PARTIAL PURIFICATION AND CHARACTERIZATION OF A UNIQUE PROLINE DEHYDROGENASE FROM CLOSTRIDIUM SPOROGENES

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
RONALD DALE COOPER
1976

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PARTIAL PURIFICATION AND CHARACTERIZATION OF A UNIQUE PROLINE DEHYDROGENASE FROM *CLOSTRIDIUM SPOROGENES*

By

Ronald Dale Cooper

A THESIS

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ABSTRACT

PARTIAL PURIFICATION AND CHARACTERIZATION
OF A UNIQUE PROLINE DEHYDROGENASE
FROM CLOSTRIDIUM SPOROGENES

By

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An NAD^+-dependent dehydrogenase catalyzing the conversion of L-proline to Δ^1-pyrroline 5-carboxylic acid (P5CA) was partially purified from Clostridium sporogenes. The enzyme is specific for L-proline and NAD^+. It is not an oxidase, and it is not affected by oxygen. Apparent K_m values measured at pH 8.0 for L-proline and NAD^+ were 33 mM and 1.2 mM, respectively. However, these may be erroneous due to the presence of an NADH-dependent P5CA reductase in the extracts. The optimum temperature for the dehydrogenase is approximately 50C, and the optimum assay pH is approximately 10. The conversion of L-proline to P5CA is strongly inhibited by low concentrations of L-glutamate. The presence of excess L-proline in the growth medium increases the levels of enzyme activity in cell extracts. Cells grown with D-glucose have lowered proline dehydrogenase activity. The partially purified proline dehydrogenase preparation contained a very low activity of glutamate dehydrogenase, which tended to copurify with the proline dehydrogenase. Also, the P5CA reductase mentioned

above copurified with proline dehydrogenase. The activity of this enzyme is not affected by L-glutamate but is inhibited by L-proline at high concentrations. Studies with labeled proline and ornithine indicate that the proline dehydrogenase may function in the conversion of proline to glutamate in C. sporogenes.

DEDICATION

I dedicate this thesis to my wife, Mary, for all her love, patience, and understanding during the completion of my graduate education at Michigan State University.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my major professor, Dr. R. N. Costilow, for his guidance and encouragement throughout the course of this investigation and the preparation of this thesis.

I would also like to express my sincere thanks to Dr. H. L. Sadoff and Dr. R. R. Brubaker for the use of their laboratory facilities and to Ms. Barbara Goelling for her technical assistance.

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INTRODUCTION

During studies of the conversion of ornithine to proline by Clostridium botulinum and Clostridium sporogenes, Costilow and Laycock (10) discovered an enzyme that appeared to catalyze the interconversion of Δ^1-pyrroline 5-carboxylic acid (P5CA) and proline. Both NAD^+-dependent proline dehydrogenase and NADH-dependent P5CA reductase activities were demonstrated. The overall equilibrium of the interconversion was far in the direction of proline. Further studies showed that the true intermediate between ornithine and proline was Δ^1-pyrroline 2-carboxylic acid rather than P5CA (23). Therefore, it was postulated that P5CA may be an intermediate in the interconversion of glutamate and proline in C. sporogenes as it is in many other microorganisms and animals.

The purpose of this investigation was to study the enzyme described above, which will be referred to by the trivial name of proline dehydrogenase. Experiments were designed to: (a) determine the physiological role of the enzyme; (b) partially purify and determine optimal conditions for dehydrogenase activity; and (c) study the inhibition of the enzyme by glutamate.

LITERATURE REVIEW

The Interconversion of Proline and Glutamic Acid

The metabolic relationship between glutamic acid and proline has long been an area of interest. As early as 1912, Abderhalden observed that an alcohol-extracted casein hydrolysate, presumably free of proline and rich in glutamate, permitted the normal growth of dogs (38). Somewhat later, Womack and Rose (50) recovered ^{14}C-proline from rats fed with ^{14}C-glutamic acid, indicating a possible metabolic pathway between these two amino acids.

The intermediates in the pathway of the conversion of proline to glutamic acid were first worked out in mammalian systems. In 1944, Stetten and Schoenheimer (36) isolated significant amounts of ^{15}N-glutamate from rats fed with ^{15}N-proline. Other evidence from their work indicated that proline was oxidized to a pyrroline carboxylic acid which was presumably futher oxidized to glutamate. Vogel and Davis (47) subsequently showed that the product of oxidized proline was most likely Δ^1-pyrroline 5-carboxylic acid (P5CA), which was in spontaneous equilibrium with glutamic γ-semialdehyde (GSA), as determined *in vitro* with chemically synthesized P5CA. Strecker (41), a major contributor to the study of the interconversion of glutamate and proline, showed that with partially purified rat liver and calf liver enzymes and pure chemically synthesized P5CA, the enzymatic conversion of proline

to glutamate proceeds via two distinct one-way steps: (a) the oxygen-dependent oxidation of proline, catalyzed by a "proline oxidase," and (b) the spontaneous conversion of the P5CA formed to GSA and the subsequent oxidation of this compound to glutamate, catalyzed by an $NAD(P)^+$-dependent "P5CA dehydrogenase." This pathway of proline catabolism may be a universal one in animals, having already been identified in guinea pig kidney, rabbit liver and kidney, human liver, brain and kidney, and rat brain (42). There is no evidence as yet for any other pathway of proline catabolism in animals.

