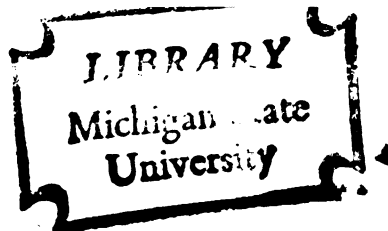


THE PURIFICATION, CHARACTERIZATION,
AND MECHANISM OF ACTION OF
ORNITHINE CYCLASE (DEAMINATING)
FROM CLOSTRIDIUM SPOROGENES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
WILLIAM LAWRENCE MUTH
1972



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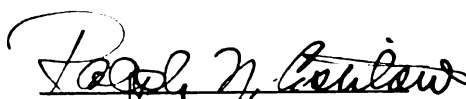
The Purification, Characterization, and Mechanism
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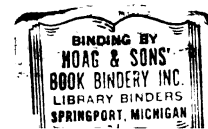
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ABSTRACT

THE PURIFICATION, CHARACTERIZATION, AND MECHANISM OF ACTION OF ORNITHINE CYCLASE (DEAMINATING) FROM CLOSTRIDIUM SPOROGENES

By

William Lawrence Muth

Ornithine cyclase (deaminating) from Clostridium sporogenes was purified to electrophoretic and ultracentrifugal homogeneity. The pure cyclase had a sedimentation coefficient ($s_{20,w}$) of 5.6S measured by high speed sedimentation velocity centrifugation, and a molecular weight of 81,000 calculated from high speed equilibrium sedimentation. Amino acid analysis calculations indicated a molecular weight of 80,000 and a partial specific volume of 0.733 cm³ per gm for the protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated the enzyme was made up of two equal subunits with a molecular weight of 41,500. The ultraviolet absorption spectrum had a major absorption peak at 267 nm which suggested the presence of a nucleotide. Assay of the pure enzyme for bound NAD⁺ demonstrated the presence of approximately 1 mole of bound NAD⁺ per mole of enzyme. Pure enzyme was stimulated by

NAD^+ and either ADP or ATP. The enzyme was inhibited by several sulfhydryl group inhibitors. Titration of the enzyme with p-chloromercuribenzoate established the presence of four sulfhydryl groups per mole. The conversion of L-ornithine to L-proline by ornithine cyclase involves the deamination of the $\alpha\text{-NH}_3$ group prior to cyclization. Therefore, 2-keto-5-aminopentanoic acid and Δ^1 -pyrroline-2-carboxylic acid are likely intermediates in the conversion. Pure cyclase failed to incorporate any tritium from NADT into proline when incubated in the presence of ornithine alone or with Δ^1 -pyrroline-2-carboxylic acid.

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By

William Lawrence Muth

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DEDICATION

This thesis is dedicated to my wife and
parents, who through their encouragement
and support made my entire doctoral
program possible.

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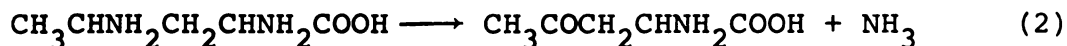
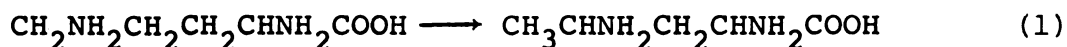
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INTRODUCTION

The putrefactive anaerobes of the genus Clostridium ferment amino acid pairs as sole substrates for the production of energy. Early work indicated that when such amino acids as alanine, leucine, and valine were oxidized there was a concomitant reduction of glycine or proline. Ornithine has been found to function as a hydrogen acceptor for Clostridium sporogenes and is reduced to δ -aminovaleric acid. However, in C. sticklandii it is primarily oxidized to NH_3 , CO_2 , and volatile fatty acids. C. botulinum can ferment ornithine as a single substrate to CO_2 , NH_3 , volatile fatty acids, and δ -aminovaleric acid utilizing both oxidative and reductive pathways.

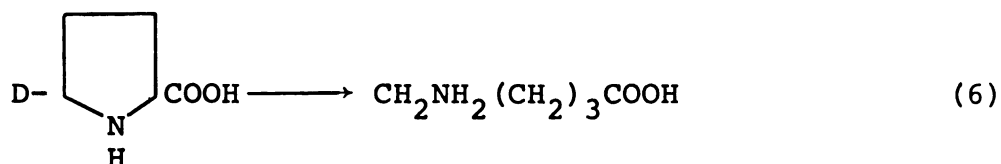
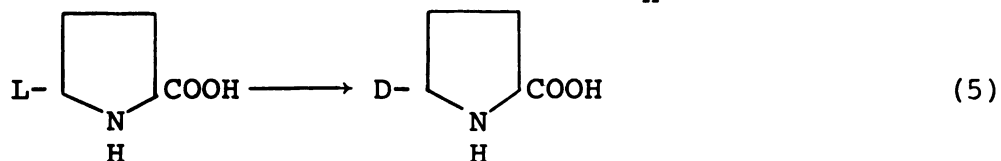
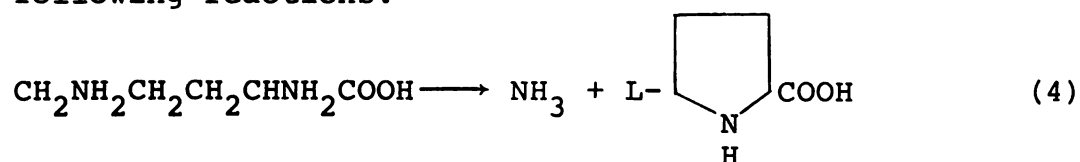
The oxidation of D-ornithine by C. sticklandii proceeds as follows:



Reaction (1) involves a cobamide and pyridoxal phosphate-requiring mutase enzyme which transfers the δ -amino group

to carbon 4 forming 2,4-diaminopentanoic acid. In reaction (2) an NAD^+ -requiring dehydrogenase yields 2-amino-4-ketopentanoic acid and ammonia. The enzymes catalyzing the conversion indicated in (3) have not been studied. Presumably, there is a CoA-dependent thiolytic cleavage of the keto-acid to form acetyl-CoA and alanine followed by phosphorolysis of the acetyl-CoA to acetyl phosphate with subsequent transfer of the high energy phosphate to ADP to form ATP.

The reduction of L-ornithine by C. sporogenes involves the following reactions:



L-ornithine is converted in a complex NAD^+ -requiring reaction to ammonia and L-proline (reaction 4) which is racemized to the D-form (reaction 5). The final step (reaction 6) involves a cleavage of the proline ring with an NADH-dependent reductase to form δ -aminovaleric acid.

The enzyme catalyzing the conversion of ornithine to proline (ornithine cyclase) can be obtained from

either C. botulinum or C. sporogenes in good quantity. It has been purified about 2-fold by fractionation with ammonium sulfate and dialysis against ornithine-phosphate buffer with no loss of activity. Further purification using ion exchange and molecular sieving columns has resulted in considerable purification but extreme loss of activity in spite of numerous attempts at stabilization of the enzyme with substrate, cofactor, reducing agents, and glycerol. The cyclase is stimulated by both NAD^+ and ADP. However, the partially purified enzyme appears to contain tightly bound NAD^+ . No other cofactors have been identified.

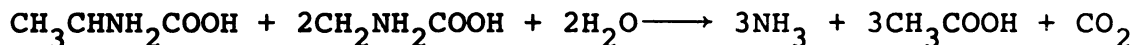
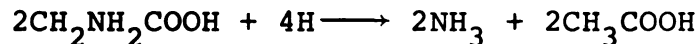
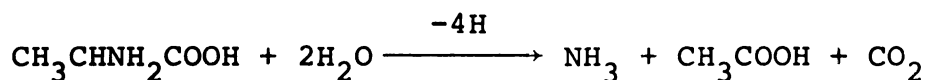
Specific transaminase activity was not found in partially purified preparations catalyzing the conversion. Therefore, ornithine is probably deaminated and cyclized by an oxidation-reduction mechanism. However, any semi-aldehyde or α -keto acid intermediate formed in the course of this reaction was probably bound to the enzyme, since attempts to trap such intermediates were unsuccessful. The conversion of ornithine to proline by a single enzyme or enzyme complex appeared to be both unique and complex. Therefore, we decided to purify the cyclase to homogeneity and examine some of its more salient physical properties. In addition, some aspects of the catalytic mechanism of the cyclase were examined.

This thesis is organized into three sections. The first section is a review of the pertinent literature in which ornithine cyclase is discussed in relation to the pathways involved in ornithine fermentation, energy production, and possible mechanisms for the reaction. The second section is a manuscript describing the purification and properties of the enzyme which has been prepared for publication. The final section contains a manuscript describing experiments to determine the mechanism of action of the cyclase.

LITERATURE REVIEW

Part I The Stickland Reaction

The non-saccharolytic clostridia are known to ferment amino acid pairs as sole substrates for the production of energy (Stickland, 1934). These fermentations are collectively known as Stickland reactions, and involve the oxidation of one of the amino acids in the pair while the other is reduced (Woods, 1936). For each mole of amino acid such as alanine, leucine, isoleucine, valine, etc. that is oxidized, two moles of glycine, proline or hydroxyproline are reduced (Stickland, 1935). For instance:



For a more complete review of Stickland reactions, see recent reviews by Nisman (1954) and Barker (1961).

Part II Energy Considerations

It is possible for cells to derive energy in two ways from Sticland reactions. The first mechanism has not been extensively studied but it probably involves substrate level phosphorylation of one or more of the products of an oxidative pathway. For example, when alanine is oxidized and deaminated, pyruvate is formed. Pyruvate is presumably thiolytically cleaved by pyruvate dehydrogenase forming $\text{CH}_3\text{COOSCoA}$ and CO_2 since CoASH is required for activity of the enzyme. Inorganic phosphate could then be incorporated as $\text{CH}_3\text{COO}(\text{P})$ by phosphate acetyltransferase with the loss of CoASH. The high energy phosphate may be transferred to ADP to form ATP and CH_3COOH by acetate kinase.

The second mechanism for the production of energy occurs with the reduction of one of the amino acids in the pair (Stadtman, 1967). The reduction of glycine has been carefully shown to be linked to the esterification of orthophosphate into ATP probably through an anaerobic electron transport system containing ferredoxin and an acidic low molecular weight protein (Stadtman et al., 1958; and Stadtman, 1966). Early papers describing the proline reductase step either did not discuss or disclaimed any ATP formation associated with this step (Stadtman, 1956; Stadtman and Elliot, 1957). However, more recent data indicate that ATP

is formed during reduction of proline to δ -aminovaleric acid (Stadtman, 1962; Stadtman, 1967). The system appears to be so labile that the stoichiometry is poor and the mechanism is not understood. Hydroxyproline or tryptophan reduction have not been examined as sources of orthophosphate esterification.

Part III Pathways for the Fermentation of Ornithine

Ackermann (1907) showed that ornithine was converted to δ -aminovaleric acid when added to mixed cultures of putrefactive bacteria. Woods (1936) clearly demonstrated ornithine to be a hydrogen acceptor when fermented with alanine by C. sporogenes and that δ -aminovaleric acid and ammonia were the end products. In contrast, ornithine was fermented primarily as an electron donor by C. sticklandii in the presence of lysine (Stadtman, 1954; Stadtman and White, 1954). In whole cells and extracts of C. botulinum, ornithine was fermented as a single substrate with the production of CO_2 , NH_3 , acetate, propionate, butyrate, valerate, and δ -aminovaleric acid (Mitruka and Costilow, 1967).

