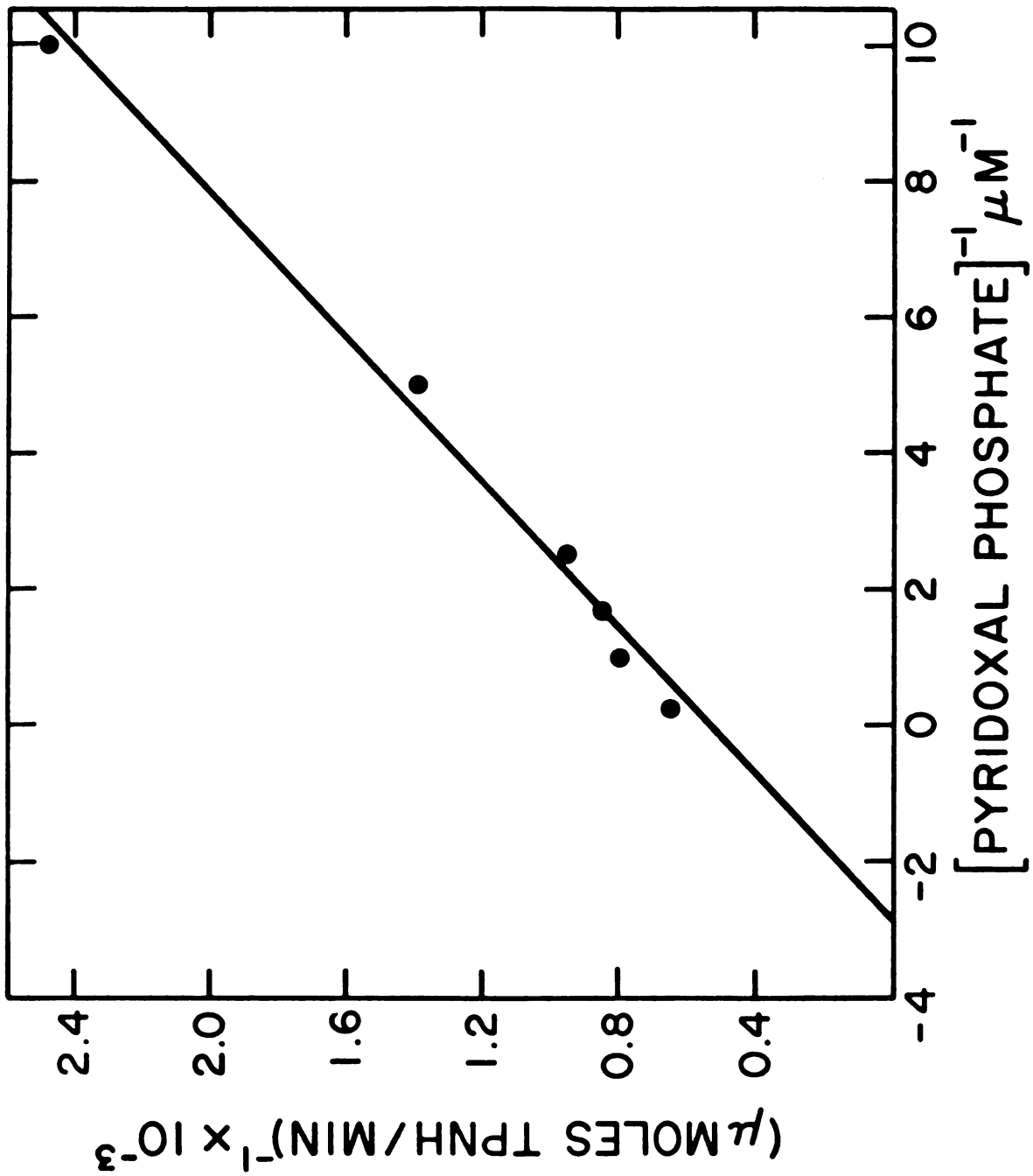
(μMOLES TPNH/MIN)$^{-1}$ x 10^{-3}
2.4
2.0
1.6
1.2
0.8
0.4
-4
-2
0
2
4
6
8
10
[PYRIDOXAL PHOSPHATE]$^{-1}$ μM^{-1}

FIGURE 7.

FIGURE 8: Conversion of ornithine to 2-amino-4-ketopentanoic acid by mutase and C_4 dehydrogenase. The standard radiochemical assay mixture with 8.5 µg of pure mutase (specific activity, 4.3 units per mg) was used. Aliquots of 20 µl were removed from the reaction mixture at the indicated times, diluted 1:1 with distilled H_2O, boiled for 30 seconds, and centrifuged. Volumes of 20 µl were then chromatographed on silicic acid to assay for 2,4-DAP as described under "Methods" (●). Volumes of 5 µl were employed in the C_4 dehydrogenase assay with pure dehydrogenase and the initial rate of TPN^+ reduction recorded (o).