The biosynthesis of proline in animals has been shown to proceed from L-glutamate through the same intermediates (P5CA ⇌ GSA), using a distinctly different system of enzymes catalyzing essentially irreversible reactions. One enzyme (not yet isolated) catalyzes the conversion of L-glutamate to GSA, and another enzyme, an NAD(P)H-dependent P5CA reductase, reduces P5CA to proline (25).

In microorganisms, most of the early work on the interconversion of proline and glutamate was concerned with the biosynthesis of proline from glutamate in Escherichia coli (44) and Neurospora (6). Vogel and Davis (47) first demonstrated that the pathway from glutamate proceeds via the same intermediates as those in animal systems by showing that chemically synthesized GSA and/or P5CA could satisfy the proline requirements of E. coli proline auxotrophs. The present accepted pathway of proline biosynthesis in E. coli includes

an as yet unisolated phosphorylated intermediate between glutamate and GSA, as proposed by Baich (2).

The catabolism of proline in microorganisms has been studied extensively in recent years. Early work by Bernheim (5) and Stone and Hoberman (39) showed that L-proline could be utilized as a single carbon source in E. coli in the presence of oxygen. Frank and Rybicki (15) later demonstrated that the degradation of L-proline to glutamate in E. coli proceeds, as is the case in animals, via the same intermediates as those for the biosynthesis of L-proline from glutamate (P5CA ⇌ GSA). In a follow-up study, Frank and Ranhand (16) showed that the enzymes involved in L-proline catabolism were not reversible by demonstrating that mutant strains of E. coli B4, blocked in the enzymes for proline biosynthesis from L-glutamate, could still convert L-proline to glutamate. This system of separate enzymes for the biosynthesis and catabolism of L-proline is comparable to the mechanism of interconversion of proline and glutamate in animals.

Little information is available concerning the metabolic relationship of proline and glutamate in plants. Very recently, a NAD^+-dependent proline dehydrogenase and a NAD(P)H-dependent P5CA reductase have been described in Chlorella pyrenoidosa (21) and in cotyledons from the pumpkin, Cucurbita moschata (31). The proline dehydrogenase described in these organisms is not an oxidase and can function normally in the absence of oxygen, unlike the proline oxidizing enzymes described thus far in animals and microorganisms. Also,

some evidence is presented by Rena and Splittstoesser (32) that proline dehydrogenase and P5CA reductase activity may occur on the same protein molecule in pumpkins.

The only other NAD^+-dependent proline dehydrogenase reported in the literature is the one found in Clostridium sporogenes by Costilow and Laycock (10). Further investigation of this enzyme is the subject of this thesis.

Regulation of Proline-Glutamic Acid Interconversion in Microorganisms

Since most of the work concerning the investigation of the regulation of proline-glutamic acid interconversion has been done with bacteria and yeast, only these systems will be discussed.

Synthesis of L-proline from L-glutamate in E. coli has been shown to be regulated by feedback inhibition. Baich (2) and Baich and Pierson (3) demonstrated that L-proline inhibits as well as represses the conversion of L-glutamate to GSA. The reduction of P5CA to L-proline is neither inhibited nor repressed in this organism, since the bacterium will excrete L-proline when supplied with exogenous P5CA (3). Berg and Rossi (4) showed that E. coli will also excrete L-proline when the conversion of L-glutamate to GSA is blocked and P5CA is formed by an alternate route (from N-acetylglutamate). Kuo and Stocker (17) obtained the same results with Salmonella typhimurium.

The inducibility by L-proline (and not P5CA) of both

enzymes of the catabolic pathway from L-proline to glutamate has been demonstrated in a number of bacteria, all growing aerobically (12, 15, 24). In all of these instances, catabolite repression of proline oxidase and P5CA dehydrogenase by D-glucose was also observed. Prival, Brenchley, and Magasanik (7, 27, 28) presented evidence that in Klebsiella aerogenes, proline oxidase is repressed by glucose only when the supply of nitrogen is abundant. In the presence of limiting nitrogen, glucose does not repress the enzyme, presumably due to increased activities of glutamine synthetase under these conditions. Adenosine 3',5'-monophosphate (cyclic AMP) does not appear to play a role in this relief of catabolite repression.

Laishley and Bernlohr (18) reported that the enzymes involved in arginine breakdown (arginase, ornithine δ-transaminase, and P5CA dehydrogenase) could be coincidentally induced by arginine or ornithine and could be repressed by glucose in Bacillus licheniformis. Also, proline oxidase and P5CA dehydrogenase were induced by L-proline, and the dehydrogenase was found to be under catabolite repression control. They suggest that P5CA could be a common in vivo inducer of arginine and proline catabolism in this organism.