The oxidative pathway for ornithine utilization has been partially elucidated in C. sticklandii (Figure 1). The first reaction in the sequence is a cobamide-requiring mutase step which transfers the δ -amino group to carbon 4 forming 2,4-diaminopentanoic acid (Dyer and Costilow, 1970;

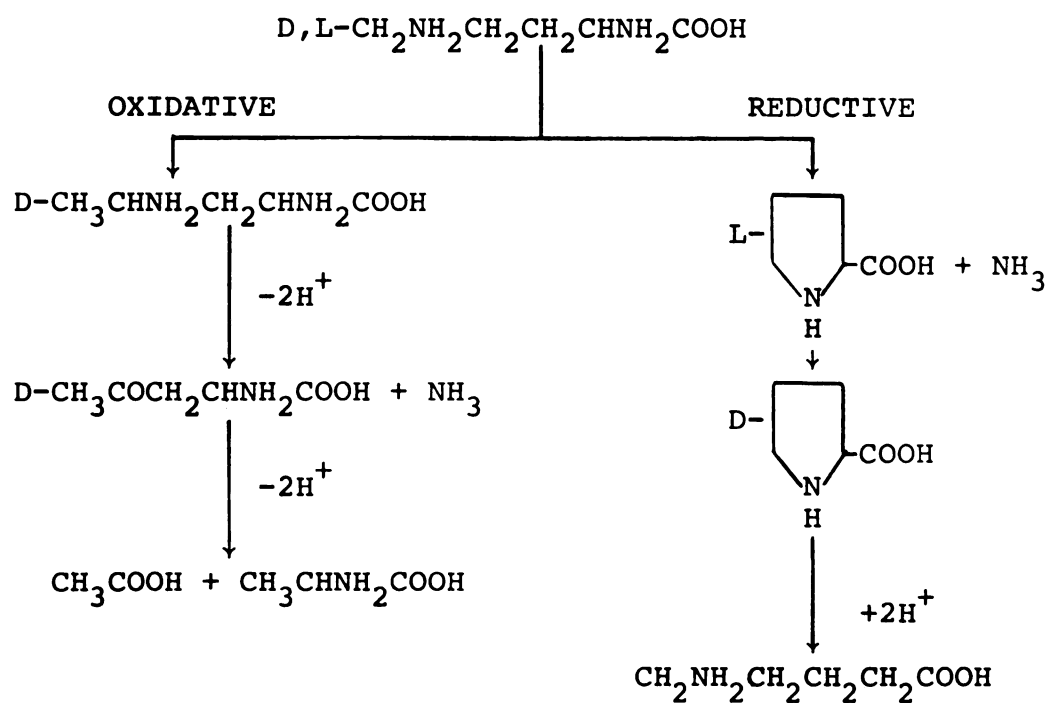


FIGURE 1. Oxidative and reductive pathways for the fermentation of ornithine

Tsuda and Friedmann, 1970; Somack et al., 1971; Somack and Costilow, submitted for publication). The second step is an NAD^+ -requiring oxidative deamination yielding 2-amino-4-ketopentanoic acid and ammonia (Somack and Costilow, 1972). Previously, a pyridoxal phosphate stimulated epimerization was postulated to occur prior to the C_4 dehydrogenase step (Tsuda and Friedmann, 1970), but more recent evidence indicates that the pyridoxal phosphate is a cofactor in the mutase reaction (Somack and Costilow, submitted for publication). The final step is the CoA-requiring thiolytic cleavage of 2-amino-4-ketopentanoic acid by several enzymes to form the primary products, acetate, alanine, and ATP (Dyer and Costilow, 1968).

The reductive pathway of ornithine utilization has been elucidated in extracts of C. botulinum, C. sporogenes and C. sticklandii cells (Figure 1). The first step of the sequence involves a complex NAD^+ -requiring deamination and cyclization to form L-proline (Costilow and Laycock, 1968, 1969, 1971). More will be said concerning this reaction later (see Part IV below). The second step is the racemization of L-proline to D-proline (Stadtman and Elliott, 1957). The final step involves the cleavage of the proline ring and reduction to δ -aminovaleric acid (Stadtman, 1956; Stadtman and Elliott, 1957). This final reaction is complex and requires a number of cofactors for activity (Stadtman, 1962; Hodgins and Abeles, 1967).

Part IV
Conversion of Ornithine to Proline

The conversion of ornithine to proline in fresh extracts of C. botulinum cells was first reported by Costilow and Laycock (1968). Considerable evidence was presented that proline was an intermediate in the conversion of ornithine to δ -aminovaleric acid. Thus, (a) proline accumulated in reactions containing excess ornithine but limited reducing power, but did not accumulate with limited substrate and excess reducing power; (b) the rate of accumulation of proline plus δ -aminovaleric acid in a system with limited reducing power was the same as the accumulation of δ -aminovaleric acid when excess reducing capability was present; (c) when proline reductase was inhibited, proline accumulated at the same rate as δ -aminovaleric acid with no inhibitor present; (d) when unlabelled proline and [^{14}C]-U-ornithine were added to reaction mixtures, radioactive proline accumulated until the exogenous proline supply was exhausted; and (e) when small amounts of extract and limited reducing supply were incubated for short periods of time (2 min) with high specific activity [^{14}C]-ornithine, a small but significant amount of radioactive proline accumulated, whereas essentially no δ -aminovaleric acid was formed.

Compounds which stimulated the conversion of ornithine to proline included NAD^+ and ADP (Costilow and

Laycock, 1968), although ADP stimulation could no longer be demonstrated after a 2-fold purification. No other cofactor was found, but FAD and NADP appeared to partially substitute for NAD^+ in stimulating the activity in more purified enzyme preparations (Costilow and Laycock, 1971).

The conversion of ornithine to proline in a number of other microorganisms and animal tissues proceeds via ornithine- δ -transaminase to glutamic- γ -semialdehyde, which is in equilibrium with Δ^1 -pyrroline-5-carboxylic acid (5-PCA), and thence, through NADH or NADPH-dependent reduction to proline (Stetten, 1955; Scher and Vogel, 1957). Evidence for a similar pathway was sought in C. botulinum and C. sporogenes without success. Although 5-PCA was produced from proline in reaction mixtures using crude extracts, and 5-PCA could be rapidly reduced by these extracts when NADH was present, further purification of the extract demonstrated that 5-PCA reductase was not involved in the conversion of ornithine to proline (Costilow and Laycock, 1969, 1971). The formation of proline from ornithine was shown to be carried out by a single enzyme or enzyme complex, and the enzyme was given the trivial name of ornithine cyclase (deaminating). The catalytic protein was unstable after purification through DEAE cellulose column chromatography. The enzyme at this stage of purity was characterized as being specific for L-ornithine and

producing L-proline in an essentially irreversible reaction. The apparent K_m for ornithine was 11.1 mM which indicated a rather low affinity of the enzyme for substrate. On the other hand, the apparent K_m for NAD^+ was 6.1×10^{-3} mM indicating a strong affinity. The enzyme was most active at pH 8.0 and at 44°. Increasing levels of D-ornithine appeared to inhibit the enzyme but L-lysine, L-alanine, L-leucine or isoleucine and L-proline did not significantly affect the activity. No tritium exchange took place when the reaction was run in the presence of T_2O . No intermediate of a pyrroline nature could be trapped in the course of the reaction. The presence of hydrazine or semicarbazide did not inhibit the reaction, thereby indicating a lack of either pyridoxal phosphate or bound pyruvate which could possibly be implicated in Schiff base formation.

Part V Possible Roles for NAD^+ in Enzyme Reactions

NAD^+ is involved in reactions catalyzed by a number of different kinds of enzymes including dehydrogenases, epimerases, and oxidoreductases. Its action may be catalytic in nature as it is in the vast majority of enzymes in which NAD^+ is found. However, NAD^+ may also function as an activator for an enzyme and not be directly involved with catalysis. For instance, nicotinamide

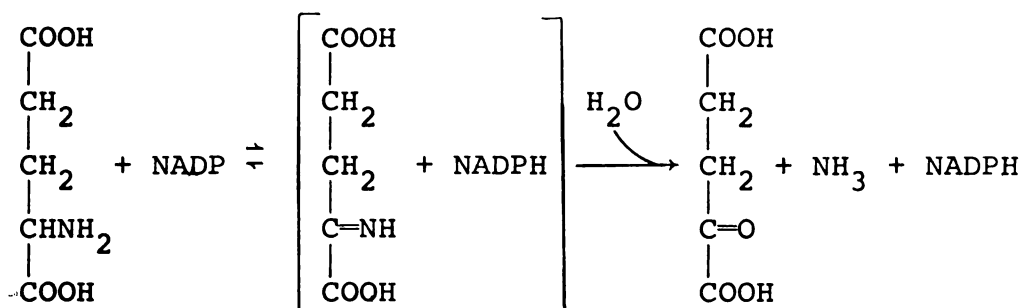
adenine dinucleotide phosphate-ferredoxin reductase from C. kluyveri which catalyzes the reduction of ferredoxin by NADPH is controlled by the oxidation state of the NAD^+ -NADH couple (Thauer et al., 1971). NAD^+ serves as an obligatory activator for this enzyme. As expected, NADH is an inhibitor, but is competitive with NAD^+ rather than NADPH.

Most NAD^+ -dependent enzymes have an apparent K_m for the cofactor in the range of 0.01 to 1.0 mM; and thus, have a relatively low affinity for it. On the other hand, there are several enzymes which display very low apparent K_m 's for their cofactors (0.1 μM to 1 μM), and have NAD^+ tightly bound to the enzyme. Velick et al. (1953) have shown that NAD^+ is tightly bound to glyceraldehyde-3-phosphate dehydrogenase. Other enzymes known to contain tightly bound NAD^+ include UDP-D-glucose 4'-epimerase from either E. coli (Wilson and Hogness, 1964, 1969) or yeast (Darrow and Rodstrom, 1968) and TDP-D-glucose oxidoreductase (Wang and Gabriel, 1969; Zarkowsky and Glaser, 1969). In these cases in which NAD^+ is tightly bound to the enzyme, a catalytic role has been ascribed to the cofactor.

Part VI Possible Mechanisms for the Ornithine Cyclase Reaction

The conversion of ornithine to proline must proceed via either a specific transaminase and a reductase or an

oxidative deamination and a reduction (Meister, 1965; Costilow and Laycock, 1969). Costilow and Laycock (1969, 1971) eliminated the possibility of a specific transaminase, since they were unable to identify either pyridoxal phosphate or any α -keto acid involvement in the conversion. The possibility that NAD^+ participates in the reaction as an essential activator controlling the reaction is remote, since the apparent K_m for NAD^+ indicates a strong interaction between enzyme and cofactor (Costilow and Laycock, 1971), whereas a cofactor which appears to have an activator function usually has a much larger apparent K_m (Thauer et al., 1971). In addition, the cyclase displayed normal saturation kinetics with NAD^+ , and thus it is not likely that NAD^+ functions as an allosteric effector. Costilow and Laycock (1971) have shown that the conversion of ornithine to proline proceeds via a single enzyme or enzyme complex. Since a transamination has been ruled out, and the status of NAD^+ involvement as an activator is highly questionable, the most logical mechanism for the conversion involves an oxidative deamination and reduction in which NAD^+ participates as a coenzyme. The oxidative deamination may occur in a fashion similar to that which occurs in glutamate dehydrogenase (Meister, 1965) which may be outlined as follows:



Glutamate is first oxidized by NADP to an imino acid. The imino group is replaced by oxygen originating from H_2O , and ammonia is released.

Two good examples of enzymes that carry out a sequential oxidation and reduction are the nucleotide-sugar epimerase, UDP-D-glucose 4'-epimerase (Glaser, 1963), and the nucleotide-sugar oxidoreductase, TDP-D-glucose oxidoreductase (Zarkowsky and Glaser, 1969). The postulated mechanisms for these reactions are shown in Figure 2-A and 2-B, and are the net result of data gathered in several laboratories (see review by Glaser, 1972, for the epimerase; and review by Glaser and Zarkowsky, 1972, for the oxidoreductase).

The similarity between what is currently known about the cyclase and several facts which are consistent with the mechanisms proposed for the epimerase and the oxidoreductase may be valuable to this discussion of possible mechanisms for the cyclase. For instance, neither the epimerase nor the cyclase incorporate tritium into the product when the reactions are carried out in tritiated water. Also, one

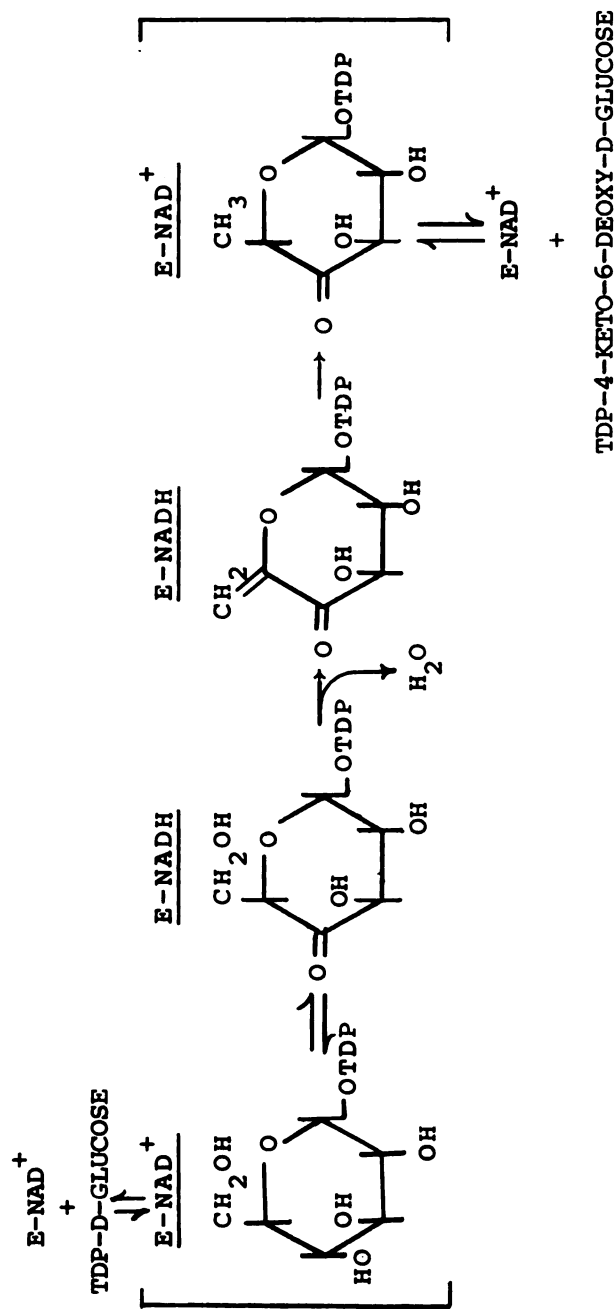
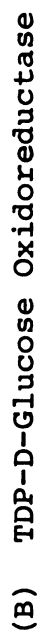
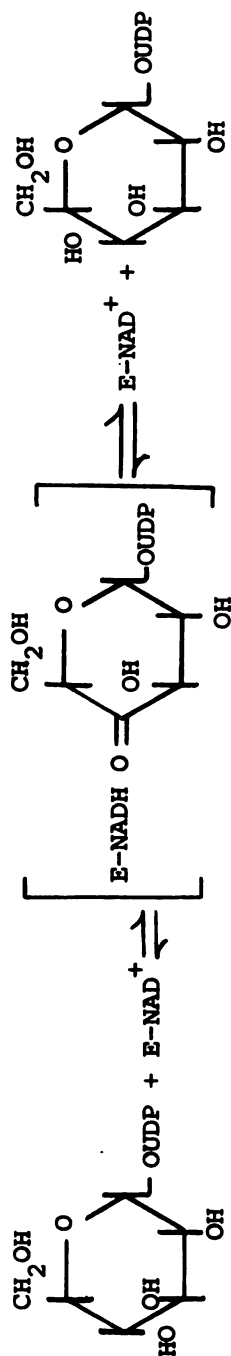
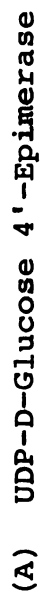