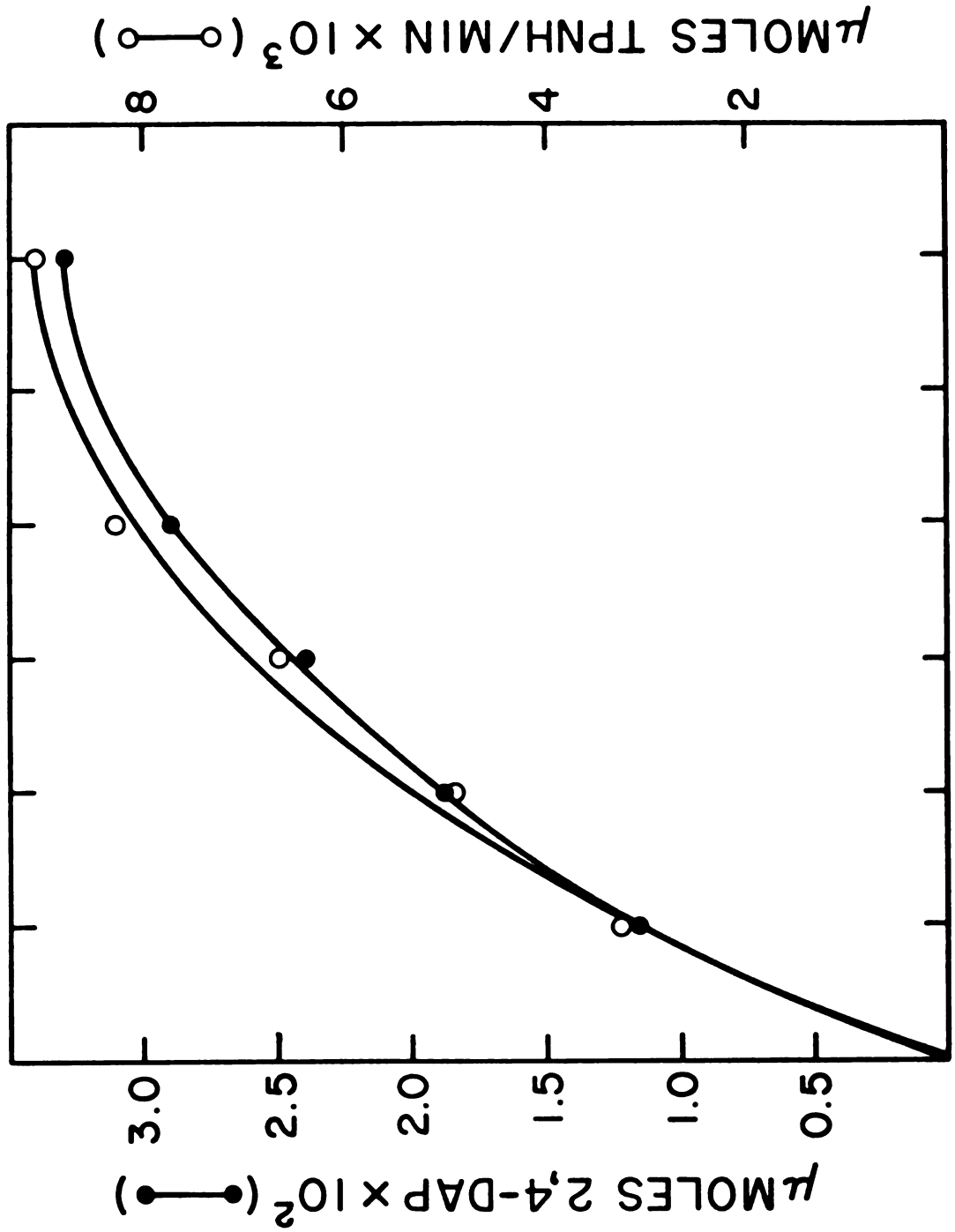
μMOLES TPNH/MIN × 10³ (○—○)
8
6
4
2
30
25
20
15
10
5
MINUTES
3.0
2.5
2.0
1.5
1.0
0.5
μMOLES 2,4-DAP × 10² (●—●)

FIGURE 8.