Lundgren and Ogur (20) showed that a Saccharomyces glutamate auxotroph blocked in the tricarboxylic acid cycle possessed a catabolic pathway to glutamate from proline, arginine, and glutamine, and grew on any of these amino acids in a minimal medium. The mutant did not, however, grow on these amino acids in a medium containing a full complement of common

amino acids minus glutamate. Their investigation showed that the P5CA dehydrogenase (and not the proline oxidase) in this organism is significantly inhibited by a wide variety of amino acids, including glutamate. They suggested that there may be a non-specific regulatory mechanism in yeast in which the size of the amino acid pools controls glutamate formation from proline and arginine.

Relationship of Ornithine to Proline and Glutamic Acid Metabolism

The recognized biosynthetic and catabolic routes for the interconversions of ornithine, proline, and glutamate are shown in Fig. 1 (1, 29, 30, 33, 37). In E. coli, the major pathway of ornithine synthesis from glutamate proceeds via acetylated intermediates, while acetylation is not required in Neurospora crassa, Torulopis utilis, or animal tissues (46). Little information is available on the interconversion of these amino acids in plants.

The conversion of ornithine to glutamate γ-semialdehyde is catalyzed by ornithine δ-transaminase with pyridoxal phosphate required for the transamination. In E. coli, the equilibrium of this reaction is far in the direction of the semialdehyde, presumably due to the tendency of the semialdehyde to cyclize (46). The glutamic γ-semialdehyde is normally oxidized to glutamate, but may be reduced to proline as an alternate method of synthesizing proline in E. coli (33, 46).

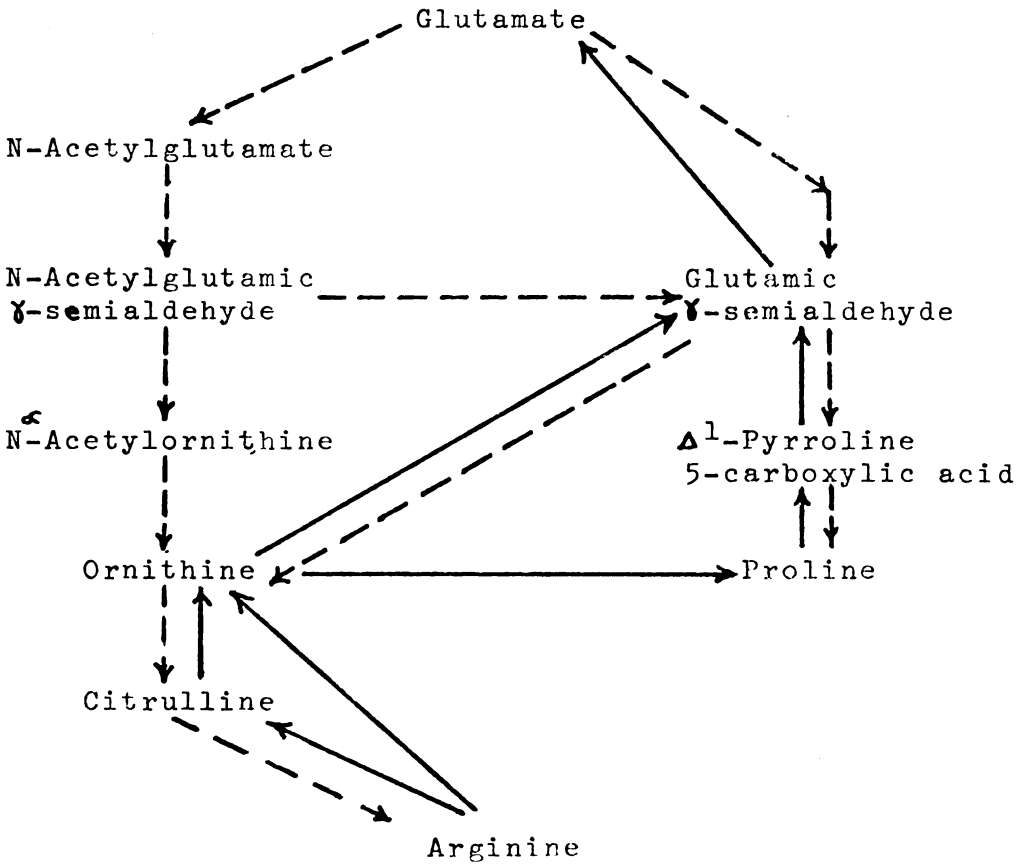

Fig. 1.--Interconversions of ornithine, proline, and glutamate in microorganisms and animals. Dashed arrows represent biosynthetic and solid arrows catabolic pathways.