FIGURE 2. Proposed mechanism of action of UDP-D-glucose 4'-epimerase (A) and TDP-D-glucose oxidoreductase (B).

mole of NAD^+ is tightly bound to both the epimerase and the oxidoreductase and Costilow and Laycock (1968, 1971) have indicated that NAD^+ may also be bound tightly to the cyclase. In addition, both the cyclase and the oxidoreductase appear to catalyze irreversible rearrangements (Costilow and Laycock, 1971; Gabriel and Lindquist, 1968). Finally, attempts to trap or isolate any of the proposed intermediates or demonstrate the formation of free NADH during reactions with the cyclase, epimerase, or the oxidoreductase have been unsuccessful (Costilow and Laycock, 1968, 1971; Glaser, 1972; Glaser and Zarkowsky, 1972). The inability to trap intermediates has been partially explained for UDP-D-glucose 4'-epimerase through changes in the conformation in the enzyme by Bertland et al. (1966), Bertland and Kalckar (1968), and Kalckar et al. (1970). These authors have demonstrated that the enzymatic reduction of enzyme bound NAD^+ to bound NADH occurs with a gross change in the tertiary structure of the enzyme as measured by circular dichroism, optical rotary dispersion, or the rate of exchange of hydrogens in the epimerase backbone with those in the medium. This conformational change in structure is probably responsible for the increased binding of the substrate analogue, 5'-UMP, to the reduced enzyme. Similarly, conformational changes may occur in the cases of the oxidoreductase and the cyclase.

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ARTICLE 1

ORNITHINE CYCLASE (DEAMINATING) :
PROPERTIES OF THE HOMOGENEOUS ENZYME¹

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FOOTNOTES

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¹The abbreviations used are: DTT, dithiothreitol; SDA, sodium dodecyl sulfate; p-CMB, parachloromercuribenzoate.

ABSTRACT

Ornithine cyclase from Clostridium sporogenes was purified to electrophoretic and ultracentrifugal homogeneity. The pure cyclase had a sedimentation coefficient ($s_{20,w}$) of 5.6S measured by high speed sedimentation velocity centrifugation, and a molecular weight of 81,000 calculated from high speed equilibrium sedimentation. Amino acid analysis calculations indicated a molecular weight of 80,000 and a partial specific volume for the protein of 0.733 cm³ per gm. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated the enzyme was made up of two equal subunits with a molecular weight of 41,500. The ultraviolet absorption spectrum had a major absorption peak at 267 nm which suggested the presence of a nucleotide. Assay of the pure enzyme for bound NAD⁺ demonstrated the presence of approximately one mole of bound NAD⁺ per mole of enzyme. Pure enzyme was stimulated by NAD⁺ and either ADP or ATP. The enzyme was inhibited by several sulfhydryl group inhibitors. Titration of the enzyme with p-chloromercuribenzoate established the presence of four sulfhydryl groups per mole.

The clostridia are known to ferment a variety of amino acid pairs as sole substrates for the production of energy (Stickland, 1934; Woods, 1936). Woods (1936) demonstrated that Clostridium sporogenes used ornithine as a hydrogen acceptor and reduced it to δ -aminovaleric acid. In contrast, ornithine is used primarily as an electron donor by C. sticklandii (Stadtman, 1954; Stadtman and White, 1954). Recently, ornithine was found to be fermented as a single substrate by whole cells and extracts of C. botulinum to CO_2 , NH_3 , volatile fatty acids, and δ -aminovaleric acid (Mitruka and Costilow, 1967).

Previous studies in this laboratory demonstrated that proline is an intermediate in the fermentative pathway which reductively converts ornithine to δ -aminovaleric acid in both C. botulinum and C. sporogenes (Costilow and Laycock, 1968; 1969). More recent data indicate the direct conversion of ornithine to L-proline and ammonia by a single protein or enzyme complex which was partially purified and characterized and given the trivial name, ornithine cyclase (deaminating) (Costilow and Laycock, 1971). The present report describes procedures for obtaining the cyclase in a homogeneous state and identifies some of the more salient properties of the pure enzyme.

EXPERIMENTAL SECTION

Culture and Cultural Conditions. A putrefactive anaerobe, C. sporogenes (National Canners Association, PA3679, American Type Culture Collection, ATCC 7955) was the source of all enzyme used in this study. The cultural conditions have been previously described (Costilow and Laycock, 1971).

Standard Buffer Preparation. All buffers used in this study were prepared in the following manner. Various levels of Tris plus 10% (w/w) glycerol were added to distilled water to which HCl was added to give the appropriate pH. After the buffer was briefly boiled to remove dissolved oxygen and cooled under argon, 10^{-2} M ornithine, 10^{-3} M DTT¹ and 10^{-4} M NAD⁺ were added as stabilizers. Wherever possible, oxygen was excluded from the buffer.

Enzyme Assay. Ornithine cyclase was assayed in a radiochemical assay utilizing L-[U-¹⁴C]-ornithine as previously described (Costilow and Laycock, 1971). One unit of enzyme activity equals one μ mole proline formed per minute. Δ^1 -Pyrroline-5-carboxylic acid reductase was assayed according to Strecker (1965) as amended by Costilow

and Laycock (1971). Protein was measured by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis. Analytical disc gel electrophoresis was carried out essentially by the technique of Davis (1964) using 7% resolving gels and 3.5% stacking gels. The lower buffer reservoir and gels were cooled with tap H₂O (14-15°) during electrophoresis. The gels were stained in 1% amido Schwartz in 7% (v/v) acetic acid. They were destained either by diffusion or in an electrophoretic destainer with 7% acetic acid.

Preparative polyacrylamide gel electrophoresis was carried out in either a Buchler "PolyPrep-100" unit or a Canalco "Prep-Disc" apparatus with the PD-2/150 lower column insert. The gel system was that of Davis (1964) as modified by Gilpin and Sadoff (1971) except that (i) the gel contained 10% (w/w) glycerol, (ii) the upper buffer reservoir buffer contained 10^{-3} M DTT and (iii) the elution buffer contained both 10% (w/w) glycerol and 10^{-3} M DTT. Each collection tube contained 0.2 ml of 0.1 M ornithine. The flow rate was 0.9 ml/min and 1.8 ml fractions were collected. The 7% resolve gel was 40 mm in length whereas the 3.5% stacking gel was 10 mm in length. A constant current of 20 or 25 mA (Buchler system) or 10 mA (Canalco system) was applied.

The gel was cooled during electrophoresis by means of a circulating ice water bath.

Subunit analysis of the homogeneous enzyme was accomplished with SDS polyacrylamide gel electrophoresis by a modification of the technique of Weber and Osborn (1969) using 5% gels, 10 cm in length. Ovalbumin (43,000), D-amino acid oxidase (37,000), chymotrypsin A (25,000), yeast alcohol dehydrogenase (37,000) and glutamate dehydrogenase (53,000) were employed as standard polypeptide markers (polypeptide chain molecular weights in parentheses). Approximately 5 μ g of each marker and ornithine cyclase were used in each gel. The gels were stained with 0.4% coomassie blue in 10% trichloroacetic acid-33% methanol and were destained in the same solvent mixture.

Ultracentrifugation Studies. All measurements were carried out in a Spinco model E analytical ultracentrifuge equipped with an RTIC temperature control unit and either Rayleigh interference optics and 12 mm double sector cells or Schlieren optics and 30 mm double sector cells. High speed sedimentation equilibrium analysis was carried out at 4.7° at a rotor speed of 25,978 rpm. The molecular weight calculations according to the meniscus depletion technique of Yphantis (1964) were based on the fringe displacement after equilibrium was achieved. Sedimentation velocity analysis was conducted at 5.4° at a rotor speed of

47,544 rpm. The sedimentation coefficient was calculated by the method of Schachman (1957) and corrected to the viscosity and density of water at 20°. Enzyme samples used in these studies were dialyzed against 1 liter standard 0.1 M Tris chloride buffer (pH 8.0) prior to use. Dialysate was used in the reference cells and in making all necessary dilutions.

Amino Acid Analysis. The pure enzyme was exhaustively dialyzed against three 1 liter changes of distilled-deionized water prior to hydrolysis of 500 µg samples of protein in constantly boiling HCl for 24 hours. The analyses of aliquots of sample equivalent to 25 or 50 µg of protein were conducted with an ultrasensitive amino acid analyzer according to the procedure outlined by Robertson et al. (1971). Tryptophan content was measured by the method of Beaven and Holiday (1952). Approximate corrections for decomposition of serine and threonine were made as outlined by Moore and Stein (1963). The half-cystine content was below the limit of resolution of the analyzer. Therefore, these residues were estimated by titration of the sulfhydryl groups contained in the dialyzed native enzyme with 0.665 mM p-CMB according to the procedure of Finlay and Adams (1970) except that 0.1 M Tris chloride buffer (pH 7.0) was substituted for 50 mM potassium phosphate buffer. The absorbance

difference at 250 nm for the free reagent and its mercaptide was taken as $7.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (Boyer, 1954).

Absorption Spectrum and Determination of Protein-Bound NAD⁺. One mg pure cyclase (in 1 ml) was dialyzed against three 500 ml changes of standard 0.1 M Tris chloride buffer minus NAD⁺. Dilution of the NAD⁺ present in the original sample was sufficient to bring the concentration to 8.0×10^{-13} M. The spectrum was then taken from 240 to 700 nm on a Cary model 15 recording spectrophotometer using the last dialysate as blank. To determine bound NAD⁺, an aliquot of enzyme was acidified with HCl to approximately pH 1.0 and incubated at 37° for 15 min, and then neutralized with NaOH. Standard NAD⁺ solutions were treated identically. The NAD⁺ released by this technique was assayed by the procedure described by Maxwell and deRobichon-Szulmajster (1960) in which NAD⁺ is detected by the disappearance of pyruvate in a cyclic coupled enzymatic system containing pyruvate and an excess of lactic dehydrogenase, alcohol and alcohol dehydrogenase. The remaining pyruvate was determined spectrophotometrically with fresh lactic dehydrogenase and NADH.

RESULTS

Enzyme Purification. Ornithine cyclase was purified in the presence of standard buffer unless otherwise noted. All operations were carried out in a cold room (5°) or in an ice bath (0°). Wherever feasible, columns and buffer reservoirs were maintained under argon. Fractions of eluate from each column containing cyclase activity were purged of air with a stream of argon and stoppered as soon as possible after collection. Preparation of the crude extract, treatment with streptomycin sulfate and ammonium sulfate fractionation were conducted as described by Costilow and Laycock (1971). A summary of the overall purification is presented in Table I.

DEAE-Cellulose Chromatography. Ammonium sulfate treated extract (19.2 ml) was placed on a large DEAE-cellulose (DE-52) column (3.5 x 15 cm) which had been equilibrated with standard 0.15 M Tris chloride buffer (pH 7.5). The column was washed with the equilibrium buffer until the A_{280} was less than 0.3. The column was then washed with standard 0.25 M Tris chloride buffer (pH 7.5) to elute the cyclase rapidly. Fractions containing the cyclase activity

were pooled and concentrated by ultrafiltration through a Diaflo UM-10 membrane (Amicon Corporation, Cambridge, Massachusetts) using argon as the pressurizing gas. The concentrated preparation was then diluted with distilled water containing the standard stabilizers to a Tris chloride concentration of 0.15 M and placed on a smaller DEAE-cellulose column (1.0 x 25 cm) which had been equilibrated with standard 0.15 M Tris chloride buffer (pH 7.5). The column was washed with the equilibration buffer until the A_{280} reached 0.15. Then a 500-ml linear gradient from 0.15 M to 0.25 M Tris chloride (pH 7.5) (250 ml of each) was used to elute the enzyme from the column. Fractions of 7 ml were collected at a flow rate of approximately 1.7 ml per min. Fractions were assayed and the cyclase was found to elute at approximately 0.21 M Tris chloride concentration. The active fractions were pooled and concentrated as described above.