FIGURE 9: Evidence for mutase-bound cobamide. Extract from step 4 of purification (250 μg of protein) was applied to a 7% analytical polyacrylamide gel and electrophoresed with a gel containing no protein for 2.5 hr as described under "Methods." Readings at the indicated wavelengths were made using the Gilford linear transport apparatus by focusing on the blank gel in electrophoresis buffer and repeated after focusing on the pink mutase band in the gel electrophoresed with protein. The spectrum is derived from the difference at each wavelength between absorbance of protein and blank gel (o). For the other spectrum pure mutase (0.7 mg per ml, 3.5 units per mg) was dialyzed against 0.05 M potassium phosphate buffer, pH 8.0, containing 0.1 M KCl. The spectrum (trace line without points) was determined using 1 cm quartz cuvettes with the dialysis buffer as a blank.

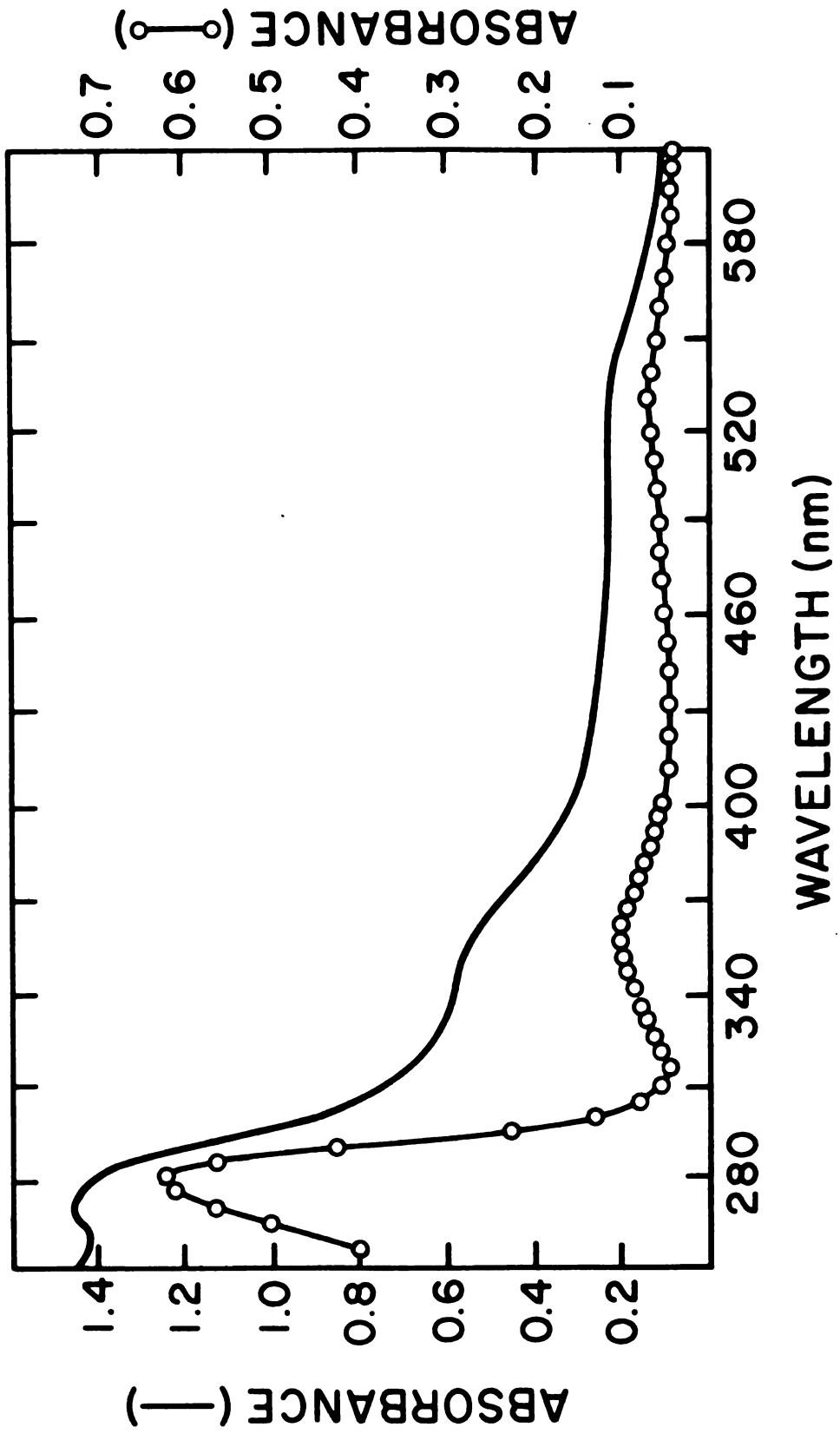
ABSORBANCE (—)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
280
340
400
460
520
580
WAVELENGTH (nm)
ABSORBANCE (—o—)
0.7
0.6
0.5
0.4
0.3
0.2
0.1

FIGURE 9.