Species of Clostridium catabolize ornithine by two pathways, both different from those described above. Dyer and Costilow (13, 14) and Tsuda and Friedman (45) have reported that Clostridium sticklandii oxidized L-ornithine as a single substrate to ammonia, alanine, acetate, and carbon dioxide by a coupled oxidation-reduction with proline as the electron acceptor. Costilow and Laycock (8, 9, 10) also showed that both C. botulinum and C. sporogenes converted L-ornithine to L-proline by a single protein, ornithine cyclase (deaminating). The intermediates in this pathway are α-keto-δ-aminovaleric acid in spontaneous equilibrium with Δ^1-pyrroline 2-carboxylic acid (23). They (10) also found significant levels of a presumably reversible enzyme that catalyzes the interconversion of P5CA and L-proline which was not involved in the conversion of ornithine to proline.

MATERIALS AND METHODS

Culture and Cultural Methods

The organism used in this study was a putrefactive anaerobe, Clostridium sporogenes (NCA Clostridium PA 3679, ATCC 7955).

Two basic kinds of media were used for the study of the proline dehydrogenase enzyme. The "standard trypticase" medium consisted of 4.0% trypticase, 2 ppm thiamine hydrochloride, and 0.05% sodium thioglycollate as a reducing agent. The other medium was a synthetic one containing salts, vitamins, and 12 amino acids adapted from the medium of Perkins and Tsuji (26). Both kinds of media were adjusted to pH 7.4 before autoclaving (15 psi for 20 min).

Growth cultures were routinely started by inoculating a stock suspension of spores (kept at 4C) into a 10 ml tube of standard trypticase medium. This suspension was heat shocked for 10 min in a 60C water bath and incubated in an anaerobic jar under a hydrogen atmosphere. Tube cultures prepared in this manner were used as 2% inocula into the appropriate media. All cultures were incubated between 15 and 18 h.

Enzyme Assays

Three assay procedures for proline dehydrogenase were used in this study. One procedure was essentially that described by Costilow and Laycock (10) which measures the amount of Δ^1-pyrroline 5-carboxylic acid (P5CA) formed from proline

during a 15 min incubation in the presence of o-aminobenzaldehyde. The colored reaction product, a P5CA-o-aminobenzaldehyde complex, is measured by absorbance at 443 nm, with a mM extinction coefficient of 2.71 mM^{-1} cm^{-1} (25). Previously reported data demonstrated that the proline dehydrogenase can readily be detected by trapping off the P5CA product by this method (10). Reaction mixtures of 0.5 ml total volume contained 25 mM Tris-HCl, pH 8.0, 0.3 M L-proline, 2 mM NAD^+, 20 mM sodium pyruvate, 0.01 mg rabbit lactic dehydrogenase (65 units/mg), and enzyme. The mixture without enzyme was saturated with o-aminobenzaldehyde and placed into 13 x 100 mm tubes. After equilibration for 5 min in a 4CC water bath, enzyme was added, and the mixture incubated for 15 min and stopped with 0.5 ml 10% trichloroacetic acid (TCA). The TCA-insoluble material was centrifuged out, and the absorbancy of the supernatant solution was read at 443 nm against pre-acidified controls.

In the second procedure, the formation of the colored reaction product of P5CA and o-aminobenzaldehyde was measured by monitoring the rate of increase in absorbancy at 443 nm. Reaction mixtures of 1.0 ml total volume contained 0.1 M Tris-HCl (saturated with o-aminobenzaldehyde), pH 8.0, 0.45 M L-proline, 10 mM NAD^+, and enzyme. The mixture without enzyme was equilibrated to 37C in a cuvette using a Haake constant temperature regulator. After addition of enzyme, the reaction was monitored immediately by measuring the increase in absorbancy at 443 nm with time.

The third proline dehydrogenase assay procedure was conducted by measuring the rate of proline-dependent reduction of NAD^+ as the increase in absorbancy at 340 nm with time. The reaction mixture for this assay was exactly the same as that for the second procedure (above), but without o-aminobenzaldehyde. Enzyme was added to start the reaction after 5 min equilibration at 37C, and the rate of absorbancy was monitored immediately. This third assay procedure was not used for the less purified enzyme fractions due to a significant amount of background reduction of NAD^+ with these preparations.

To assay for P5CA reductase, the P5CA-dependent oxidation of NADH was measured by monitoring the loss of absorbancy at 340 nm with time. Reaction mixtures of 1.0 ml total volume contained 0.1 M Tris-HCl, pH 8.0, 0.1 mM NADH, 1.3 mM P5CA, and enzyme. After 5 min equilibration at 37C in a cuvette, enzyme was added to start the reaction which was monitored immediately.

Glutamate dehydrogenase assays were conducted by measuring the α-ketoglutarate-dependent oxidation of NADH as the rate of loss of absorbancy at 340 nm by the procedure of Winnacker and Barker (49). A 1.0 ml reaction mixture contained 0.15 M Tris-HCl, pH 8.0, 0.18 M ammonium chloride, 0.1 mM NADH, 5 mM α-ketoglutarate, and enzyme. After 5 min equilibration in a cuvette at 37C, NADH was added to start the reaction.