Sephadex Gel Filtration. The 3.0 ml concentrated sample from the second DEAE-cellulose column was applied to the top of a 2.5 x 100 cm Pharmacia column which was packed with Sephadex G-200 supported on siliconized glass beads according to the technique of Sachs and Painter (1971) and equilibrated with standard 0.1 M Tris chloride buffer (pH 8.0). Fractions of 3.8 ml were collected at a flow

rate of 40 ml per hr. A large protein peak eluted with the void volume (fractions 16-18). The enzyme was eluted in a second peak (fractions 25-30). Much contaminating protein was removed by this step although there was but little increase in the specific activity of the cyclase. One contaminating protein which migrated on disc gel electrophoresis with an R_F almost coincident with the cyclase was totally removed. The active fractions were pooled and concentrated to 3 ml by ultrafiltration as before.

Preparative Polyacrylamide Gel Electrophoresis. Two ml of the concentrated cyclase activity from the gel filtration step were applied to a preparative gel column described in the Experimental Section along with 0.2 ml glycerol and 0.05 ml 0.1% bromphenol blue. The enzyme was eluted 6 hours after application to the column.

The elution profile of the cyclase from preparative polyacrylamide gel electrophoresis is illustrated in Figure 1. The first peak corresponds to the tracking dye and the second was primarily NAD^+ which was present in the sample when applied to the column. The fractions corresponding to the shoulder on the trailing edge of the NAD^+ peak contained the cyclase free of other protein. The specific activity of the final preparation was about 36-fold that of the crude extract and about 3.7% of the total activity in the crude extract was recovered.

Homogeneity. The ornithine cyclase prepared as described above was homogeneous by several criteria. A single protein band was observed following analytical disc gel electrophoresis (Figure 2). A single symmetrical Schlieren peak was obtained from high speed sedimentation velocity centrifugation. One subunit species was obtained from SDS polyacrylamide gel electrophoresis. A plot of the fringe displacement upon high speed equilibrium sedimentation vs. the square of the radius was linear (see below). In addition, no Δ^1 -pyrroline-5-carboxylic acid reductase activity (an enzyme earlier thought to be involved in the conversion of ornithine to proline) was present. Also, neither glutamate dehydrogenase nor NADH oxidase activities (enzymes present in large quantities in extracts of this Clostridium) were found. Cyclase of this purity is stable for at least 6 months when stored in sealed ampoules under argon in liquid nitrogen. Cyclase stored at 4° under argon generally lost at least 50% of the original activity within 3 days. Freezing the enzyme (-14°) did not appreciably increase the stability.

Molecular Weight and Subunit Analysis. The equilibrium sedimentation analysis for determination of the molecular weight was carried out on two samples, one of 0.9 mg/ml and the other of 0.45 mg/ml. A plot of the

fringe displacement ($3 + \log \Delta Y$) vs. the square of the distance from the center of rotation (radius²) was linear (Figure 3). The weight-average molecular weight averaged from the two runs was 81,040.

Analysis of the plates taken during sedimentation velocity centrifugation indicated a single symmetrical peak. Using a partial specific volume calculated from amino acid analysis data of $0.733 \text{ cm}^3/\text{gm}$, a corrected Svedburg constant ($\bar{s}_{20,w}$) of 5.6S was calculated for the enzyme, which corresponds roughly to a molecular weight of 83,000.

The results of two separate SDS polyacrylamide gel electrophoresis experiments (see Experimental Section for details) indicated that the cyclase consists of two identical subunit species. The average molecular weight of the subunits estimated by this procedure was 41,500. No minor or additional bands were observed.

Amino Acid Analysis. The analysis of the average of 3 replicate samples of cyclase is shown in Table II. The calculations for each amino acid are based on the number of residues per 32 glycine residues. Calculations based on residues per 12 tyrosine, 24 alanine or 24 isoleucine residues yielded similar results. A molecular weight of 80,220 is indicated. The partial specific volume calculated from the amino acid data by the procedure of McMeekin and Marshall (1952) was $0.733 \text{ cm}^3/\text{gm}$.

Absorption Spectrum and Determination of Protein-Bound NAD⁺. Previous studies (Costilow and Laycock, 1969; 1971) indicated that the conversion of ornithine to proline was NAD⁺ dependent. However, the highly purified cyclase was not completely dependent on added NAD⁺. Thus, it was of interest to look for enzyme-bound NAD⁺. The spectrum from 340 to 700 nm was not distinctive and decreased slowly from an absorbance of ~0.1 at 340 nm to 0 at 700 nm. Addition of ornithine to the buffer had no effect on the absorbance at 340 nm. However, in the ultraviolet range, the standard protein peak at 280 nm is little more than a shoulder on a peak that has a maximum at 267 nm (Figure 4). The peak is broad and exhibits a minimum at 245 nm.

Three aliquots of a solution containing cyclase denatured with HCl to release bound NAD⁺ were assayed. The results indicate that the purified enzyme does indeed have tightly bound NAD⁺ (Table III). The average number of moles of bound NAD⁺ per mole of enzyme was calculated to be 1.10.

Effect of Various Compounds on Enzyme Activity.

The data presented in Table IV indicate that NAD⁺, ADP, and ATP are stimulatory to the pure enzyme. AMP and Pi had no effect. Several divalent and trivalent cations (Mg⁺⁺, Mn⁺⁺ and Fe⁺⁺⁺) were inhibitory at the concentrations employed (10 mM to 10 μ M final concentration. The

apparent K_m for L-ornithine was not changed significantly by the presence of 5 mM ADP in the reaction mixture. The values with and without ADP were 6.2 mM and 5.5 mM respectively.

Effect of Sulfhydryl Group Inhibitors on Enzyme Activity. Table V illustrates the effect of p-CMB, iodoacetate, n-ethylmaleimide or arsenite on cyclase activity. p-CMB and n-ethylmaleimide inhibited activity 100% at the highest levels used. Iodoacetate inhibited to a lesser extent and arsenite had but little effect on enzyme activity.

DISCUSSION

These results establish with a high degree of certainty that the conversion of ornithine to proline is catalyzed by a single enzyme. The fact that a single subunit species of one-half the molecular weight of the holoenzyme was observed in SDS polyacrylamide gels essentially eliminates the possibility of an enzyme aggregate. Previous data (Costilow and Laycock, 1971) indicated that this was probably true, but failed to eliminate the possibility of a complex.

It is apparent that the purified protein obtained in this study did not retain maximum specific activity. At least one preparation of the partially pure enzyme studied earlier by Costilow and Laycock (1971) had a specific activity of 4.8 units per mg of protein while that of the homogeneous preparation obtained in this study was only 1.8. However, the previous report also notes that there was a great loss in specific activity during further purification of their enzyme preparation. The reason for this loss of activity is unknown. We have attempted to reactivate the pure enzyme by adding heat inactivated crude or partially purified extracts, ashed residues of crude

extracts, or various mineral ions without success. It appears probable that much of the protein is denatured during the final phases of purification.

Previous papers (Costilow and Laycock, 1968; 1969; 1971) have indicated that NAD^+ is a required cofactor for this enzyme. The apparent K_m for NAD^+ obtained with a partially purified preparation was 6.1×10^{-3} mM. However, no absolute requirement for NAD^+ was demonstrable when more highly purified preparations were used. We also failed to find a complete dependence on added NAD^+ by the homogeneous enzyme. However, an analysis of an acid-denatured enzyme preparation using a dehydrogenase assay demonstrated the presence of approximately 1 mole of NAD^+ per mole of enzyme. Also, the absorption spectrum of an exhaustively dialyzed preparation indicated the presence of a purine and/or a pyrimidine base. The major absorption peak was at 267 nm with only a shoulder at 280 nm. One would not expect this much of a shift in the absorption maximum of an average protein by the presence of 1 mole of NAD^+ per mole enzyme. The $A_{280}:A_{260}$ for the cyclase was only 0.92, as compared to an $A_{280}:A_{260}$ of 1.19 for uridine diphosphogalactose 4'-epimerase which has 1 mole NAD^+ per 72,000 gm enzyme (Wilson and Hogness, 1964). Thus, it appears that our estimate of bound NAD^+ may be low or that there are other nucleotides bound to the cyclase.

We failed to detect any evidence of reduced pyridine nucleotide by measuring the absorbance of the enzyme at 340 nm in the presence of substrate. Such an absorption peak has been observed with other enzymes containing bound NAD^+ (Taylor et al., 1948; Wilson and Hogness, 1964; Bertland and Kalckar, 1968; Zarkowsky and Glaser, 1969). Our failure to detect a 340 nm absorbance may have resulted from the low specific activity of the dialyzed preparation in the absence of added NAD^+ (~ 0.6 units/mg). Also, it is possible that NADH is oxidized as rapidly as it is formed with this enzyme, since the cyclase reaction is either irreversible or proceeds very far in the direction of proline (Costilow and Laycock, 1971). It is obvious that more extensive efforts will be required to conclusively establish a catalytic role of NAD^+ in this reaction.

Strong inhibition of enzyme activity by three sulfhydryl-binding reagents suggests the participation of sulfhydryl groups during catalysis. Titration of the sulfhydryl groups present in the enzyme by p-CMB indicates the presence of 4.1 such groups per mole. However, the low level of inhibition of activity by the arsenite anion indicates a lack of vicinal sulfhydryl groups in the active site.

Studies are currently in progress to elucidate the mechanism of the ornithine cyclase reaction and to

establish with confidence the cofactor role of NAD^+ in the reaction.

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4750.

TABLE I. Purification of Ornithine Cyclase.

Step	Preparation	Volume (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Percent Recovery
1	Crude extract	18.7	1310	65.2	.050	100
2	2.5% Streptomycin treatment	36.8	1290	74.9	.058	114.8
3	(NH ₄) ₂ SO ₄ (50-80% cut)	19.2	652.8	60.0	.092	92.0
4	DEAE I	60.0	156.0	51.4	.329	78.8
5	DEAE II	4.7	21.15	25.7	1.215	39.4
6	Sephadex G-200	3.0	9.9	14.7	1.480	22.5
7	Preparative poly- acrylamide gel	2.0	1.33	2.4	1.80	3.7

TABLE II. Amino Acid Analysis of Ornithine Cyclase.^a

Amino Acid	Residues per 32 Glycine	Residues per Subunit
Asp	38.68	39
Thr ^b	20.88	21
Ser ^b	25.76	26
Glu	42.56	43
Pro	14.56	15
Gly	32.00	32
Ala	23.84	24
Val	21.60	22
Met	18.56	19
Ile	24.24	24
Leu	29.44	29
Tyr	11.56	12
Phe	10.28	10
Lys	25.28	25
His	5.80	6
Arg	9.44	9
Cys ^c		2
Try ^d		3

^aThe data were obtained from the average of triplicate analyses of 25 or 50 μ g cyclase samples which had been hydrolyzed for 24 hours in constantly boiling HCl in sealed, evacuated tubes at 110°. ^bApproximate corrections for decomposition of these amino acids were made according to Moore and Stein (1963). ^cTitrated as free sulfhydryl groups with p-CMB. ^dEstimated by spectral means described in the Experimental Section (Beaven and Holiday, 1952).

TABLE III. Estimation of Protein-bound NAD^+ .^a

Addition	Pyruvate Reduced (μmoles)	Moles NAD^+ per Mole Enzyme
None	0.0000	-
0.167 nmole NAD^+	0.056	-
0.333 nmole NAD^+	0.112	-
0.500 nmole NAD^+	0.167	-
0.677 nmole NAD^+	0.222	-
Acid treated enzyme:		
0.101 nmoles	0.041	1.21
0.203 nmoles	0.073	1.08
0.305 nmoles	0.104	1.02

^aPreincubation mixture contained 100 μl of 0.02M potassium phosphate buffer (pH 7.4), 20 μl of 95% ethanol, 10 μl each of 0.025 M sodium pyruvate, lactic and alcohol dehydrogenases and either standard samples of NAD^+ or acid treated enzyme solutions and sufficient water to bring the total volume to 0.2 ml. The mixtures were incubated at 25° for 10 min and then plunged into boiling H_2O for 2 min to inactivate. Residual pyruvate was determined spectrophotometrically by addition of fresh LDH and NADH.

TABLE IV. Effect of Various Compounds on Enzyme Activity.^a

Addition	Activity ₃ (units x 10 ³)	Percent of Control
Experiment 1:		
None	18.4	100
2.0 μ M NAD ⁺	21.2	115.2
20.0 μ M NAD ⁺	24.5	133.0
200.0 μ M NAD ⁺	25.3	137.5
Experiment 2:		
None	11.3	100
1.0 mM AMP	11.0	97.2
5.0 mM AMP	11.8	105.0
1.0 mM ADP	13.6	120.7
5.0 mM ADP	15.4	137.1
10.0 mM ADP	14.7	130.1
Experiment 3:		
None	8.1	100
5.0 mM ADP	13.4	164.6
1.0 mM Pi ^b	8.6	106.9
5.0 mM Pi	9.2	113.9
1.0 mM Pi + 1.0 mM ADP	10.6	130.0
5.0 mM Pi + 5.0 mM ADP	11.5	141.3
1.0 mM ATP	11.7	143.8
5.0 mM ATP	12.2	149.3

^aThe standard radiochemical assay was used in each experiment except that no NAD⁺ was added to the reaction mixtures in Experiment 1 unless indicated. In each case the added compound was pre-incubated with enzyme for 10 min at 39° before adding substrate. In Experiment 1, 29.5 μ g

TABLE IV.--Continued.

protein (specific activity = 0.857) was used which had been exhaustively dialyzed to remove NAD^+ prior to assay. In Experiments 2 and 3, 20 and 15 μg enzyme (specific activity = 0.565) were used. Reaction mixtures in Experiments 2 and 3 contained 1×10^{-4} M NAD^+ . ^bInorganic phosphate was potassium salt at the pH of the reaction mixture.