FIGURE 10: Extent of conversion of ornithine to 2,4-DAP. The standard radiochemical assay was employed with 17 μg of pure mutase (specific activity, 3.0 units per mg) and 20 mM D-ornithine (●), or 8.5 μg of pure enzyme and 0.7 mM 2,4-DAP (o). At the indicated times aliquots were boiled, diluted as necessary, and added to cuvettes containing the components of the coupled assay with 0.005 units of pure C_4 dehydrogenase. The percentage 2,4-DAP generated or lost was estimated from the initial rate of TPN^+ reduction relative to a standard curve constructed from a control reaction mixture containing 2,4-DAP at various concentrations but no mutase.

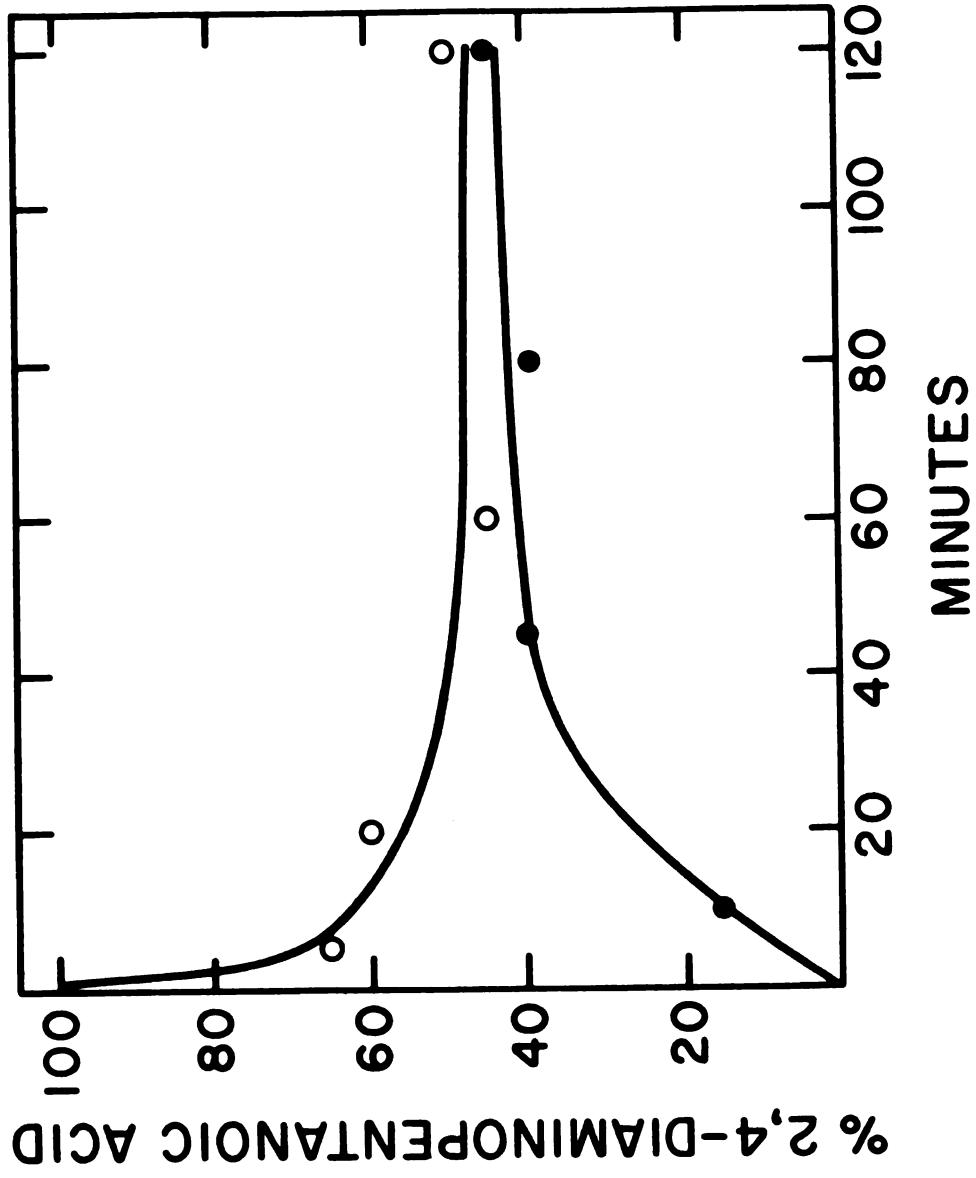
% 2,4-DIAMINOPENTANOIC ACID
100
80
60
40
20
MINUTES
20
40
60
80
100
120

FIGURE 10.


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