For all of these enzyme assays, a unit of enzyme is

defined as that amount of enzyme necessary to convert 1 μmole of substrate to 1 μmole of product in one minute. And specific activity is defined as units of enzyme per mg of protein. For the reactions monitored at 340 nm, NADH was taken to have a millimolar extinction coefficient of 6.2 mM^{-1} cm^{-1}. All reactions were read on a Gilford multisample recording spectrophotometer. Protein concentrations were estimated by the method of Lowry, et. al. (19), using bovine serum albumin as a standard, or by reading absorbancy at 260 nm and 280 nm and using a nomograph.

Preparation of Δ^1-Pyrroline 5-carboxylic Acid

Two methods were used to prepare P5CA. In one method it was formed by the transamination of L-ornithine, using partially purified rat liver ornithine δ-transaminase according to the method of Strecker (40). Livers extracted from adult rats were partially homogenized in 0.25 M sucrose, washed in 0.05 M potassium phosphate, homogenized again in a Waring blendor, precipitated with ammonium sulfate, and dialyzed. To generate the P5CA, the reaction mixture adapted from Strecker contained 40 mM L-ornithine (L-[U-^{14}C]-ornithine when [U-^{14}C]-P5CA was needed), 8 mM α-ketoglutarate, pH 7.1, 50 mM potassium phosphate, pH 7.1, and the partially purified ornithine δ-transaminase, in a total volume of 10 ml. The reaction was run with shaking for 35 min at 37C and stopped with an equal volume of 10% TCA.

The P5CA prepared by this method was separated from

the other amino acids in the final reaction mixture by the method of Costilow and Laycock (9).

P5CA was also prepared by another method described by Strecker (42), using a rat liver mitochondrial preparation and cytochrome c with L-proline as substrate.

In Vitro Interconversion of Proline and Glutamate

To attempt to show the *in vitro* conversion of L-proline to glutamate, reaction mixtures of 0.6 ml total volume were combined in 13 x 100 mm tubes to contain 25 mM potassium phosphate, pH 7.5, 15 mM ATP, 1 mM magnesium chloride, 15 mM NAD^+, 130 mM L-[U-^{14}C]-proline (specific activity = 0.0032 µCi/µmole), and either whole cells or crude extract of *C. sporogenes* grown in a synthetic medium. Likewise, to attempt to show the *in vitro* conversion of L-glutamate to proline, similar reaction mixtures of 0.6 ml total volume were combined in 13 x 100 mm tubes to contain 25 mM potassium phosphate, pH 7.5, 15 mM ATP, 1 mM magnesium chloride, 15 mM NADH, 30 mM L-[U-^{14}C]-glutamate (specific activity = 0.02 µCi/µmole), and whole cells or crude extracts of cells grown in synthetic media.

In both cases, the reaction mixtures were equilibrated in a water bath to 40C for 5 min. Whole cells or crude extract was added to start the reactions, which were stopped after 1 h with an equal volume of 10% TCA. A cold TCA extraction was carried out in an ice bath for 30 min, after which the TCA insoluble material was centrifuged out

and the supernatant solutions extracted three times with ether. These solutions from the reactions and the pre-acidified controls were adsorbed on Pasteur pipette columns of Dowex 50W-X4 H^+-form cation exchange resin, washed with water, eluted with 1 M ammonium hydroxide, and concentrated to dryness under vacuum. The dry amino acids were dissolved in 0.1 ml water, and 20 µl samples were electrophoresed (35 volts/cm) on Whatman No. 1 paper using a buffer system of 0.25 M sodium acetate, pH 4.3, with which glutamate could be readily separated from proline and ornithine. Following electrophoresis, the paper was dried, and the amino acid spots were developed with 0.01% ninhydrin in acetone in a drying oven. Spots corresponding to the standards (L-glutamate, L-proline, δ-aminovaleric acid, and L-ornithine) were cut out, folded, and covered with toluene-based scintillation fluid (8) in glass vials. All counting was done on a Packard Tri-Carb Liquid Scintillation Spectrometer.

Since proline could not be separated from other neutral amino acids by electrophoresis, the amino acids from the above reaction mixtures containing L-[U-^{14}C]-glutamate as substrate were also chromatographed (descending) on Whatman No. 3 paper with a butanol-acetic acid-water (60:15:25) solvent system. Amino acid spots were developed and cut out for counting as above.

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to glutamate, [U-^{14}C]-P5CA prepared from L-[U-^{14}C]-ornithine using rat liver ornithine δ-transaminase was dissolved in 250 mM potassium phosphate, pH 7.5, to give a 15 mM solution of the labeled P5CA. Reaction mixtures of 100 µl total volume contained 25 mM potassium phosphate, pH 7.5, 10 mM ATP, 10 mM NAD^+, 1 mM magnesium chloride, 400 mM [U-^{14}C]-P5CA (specific activity = 0.066 µCi/µmole), varying concentrations of hydrazine sulfate, pH 7.7, and 1.2 mg crude extract of C. sporogenes grown in standard trypticase medium.