TABLE V. Effect of Sulfhydryl Group Inhibitors on Enzyme Activity.^a

Addition	Activity ₃ (units x 10 ³)	Percent Inhibition
None	13.0	0
p-CMB:		
0.01 mM	11.7	10
0.1 mM	6.1	53.1
1.0 mM	0.0	100
Iodoacetate:		
0.02 mM	13.8	0
0.2 mM	10.5	19.2
2.0 mM	3.9	70.0
n-Ethylmaleimide:		
0.02 mM	12.0	7.7
0.2 mM	5.6	56.9
2.0 mM	0.0	100
Arsenite:		
0.02 mM	10.8	16.9
0.2 mM	9.6	26.2
2.0 mM	12.7	2.3

^aPure cyclase (1 mg in 1 ml) was dialyzed against 2 liters of standard Tris chloride buffer (pH 8.0) minus DTT for 12 hours. The various inhibitors were added to the standard radiochemical assay minus mercaptoethanol. Fifteen μ g protein with 1.3×10^{-2} units of activity were used in each assay.

FIGURE 1. Preparative polyacrylamide gel electrophoresis of ornithine cyclase.
The experimental details are outlined in the text. Circles, the
elution profile (A_{280}); triangles, cyclase activity.

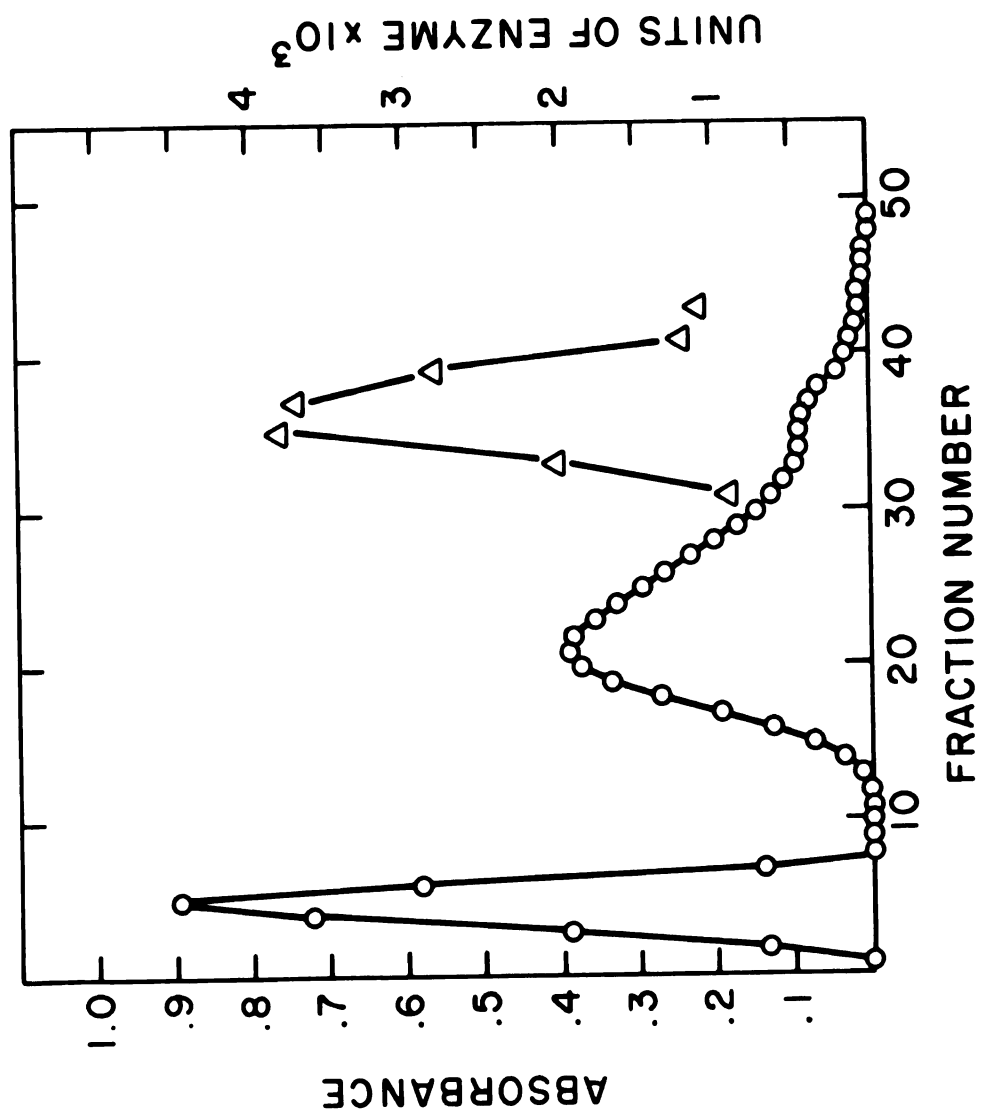


FIGURE 1

FIGURE 2. Gel scan of the purified cyclase in a 7% analytical polyacrylamide gel taken at a wavelength of 600 nm. For details, see the Experimental Section. Thirty-six μ g protein (specific activity = 1.80) were applied to the gel.

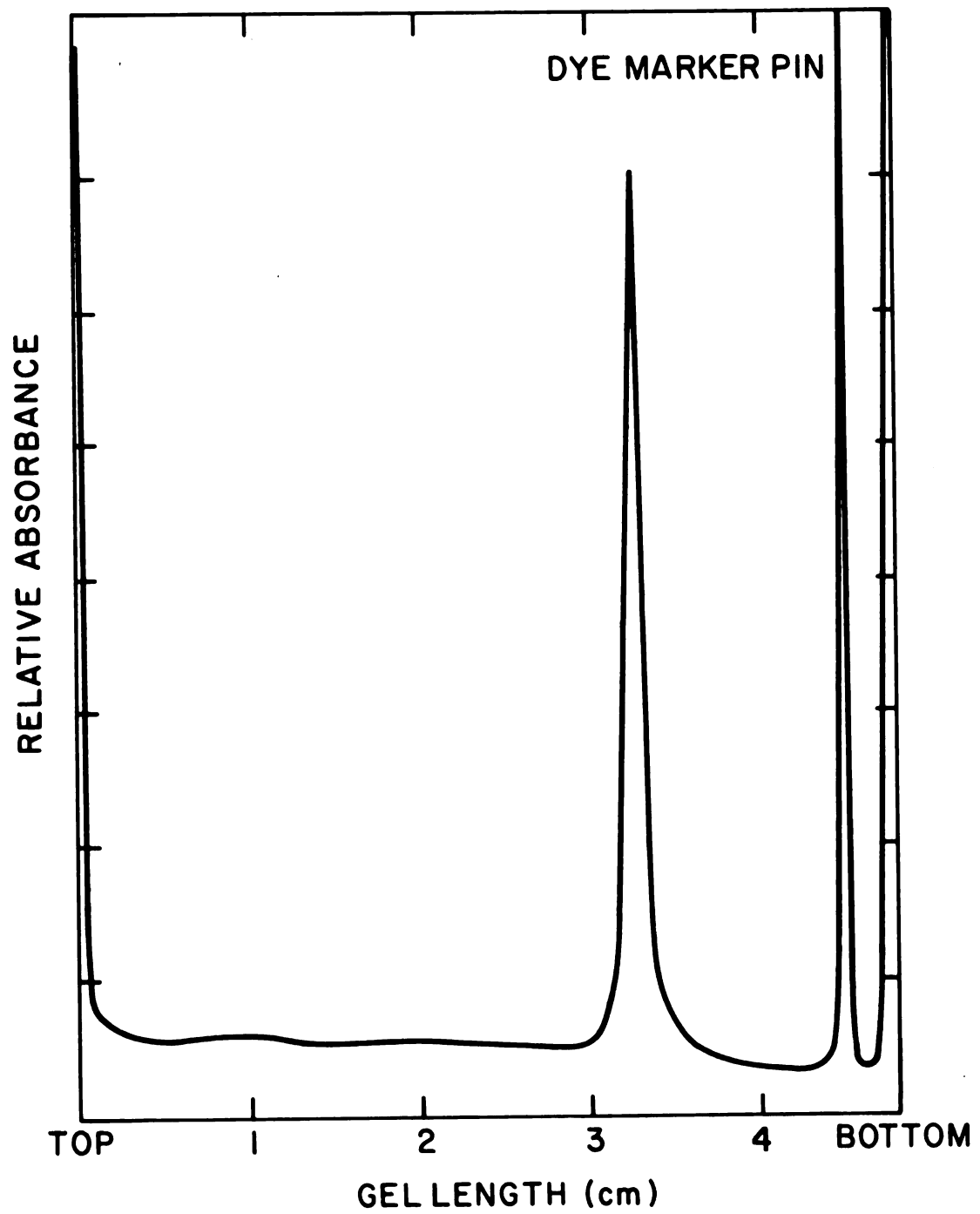


FIGURE 2

FIGURE 3. Plot of fringe displacement $(3 + \log \Delta Y)$ vs. the square of the distance from the center of rotation (radius²). See the Experimental Section for detailed procedures. The cell used for these data contained 0.45 mg purified cyclase.

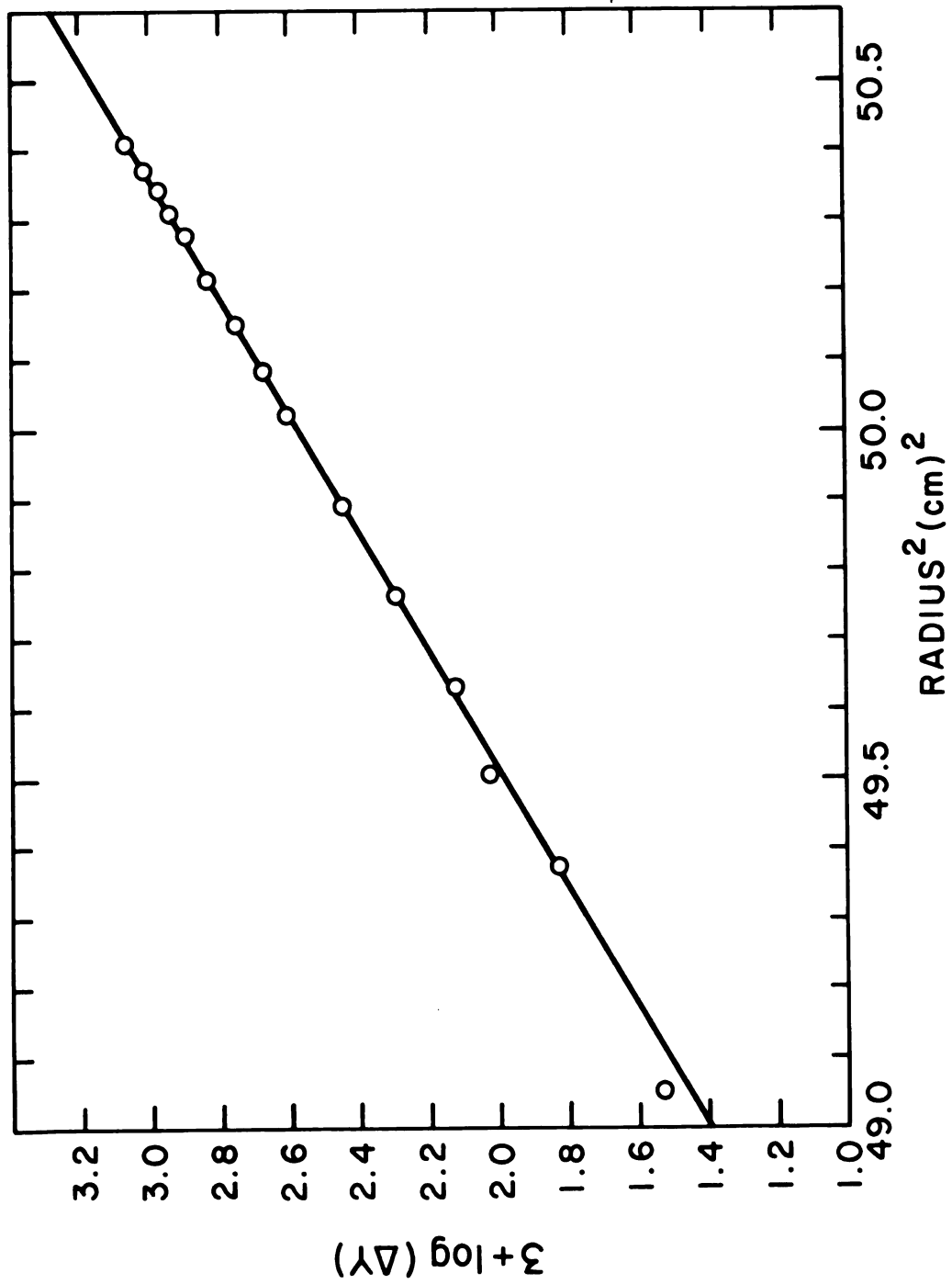


FIGURE 3



FIGURE 4. UV scan of homogeneous cyclase. The procedure is outlined in the Experimental Section. Protein concentration was 1.0 mg/ml (specific activity: 1.80 before dialysis; 0.624 after dialysis).

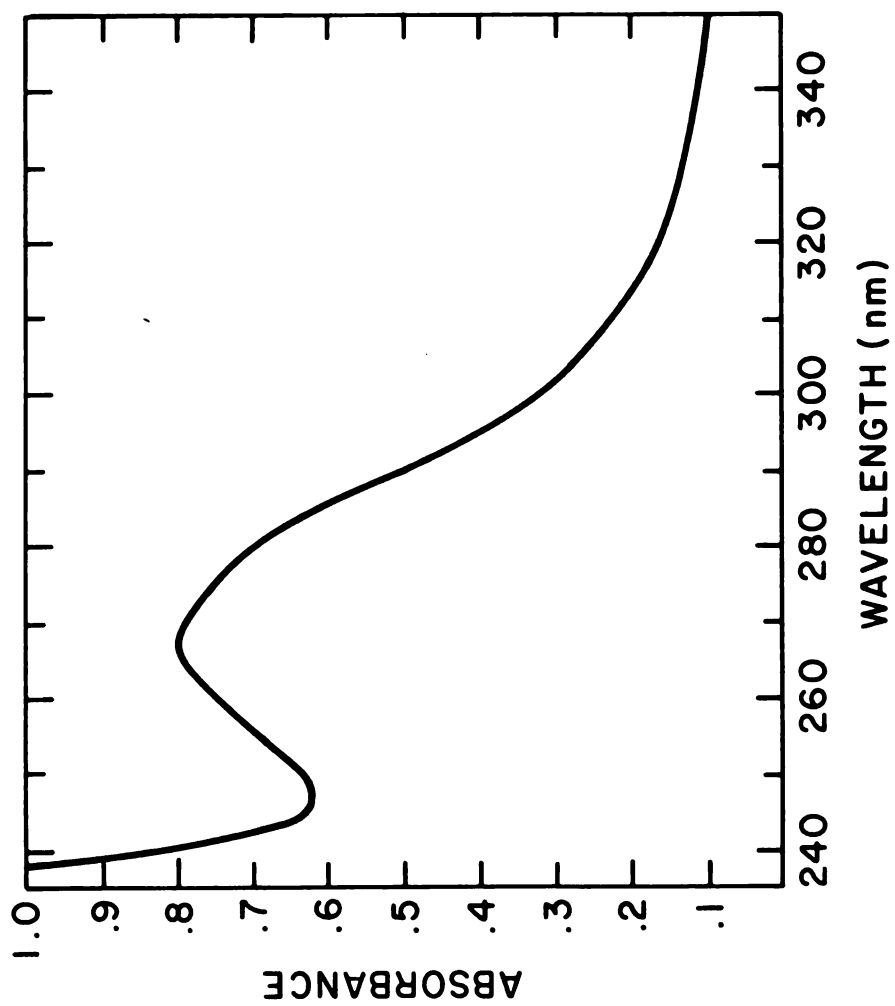


FIGURE 4

ARTICLE 2

REMOVAL OF THE ALPHA-AMINO GROUP DURING THE
CONVERSION OF ORNITHINE TO PROLINE*

By

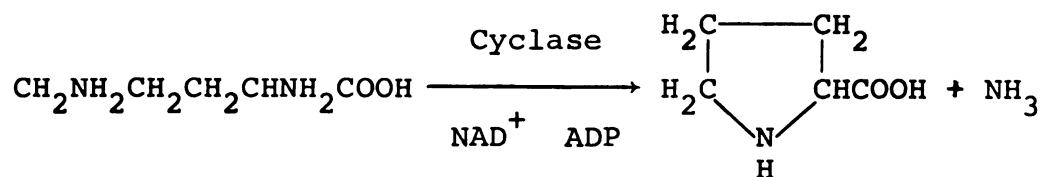
W. L. Muth[†] and R. N. Costilow

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FOOTNOTES

- * From the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823. Received _____. This investigation has been supported by Research Grant No. _____ from the National Institutes of Health. Journal Article No. _____ from the Michigan Agricultural Experiment Station.
- † Graduate Fellow under Title IV, National Defense Education Act. This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
- ¹ Abbreviations used in this paper include: MET, mercaptoethanol; 2-PCA, Δ^1 -pyrroline-2-carboxylic acid; and TMS, trimethylsilyl.

Ornithine cyclase catalyzes the transformation of ornithine to proline in a unique enzymatic reaction (Costilow and Laycock, 1971) and is outlined as follows:



The cyclase, which is a single protein, appears to catalyze a balanced oxidation-reduction reaction. It is stimulated by catalytic amounts of NAD^+ and the pure enzyme contains approximately 1 mole of bound NAD^+ per mole of enzyme (Muth and Costilow, submitted for publication). Some stimulation of the cyclase was apparent with ADP or ATP, though no evidence of allosterism was observed and the apparent K_m for ornithine was unchanged by the addition of ADP. The enzyme contains 4 labile sulfhydryl groups and is sensitive to oxygen. No other cofactors have been identified.

The present study was conducted to determine which amino group is removed from ornithine during the cyclase reaction. An attempt was also made to incorporate tritium from NADT into proline during the conversion of ornithine to proline.

EXPERIMENTAL SECTION

Synthesis of [δ - ^{15}N]-Ornithine. [δ - ^{15}N]-Ornithine was synthesized from α -aminoadipic acid and potassium azide- ^{15}N in a Schmidt degradation reaction by a procedure adapted from two sources (Adamson, 1939; Wolff, 1946). Nine ml anhydrous CHCl_3 , 3 ml concentrated H_2SO_4 , and 1.5 mmoles (241 mg) α -aminoadipic acid were added to an acid-cleaned 16 x 150 mm tube containing a small stirring bar. The reaction was started by slowly adding 1.5 mmoles (98 mg) KNN^{15}N (96.4 atom % enriched, Mallinkrodt Nuclear, St. Louis, Mo.) to the stirred mixture maintained at 43-45°. The reaction proceeded until gas evolution had ceased (5 hr). The sulfuric acid containing the newly synthesized amino acid was diluted in 297 ml cold distilled H_2O and was then applied to a small (0.9 x 6.0 cm) column containing Dowex-50 H^+ -form resin (400 mesh). The unreacted α -aminoadipic acid was eluted from the column with 1N HCl and the newly synthesized ornithine was then eluted with 3N HCl . The fractions containing ornithine were pooled and dried under a vacuum at 45° in a rotary evaporator. The amino acid was dissolved in water and dried several times to remove excess HCl . The sample was taken up in

2 ml of distilled H₂O and compared with an authentic ornithine standard by (a) ionophoresis in 0.125N sodium acetate (pH 5.2), 0.5N acetic acid (pH 2.6), and 0.2M formate (pH 2.0); (b) descending chromatography on Whatman 3 mm paper developed in butanol: acetic acid: water (120:30:50); and (c) by ascending chromatography on thin layer silica gel developed with chloroform: methanol: 15% ammonium hydroxide (36:46:20). The relative migrations of the synthesized product and the standard were identical in all cases. Assay of the product by the reduced ninhydrin method of Moore and Stein (1954) indicated that the yield was 250 μ moles or 16.6%. A small ninhydrin-positive impurity was present, but was neither identified nor removed. Samples of the synthesized product and the standard were examined in the combined gas chromatograph-mass spectrograph after preparing trimethylsilyl derivatives of each.

Conversion of Ornithine to Proline. Two reactions were carried out in this experiment, one with [δ -¹⁵N]-labelled and the other with unlabelled ornithine. Both reactions were carried out in 6 x 50 mm tubes inserted in 13 x 100 mm stoppered tubes which had been flushed with argon. The reaction mixtures were set up in a manner similar to the radiochemical assay previously described (Costilow and Laycock, 1971). Each reaction tube contained: 0.015 ml

each of 0.25M Tris chloride buffer (pH 8.0), 0.1M MET¹, 10⁻³M NAD⁺, and 6.6 x 10⁻²M CuCl₂. The Cu⁺⁺ was added to inhibit the reduction of some of the proline formed to δ-aminovaleric acid by the enzyme preparation used (Costilow and Laycock, 1968). Each reaction also received 0.075 ml (0.2 mg protein) of an ammonium sulfate-treated and dialyzed extract of Clostridium sporogenes cells (Muth and Costilow, submitted for publication). The reactions were started by adding 0.035 ml of either 0.2M [δ-¹⁵N]-labelled or unlabelled ornithine to individual reaction mixtures. The tubes were incubated at 37° for 2 hr and the reactions stopped by placing each tube in an 80° water bath for 5 min. A radiochemical assay (Costilow and Laycock, 1971) run concurrently indicated that about 3.3 μmoles of proline were formed by the enzyme.

Some of the NH₃ liberated by the cyclase reaction was trapped in glutamate by the use of glutamate dehydrogenase. The following reagents for this reaction were added to each of the two tubes: 0.030 ml each of 1.5M Tris chloride buffer (pH 8.2), 5.0 x 10⁻²M α-ketoglutarate, 2.0 x 10⁻² ADP, and 4.0 x 10⁻²M NADH. The reaction was started by adding 8.5 units (0.010 ml) of glutamate dehydrogenase. The tubes were incubated at 30° for 1 hr. Again, the protein was precipitated by heating each tube to 80° for 5 min and the tubes were centrifuged at 3,000 RPM for

15 min in a clinical centrifuge to remove the denatured protein. The supernatant solutions were removed and the pellets washed by resuspending in 0.3 ml distilled H_2O . After centrifuging again, the supernatant solutions from the first and second centrifugations were mixed. The entire solution from each reaction was applied to a small (0.9 x 6.0 cm) column containing Dowex-50 H^+ -form resin (400 mesh) and washed with 12 ml H_2O . The amino acids were eluted with 10 ml 1M NH_4OH . The eluate from each column was dried under reduced pressure at 45° in a rotary evaporator. The dried amino acids were dissolved in 0.1 ml distilled water.

Preparation of Trimethylsilyl Derivatives. Samples containing 100 μg each of [δ - ^{15}N]-labelled or unlabelled ornithine and samples of the resuspended enzymatic products estimated to contain approximately 100 μg of proline and 35 μg of glutamate were dried at 50° in small (6 x 50 mm) tubes inside 13 x 100 mm screw-capped tubes. After drying, the sample tubes were maintained under dry nitrogen or argon. The dried samples were then solublized in 0.050 ml of acetonitrile and 0.050 ml bis-(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlorosilane (Andrews et al., 1971; Gehrke, 1971). Each sample was heated to 77° for at least 30 min to facilitate the trimethylsilylation reaction.

Isotope Analysis. Aliquots (0.002-0.004 ml) of the trimethylsilyl derivatives were analyzed in an LKB-9000 combined gas chromatograph-mass spectrograph equipped with a 3.0 mm x 1.83 m silanized glass column packed with 1% (w/v) SE-30 on silanized Supelcoport (100-120 mesh, Supelco, Inc.). The column temperature was maintained at 100° until proline was eluted and then the temperature was increased at a rate of 7.3°/min to 140° to facilitate the elution of glutamate. Peaks containing ornithine derivatives eluted at 140° and at 160°. The helium carrier gas flow rate was 25 cc/min. The ion source temperature of the mass spectrograph was 290°, and the ionizing voltage was 70 eV. All determinations of intensities of the various ions were made from normalized bargraphs (Sweeley et al., 1970). Isotope incorporation was measured by determining the ratio $I_{p+1}/I_p + I_{p+1}$, where I_p is the intensity of the ion at $m/e = p$ and I_{p+1} is the intensity of the ion at $m/e = p+1$.

Chemical Reduction of NAD^+ in T_2O . The synthesis of NADT labelled with tritium in the 4-position of the pyridine ring was similar to the procedures used by Ohlmeyer (1938) and Fisher et al. (1953). One hundred mg NAD^+ (89% pure) were dissolved in 2 ml of tritiated water (total activity 250 mCi) contained in an acid-cleaned 16 x 150 mm stoppered

tube flushed with argon. Fifty mg of sodium hydrosulfite (dithionite) and 104 mg NaHCO_3 were then added and dissolved as a mixture. The tube was flushed with argon again and incubated at 30° for 3-5 hr. The reduced NAD^+ was crystallized by addition of 25 ml of absolute ethanol at -20° . A second crop of crystals was obtained by adding 11 ml of absolute ethanol to the supernatant solution. The crystals of NADT were dissolved in a small volume of H_2O and recrystallized to remove excess tritium from the compound. To remove any exchangeable tritium left, the recrystallized NADT was dissolved in 8 ml of H_2O and concentrated in a Diaflo ultrafilter using a UM-2 membrane. The concentrated compound was diluted to 10 ml and concentrated again to a volume of 2.0 ml. A small sample was counted for tritium content, and an additional sample was assayed for reduced NAD^+ content by measuring the absorbance of a diluted sample at 340 nm. The yield was 49% or 70 μmoles NADT with 15,000 cpm/ μmole . Approximately 30% of the reduced compound carried the tritium label in the A form and 70% in the B form (Colowick and Kaplan, 1957).