All reactions were run at 40C for 30 min and stopped with 60 µl 0.4 M formic acid. After centrifuging at 18,000 x g for 15 min, 15 µl samples of the reaction mixture and of a pre-acidified control were spotted on Whatman No. 3 paper and chromatographed with a descending butanol-acetic acid-water solvent system. The glutamate spots were cut out and counted for 10 min in the toluene-based scintillation fluid.

Fractionation of Cells Grown in Synthetic Media with Labeled Amino Acids

Three C. sporogenes cultures of 100 ml each were grown in synthetic medium + 0.05% sodium thioglycollate with 0.23% arginine added as a supplementary amino acid. After autoclaving, 0.5% sterile glucose was added to the media. Each flask was inoculated with 4 ml of a growing culture of C. sporogenes (in standard trypticase media), and the flasks were incubated in anaerobic jars.

After 5 h, single cultures were supplemented with 10

μCi of sterile L-[U-^{14}C]-proline, L-[U-^{14}C]-ornithine, or L-[U-^{14}C]-glutamate. Growth was allowed to continue for 15 more hours.

All cultures were harvested at 18,000 x g for 15 min, the pellets washed twice with 10 ml volumes of cold 0.05 M potassium phosphate, pH 7.5, and then treated with the following procedures: (a) cold TCA extraction; (b) hot TCA extraction; and (c) complete protein hydrolysis.

(a) Cold TCA extraction: Five ml of cold 5% TCA were added to each pellet and held at 0C for 5 min before centrifuging. The pellets were washed a second time and the two 5-ml supernatant solutions combined to give the cold TCA extracts.

(b) Hot TCA extraction: Each pellet was extracted with 2.5 ml 5% TCA at 90C for 30 min and centrifuged. The pellets were then washed with 2.5 ml warm TCA and the washings discarded.

(c) Protein hydrolysis: The pellets were then suspended in 1 ml 6 N HCl and transferred to vials. The centrifuge tubes were rinsed with another 1 ml of the HCl and added to the vials. The vials were flushed with argon, sealed, and incubated at 110C for 24 h to give the protein hydrolysate.

The cold TCA extracts were extracted three times with equal volumes of ether, adsorbed on a Pasteur pipette column of Dowex 50W-X4 H^+-form resin, washed with water, and eluted

with 1 M sodium hydroxide. The eluates were dried under a vacuum, washed once with 5 ml water, and dried again. The dried pellets were dissolved in 0.5 ml water and electrophoresed as previously described.

The protein hydrolysates were washed from their vials with 3 ml water and dried under vacuum. The pellets were picked up in 1 ml water and adsorbed on Pasteur pipette Dowex columns as described above and eluted with 1 M sodium hydroxide. These eluates were dried under vacuum, washed with water, and dried again. The dry amino acids were dissolved in 0.5 ml water and electrophoresed as described above.

The cold TCA extract and the protein hydrolysate of the cells from each culture were also chromatographed as previously described. The paper was then dried, sprayed with 0.1% ninhydrin in acetone, and the spots cut out which corresponded to amino acid standards. These spots were counted for 10 min in toluene-based scintillation fluid.

RESULTS

Studies on the Role of Proline Dehydrogenase in Cells of Clostridium sporogenes

Effect of growth medium on proline dehydrogenase activity: If proline dehydrogenase is involved in the dissimilation of proline in C. sporogenes, one might expect the enzume to be repressed in a glucose medium and induced in a medium supplemented with excess proline. Conversely, if the enzyme is involved in the biosynthesis of proline from glutamate, one might expect it to be repressed in a high proline medium. To test these possibilities, C. sporogenes cells were grown in 1 liter batches of standard trypticase and synthetic media supplemented with various amino acids and with glucose. Crude extracts of cells were prepared from each medium and these extracts assayed for proline dehydrogenase activity.

The presence of glucose in the medium resulted in a decrease of about 50% in enzyme activity (Table 1). There is also some indication that the presence of proline or of amino acids readily converted to proline (arginine and ornithine) in the medium enhanced proline dehydrogenase activity. The extract with the highest enzyme activity was of the cells grown in standard trypticase medium supplemented with 0.1% L-proline. While not definitive, these data indicate that proline dehydrogenase may be involved in the dissimilation of

Table 1.--Effect of growth medium on proline dehydrogenase activity.*

Growth Medium	Specific Activity
Standard trypticase	0.0119
Standard trypticase + 0.1% L-ornithine	0.0147
Standard trypticase + 0.1% L-arginine	0.0159
Standard trypticase + 0.5% D-glucose	0.0076
Standard trypticase + 0.5% D-glucose + 0.1% L-ornithine	0.0095
Standard trypticase + 0.1% L-proline	0.0184
Synthetic	0.0093
Synthetic + 0.5% DL-proline	0.0163
Synthetic + 0.1% L-glutamate	0.0150
Synthetic + 0.5% D-glucose	0.0058
Synthetic + 0.5% DL-proline + 0.1% L-glutamate	0.0148
Synthetic + 0.5% DL-proline + 0.5% D-glucose	0.0074

*Assays were performed by measuring the rate of increase in absorbancy at 443 nm. Enzyme was crude extract, between 0.55 and 1.3 mg protein per reaction.

proline in these cells.