Production of Δ^1 -Pyrroline-2-carboxylic Acid. Δ^1 -Pyrroline-2-carboxylate was produced by the oxidation of D-proline by D-amino acid oxidase in the presence of excess catalase in a Warburg respirometer (Strecker, 1960; Costilow and Laycock, 1969). The protein was removed by treatment of the reaction

with 5% trichloroacetic acid followed by extraction of the trichloroacetic acid with ether. Ether solublized in the water was removed by bubbling argon through the solution. Oxygen consumption data indicated that approximately 300 μ moles of 2-PCA were formed from 500 μ moles of D-proline. The yield was 60%.

Incorporation of NADT into Proline. Reactions to determine the extent of incorporation of tritium from NADT into proline by the enzyme in the presence of ornithine alone or with 2-PCA were similar to the standard radiochemical assay for the cyclase (Costilow and Laycock, 1971). Each reaction contained: 19.2 mM Tris chloride (pH 8.0), 7.7 mM mercapto-ethanol, 1.5 mM ADP, and 0.025 ml pure cyclase (specific activity 0.86) prepared as described by Muth and Costilow (submitted for publication). Where indicated, 2 μ moles of ornithine, 1.2 μ moles 2-PCA, 5 nmoles NAD^+ , and 0.35 μ moles NADT were added alone or in various combinations. The total volume of each reaction was adjusted to 0.065 ml with distilled water. Each reaction was incubated for 2 hr at 37°. Reactions were stopped and protein precipitated by addition of 0.025 ml of 0.5M formate. An aliquot (0.040 ml) of each reaction mixture was spotted on Whatman 3 mm paper and developed in descending fashion with butanol: acetic acid: water (120:30:50). The chromatogram was dried and sprayed

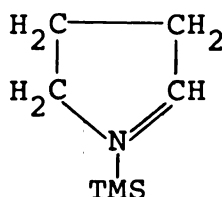
with 0.01% ninhydrin. The proline spots were cut out and the radioactivity determined in a liquid scintillation spectrometer.

RESULTS

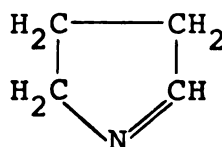
Analysis of [δ - ^{15}N]-Ornithine. Gas chromatographic analyses of TMS derivatives prepared from either [δ - ^{15}N]-labelled or unlabelled ornithine each yielded two peaks. Mass scans taken as each peak eluted from the column were consistent with a tri-TMS and a tetra-TMS derivative of ornithine corresponding to peaks B and D of Figure 1, respectively. Analysis of the parent ion and four other prominent peaks obtained from the tetra-TMS-ornithine peak indicated the labelled compound was 28.0 atom % enriched for ^{15}N . The method of synthesis insured that all label was directed to the δ -position.

Incorporation of ^{15}N into the Products of the Ornithine Cyclase Reaction. TMS derivatives of proline and glutamate formed from either labelled or unlabelled ornithine were readily separated from each other and from residual ornithine and several other compounds present in the derivatized sample by gas chromatography (Figure 1). Four ion peaks from the mass spectrum of proline were examined (Table I). The parent ion [$(\text{TMS})_2$ -proline (M)], was not included since it was either of very low intensity or absent in every mass

spectrum taken. The largest molecular ion consistently present was that at m/e 244 (Figure 2) which corresponds to M-15 (the loss of one methyl group). Other prominent ions used were those at m/e 216, 142, and 70. Each of these contain the nitrogen from the proline ring. The ion at m/e 216 may be regarded as TMS-N=CH-COO-TMS. The base peak at m/e 142 (M-117) represents one of the expected products of α -fission (Bergström, 1970):



The final ion considered is that at m/e 70 which may be regarded simply as the pyrroline ring from proline:



It is evident from a comparison of the mass spectra (Figures 2A and 2B) and the ion ratio intensities (Table I) of proline produced from unlabelled and from [δ -¹⁵N]-ornithine that the ¹⁵N and thus the δ -NH₃ group of ornithine is conserved in proline. The calculated atom-percent ¹⁵N in the proline formed was the same as that determined in the

$[\delta\text{-}^{15}\text{N}]$ -ornithine used as the substrate for the cyclase reaction.

Four ion peaks from the mass spectrum of glutamate were also examined (Table II and Figure 3). A small but consistent parent ion of m/e 363 was present which suggested a tri-TMS derivative. The readily identifiable second ion of m/e 348 (M-15) results from the loss of one methyl group from the parent ion. The third useful ion was located at m/e 246 (M-117) and corresponded to an α -fission product: $\text{di-TMS-(OOC-CH}_2\text{CH}_2\text{CHNH)}$. The last molecular ion examined was m/e 218 (M-145). The ion was regarded as a β -fission product containing both the amino group and the α -carboxyl group ($\text{TMS-}^+\text{NH=CHCOOTMS}$). Thus, all four ions contained the NH_3 group of glutamate which was generated from the ammonia liberated during the conversion of ornithine to proline by the cyclase. Comparison of the mass spectra (Figure 3A and 3B) and of the ion intensities (Table II) of glutamate from reaction mixtures in which unlabelled and $[\delta\text{-}^{15}\text{N}]$ -ornithine were substrates for the cyclase reaction show that there was no significant enrichment of ^{15}N . Therefore, the $\alpha\text{-NH}_3$ group of glutamate must be derived from the $\alpha\text{-NH}_3$ group released from ornithine by the cyclase.

Incorporation of NADT into Proline. There was no apparent incorporation of tritium from NADT into proline during the

cyclase reaction (Table III). It also appeared that the enzyme failed to incorporate tritium from NADT into proline when 2-PCA was added. Based on the control reaction, the maximum radioactivity which could have been observed in the samples examined would have been about 450 cpm above background if the A form of NADT was used and about 1000 cpm above background if the B form was used. Incorporation of very low percentages of either form could have occurred without detection in this experiment.

DISCUSSION

Previous data (Costilow and Laycock, 1969; 1971) indicated that the conversion of ornithine to proline by ornithine cyclase might proceed in one of two possible directions (Figure 4). The direction taken would obviously depend on which amino group was involved in the deamination. Loss of the δ -amino group would lead to the formation of glutamic- γ -semialdehyde which would undergo ring closure to form Δ^1 -pyrroline-5-carboxylic acid (5-PCA). Deamination in the α -position would form 2-oxo-amino-pentanoic acid and 2-PCA. The conservation of ^{15}N from [δ - ^{15}N]-ornithine in proline during the conversion of ornithine to proline by the cyclase enzyme establishes with certainty that it is the α -amino group of ornithine which is removed. Thus, it is highly probable that the latter compounds are intermediates in the reaction.

The failure of our efforts to incorporate tritium from NADT into the proline formed during the reaction was not unexpected. Some of the sugar epimerases which contain tightly bound NAD^+ and probably operate by an oxidation and reduction mechanism also fail to exchange tritium from reduced NAD^+ or from solvent during substrate catalysis

(Maxwell, 1957; Glaser, 1972). Two explanations of these results are evident. First, the enzyme-NADH-intermediate complex may be of such short duration or of such an undissociable nature that exchange simply cannot take place. Such is the case with UDP-D-glucose 4'-epimerase (Bertland and Kalckar, 1968). In the case of the cyclase, this possibility is reinforced by the fact that numerous attempts to trap an intermediate of a pyrroline nature with o-amino-benzaldehyde were unsuccessful (Costilow and Laycock, 1968). The second explanation involves isotope selection. Using UDP-D-glucose 4'-epimerase with UDP-D-glucose-4-T as substrate Beville et al. (1965) noted a large negative isotope effect. Thus, it is possible that NADT fails to exchange with enzyme bound NADH simply because of total isotope selection.

The possible role of NAD^+ in the cyclase reaction (see Figure 4) is still unresolved. Data with partially purified enzymes (Costilow and Laycock, 1969; 1971) show that the conversion of ornithine to proline is almost completely dependent on added NAD^+ . However, enzyme preparations of high purity (Costilow and Laycock, 1971; Muth and Costilow, unpublished data) were only stimulated by added NAD^+ . There is evidence that this cofactor is tightly bound to the pure enzyme.

The fact that no tritium from the medium was incorporated into proline when the reaction was carried out in

tritiated water (Costilow and Laycock, 1971) is consistent with an enzyme-bound oxidation-reduction mechanism. All known dehydrogenases add or remove hydrogens from non-exchangeable positions, while the other involved hydrogen is in a freely exchangeable position on the substrate molecule (Vennesland and Westheimer, 1954). The nucleotide-sugar epimerase also has a postulated internal oxidation-reduction mechanism and fails to exchange tritium from T_2O into the product (Kalckar and Maxwell, 1956).

Further elucidation of the mechanism of this unique enzyme may be forthcoming from experiments currently being planned.

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TABLE I. Incorporation of ^{15}N into Proline.^a

m/e	Ratios of Intensities (%)		^{15}N Content (atom %)
	$^{14}\text{N}^b [I_{p+1}/(I_p + I_{p+1})]$	$^{15}\text{N}^c [I_{p+1}/(I_p + I_{p+1})]$	
70/71	12.0 ± 6.8^d	43.5 ± 3.1	31.5
142/143	10.9^e	40.2^e	29.3
216/217	18.0 ± 0.63	46.5 ± 2.9	28.5
244/245	17.1 ± 0.6	42.2 ± 6.8	25.1
			Ave. = 28.6 ± 2.6

^aFor conditions of each reaction and purification scheme, see Experimental

Section. ^bProline produced from unlabelled ornithine by partially purified cyclase.

^cProline produced from $[\delta\text{-}^{15}\text{N}]$ -ornithine by partially purified cyclase. ^dThe error

quoted is the standard deviation of three determinations. ^eOnly a single value

could be determined from these data.

TABLE II. Incorporation of ^{15}N into Glutamate.^a

m/e	Ratios of Intensities (%)		^{15}N Content (atom %)
	$^{14}\text{N}^b [I_{p+1}/(I_p + I_{p+1})]$	$^{15}\text{N}^c [I_{p+1}/(I_p + I_{p+1})]$	
218/219	19.9 ± 3.5^d	26.0 ± 2.7	+6.1
246/247	21.2 ± 8.2	20.0 ± 7.0	-1.1
348/349	21.0 ± 0.9	21.7 ± 1.1	+0.7
363/364	20.1 ± 9.5	18.7 ± 3.4	-1.4
			Ave = $+1.1 \pm 3.9$

^aFor conditions used in each reaction and purification scheme, see Experimental Section. ^bGlutamate generated with the NH_3 released from unlabelled ornithine by partially purified cyclase. ^cGlutamate generated with the NH_3 released from [δ - ^{15}N]-ornithine by partially purified cyclase. ^dThe error quoted is the standard deviation of three determinations.

TABLE III. Incorporation of Tritium from NADT into Proline with Ornithine Alone or with Δ^1 -Pyrroline-2-carboxylic Acid (2-PCA) as Substrates.^a

	Addition (μ moles)				Proline (c.p.m.)
	Ornithine	2-PCA	NAD ⁺	Cyclase	
1.	2.0	-	-	-	320
2.	-	1.2	-	+	316
3.	-	-	-	+	327
4.	-	1.2	0.005	+	301
5.	1.0	1.2	-	+	293

^aEach reaction contained 0.005 ml each of 0.25 M Tris chloride (pH 8.0), 0.1 M mercaptoethanol, 0.02 M ADP, and 0.35 μ moles NADT (specific activity = 15,000 c.p.m./ μ mole). Cyclase (29.5 μ g, specific activity = 0.86) added to each reaction contained 0.5 μ moles ornithine and 2.5 nmoles NAD⁺. In a control reaction, 0.24 μ moles of proline were formed when 2.0 μ moles of ¹⁴C-ornithine and 0.35 μ moles NADH were added with the cyclase. For other aspects of procedure, consult the Experimental Section.

FIGURE 1. Gas chromatography of the trimethylsilylated amino acids separated from a reaction mixture containing unlabelled ornithine, proline, glutamate, and ornithine cyclase. The tracing is the total ion current detector response and the dotted line is the column temperature. For other components of the reaction mixture and isolation and preparation of samples, consult Experimental Section. Mass spectra indicated that peak A was $(\text{TMS})_2$ -proline, peak B was $(\text{TMS})_3$ -ornithine, peak C was $(\text{TMS})_3$ -glutamate, and peak D was $(\text{TMS})_4$ -ornithine.

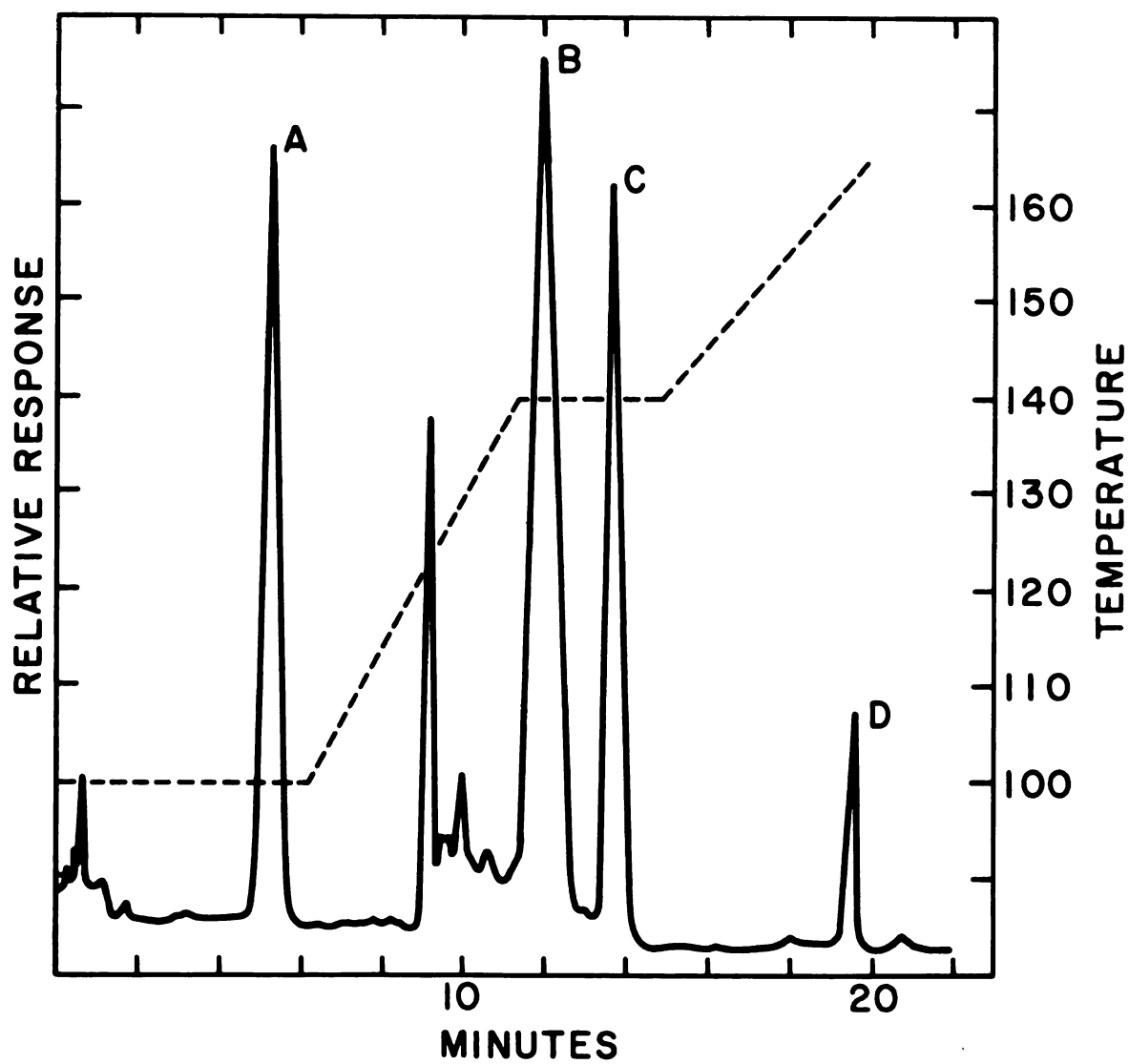


FIGURE I

FIGURE 2. Comparison of part of the mass spectrum of (TMS)₂-proline derived catalytically from unlabelled ornithine (a) with that of proline from [δ -¹⁵N]-ornithine (b).

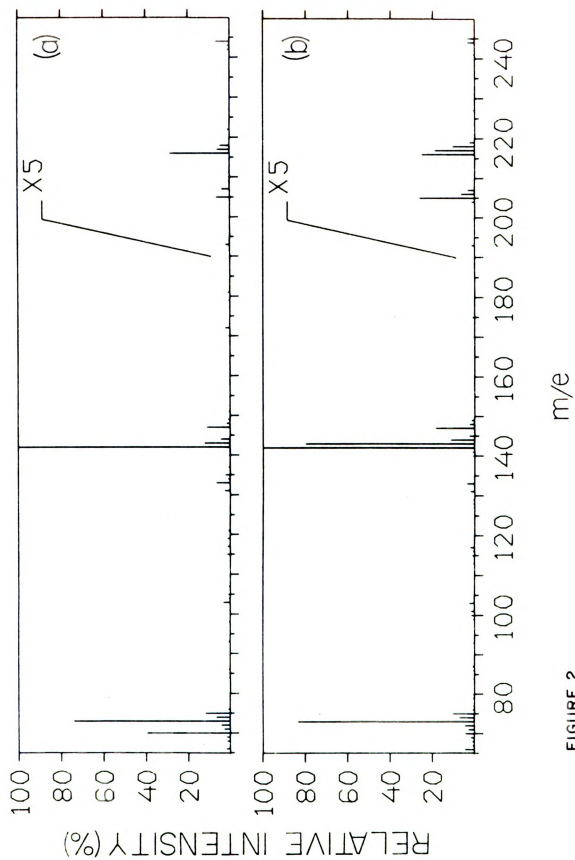


FIGURE 2

FIGURE 3. Comparison of part of the mass spectra of $(\text{TMS})_3$ glutamate derived catalytically from α -ketoglutarate and the ammonia generated from either unlabelled (a) or $[\delta\text{-}^{15}\text{N}]\text{-}$ (b) ornithine.

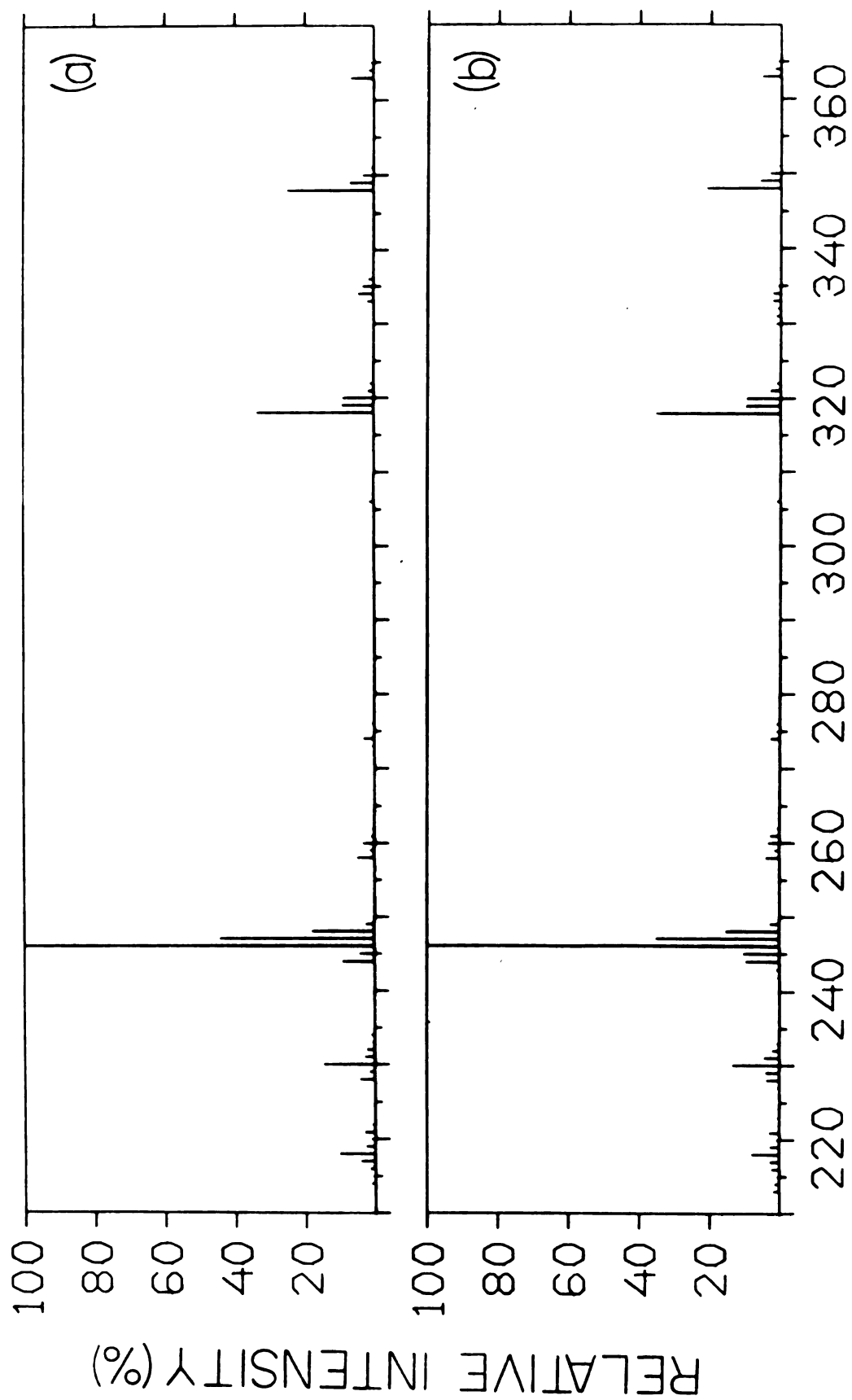
 m/e

FIGURE 3

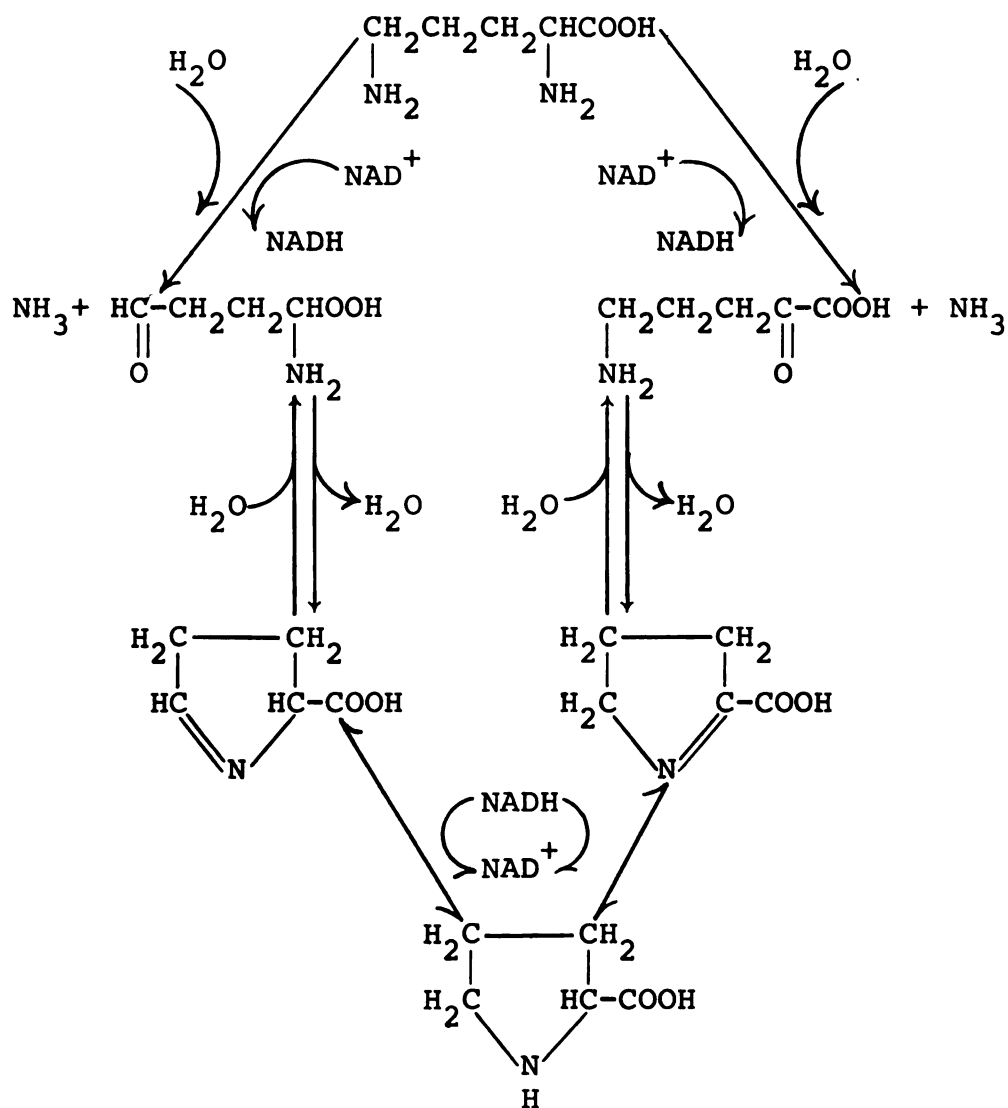


FIGURE 4. Proposed mechanism of action of ornithine cyclase.

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