Interconversion of proline and glutamate: Since the most likely function of the proline dehydrogenase in cells of C. sporogenes would be in the interconversion of proline and glutamic acid, a number of experiments were conducted to attempt to demonstrate such conversions. In an effort to show the in vitro conversion of L-[U-^{14}C]-proline to L-[U-^{14}C]-glutamate, reactions were conducted as described in MATERIALS AND METHODS with whole cells of C. sporogenes, grown in a synthetic medium supplemented with L-proline and/or D-glucose, in the presence of L-[U-^{14}C]-proline. Counting the glutamate spots from paper electrophoresis in toluene-based scintillation fluid revealed a significant conversion of the labeled proline to L-[U-^{14}C]-glutamate by cells grown in the synthetic medium + 0.5% L-proline and some conversion to L-[U-^{14}C]-glutamate by cells grown in synthetic medium + 0.5% L-proline and 0.5% D-glucose (Table 2). These data agree with the specific activities noted for the proline dehydrogenase from cells grown in different media (Table 1). The results suggest that proline dehydrogenase is indeed involved in the conversion of proline to glutamate.

To attempt to demonstrate the conversion of L-[U-^{14}C]-glutamate to L-[U-^{14}C]-proline, reactions were conducted as described above but with L-[U-^{14}C]-glutamate as substrate, using whole cells or crude extracts of cells of C. sporogenes grown in synthetic medium supplemented with a variety of amino acids, including 0.1% L-glutamate. Conversion of the labeled

Table 2.--Conversion of L-[U-^{14}C]- proline to L-[U-^{14}C]-glutamate by cells of *C. sporogenes*.*

Growth Medium	L-[U-^{14}C]-glutamate Spots	
	Reaction (cpm)	Control (cpm)
Synthetic	467	408
Synthetic + 0.5% L-proline	1544	286
Synthetic + 0.5% D-glucose	378	422
Synthetic + 0.5% L-proline + 0.5% D-glucose	897	604

*Reaction mixtures were as follows: 25 mM potassium phosphate, pH 7.5; 15 mM ATP; 15 mM NAD^+; 130 mM L-[U-^{14}C]-proline (specific activity = 0.0032 uCi/umole); and 25 mg (dry weight) whole cells of *C. sporogenes*. Controls were acidified prior to adding cells.

glutamate to labeled proline could not be shown in any instance with these reaction mixtures. Incubation was conducted both aerobically and under an argon atmosphere.

The next step was to attempt the conversion of the proline dehydrogenase reaction product, P5CA, to glutamate. Reactions were conducted as described in MATERIALS AND METHODS, with crude extracts of cells of C. sporogenes grown in standard trypticase medium, and [U-^{14}C]-P5CA in the presence of various concentrations of hydrazine sulfate. The hydrazine sulfate was used because previous experiments had demonstrated that it strongly inhibited the interconversion of L-proline and P5CA. In this case, the hydrazine was used to prevent the reduction of the [U-^{14}C]-P5CA to L-[U-^{14}C]-proline. After paper chromatography of the final reaction mixtures, counting the glutamate spots in toluene-based scintillation fluid revealed significant conversion of [U-^{14}C]-P5CA to L-[U-^{14}C]-glutamate, with the extent of conversion dropping off as the concentration of hydrazine increased (Table 3). These data indicate that C. sporogenes does possess a functional P5CA dehydrogenase which can operate in vitro.

To attempt to determine the interconversion of L-proline, L-glutamate, and L-ornithine in growing cells of C. sporogenes, cells were grown in synthetic medium supplemented with the ^{14}C-labeled amino acids and fractionated as described in MATERIALS AND METHODS. Table 4 summarizes the results of this study. The data are not altogether consistent, however there is some evidence that labeled proline may have been

Table 3.--Conversion of [U-^{14}C]-P5CA to L-[U-^{14}C]-glutamate in the presence of various concentrations of hydrazine sulfate.*

Hydrazine sulfate	L-[U-^{14}C]-glutamate Spots (cpm)
Control	174
10 mM	681
15 mM	574
20 mM	350
25 mM	320

*Reactions contained 25 mM potassium phosphate, pH 7.5, 10 mM ATP; 1 mM magnesium chloride; 400 mM [U-^{14}C]-P5CA (specific activity = 0.066 μCi/umole); 10 mM NAD^+; hydrazine sulfate as above; and 1.2 mg crude extract from cells of C. sporogenes grown in standard trypticase. The control contained 10 mM hydrazine sulfate and was acidified prior to adding cell extract.

Table 4.--Interconversion of proline, glutamate, and ornithine during growth of *C. sporogenes* in a synthetic medium.*

Fractionation step	Amino acid spot	L-[U-^{14}C]-proline 10 µl	L-[U-^{14}C]-proline 20 µl	L-[U-^{14}C]-glutamate 10 µl	L-[U-^{14}C]-glutamate 20 µl	L-[U-^{14}C]-ornithine 10 µl	L-[U-^{14}C]-ornithine 20 µl
Concentrated supernatant (electrophor.)	glutamate	240	472	5225	10455	115	230
	neutral	580	2075	320	725	1035	4120
	basic	92	170	7	10	170	335
Cold TCA extract (electrophor.)	glutamate	43	80	596	1210	80	157
	neutral	5	5	50	90	25	50
	basic	0	0	12	10	0	4
Cold TCA extract (chromatogr.)	glutamate	0	0	570	1200	65	120
	proline	0	0	0	0	0	0
	ornithine	0	0	5	5	5	10
Protein hydrolysate (electrophor.)	glutamate	0	0	4045	8360	555	1155
	neutral	320	645	55	100	605	1202
	basic	5	0	5	5	55	100
Protein hydrolysate (chromatogr.)	glutamate	75	130	4255	8735	590	1305
	proline	240	530	10	30	430	880
	ornithine	0	0	5	10	0	0

*Radioactivity was measured in cpm. Amino acid spots were cut out following paper electrophoresis or descending paper chromatography of 10 and 20 µl samples and counted for 10 min in toluene-based scintillation fluid.

partially converted to glutamate, which either was incorporated into protein or leaked back into the medium. In contrast, labeled ornithine appeared to have been converted to proline and glutamate to a rather large extent and then incorporated into protein. Relatively little conversion of glutamate to proline was indicated by the data, and there was no indication of any conversion of glutamate to basic amino acids.

Partial Purification of Proline Dehydrogenase

Production of cells and preparation of crude extract: Cells of *C. sporogenes* were grown in two 20-liter batches as described by Costilow and Laycock (10). After 17 h incubation, the cells were harvested with a Sharples continuous flow centrifuge, model AS-12; 86 g of cell paste were obtained. This pellet was suspended in an equal volume of 10 mM potassium phosphate, pH 7.5, with 10% glycerol and extracted by ultrasonic oscillation of 8 ml batches. The cell debris was removed by centrifugation at 20,000 x g for 20 min to yield 64 ml of crude extract.

Dialyzed extract: The crude extract was dialyzed against 2 liters of 10 mM potassium phosphate, pH 7.5, for 12 h, changing the buffer every 4 h, to yield 78 ml of dialyzed extract.

It was interesting that the dialysis of crude extracts of cells consistently resulted in a dramatic increase in activity of proline dehydrogenase. A possible explanation of

this phenomenon is that glutamate present in the crude extracts may have been inhibiting the enzyme, with dialysis removing the glutamate and thus relieving the inhibition. Proline dehydrogenase in crude extracts of cells prepared with the aid of a French press had the same specific activity as proline dehydrogenase from sonicated cells.

Streptomycin sulfate treatment: The dialyzed extract was diluted with 10 mM potassium phosphate, pH 7.5, with 10% glycerol, to a final protein concentration of approximately 25 mg/ml. An equal volume of 5% streptomycin sulfate in the same buffer was added slowly with stirring, and the mixture was allowed to stand overnight at 4C. After centrifuging at 36,000 x g for 30 min, 172 ml of extract were collected.

Ammonium sulfate precipitation: The streptomycin sulfate-treated extract was fractionated by precipitation with solid ammonium sulfate between 45 and 80% saturation. The ammonium sulfate was added very slowly with stirring in an ice bath and allowed to continue stirring for 30 min. This 45-80% fraction was centrifuged at 36,000 x g for 30 min and the supernatant solution (which gave a strong reaction with o-aminobenzaldehyde due to the high concentration of ammonium sulfate) was discarded. The pellet was resuspended in 20 ml of 25 mM Tris-HCl, pH 8.0, and dialyzed against 1 liter of the same buffer for 16 h, changing the buffer after 8 h. This dialysis yielded 38 ml of extract which was divided into 5 ml aliquots and frozen at -18C.

DEAE-cellulose chromatography: A column of precycled DEAE-cellulose (Whatman DE52) was poured to 1.5 x 15 cm and equilibrated with 50 mM Tris-HCl, pH 8.0. One 8-ml aliquot of the dialyzed 45-80% ammonium sulfate cut (168 mg protein) was layered onto the column and eluted with a gradient of Tris-HCl, pH 8.0, from 0.2 M to 0.3 M (100 ml of each). Five-ml fractions were collected with a flow rate of 30 ml/h. The column was further eluted with 40 ml of 0.3 M Tris and then with 40 ml of 0.35 M Tris-HCl, pH 8.0. Most of the protein was eluted with the first 50 ml of the gradient (fractions 1 to 10), as determined by absorbancy at 280 nm. The proline dehydrogenase eluted with the 0.35 M Tris (fractions 37 to 40), and these fractions were pooled, concentrated to 0.8 ml in an Amicon ultrafiltration cell, model 12, and frozen at -18C.

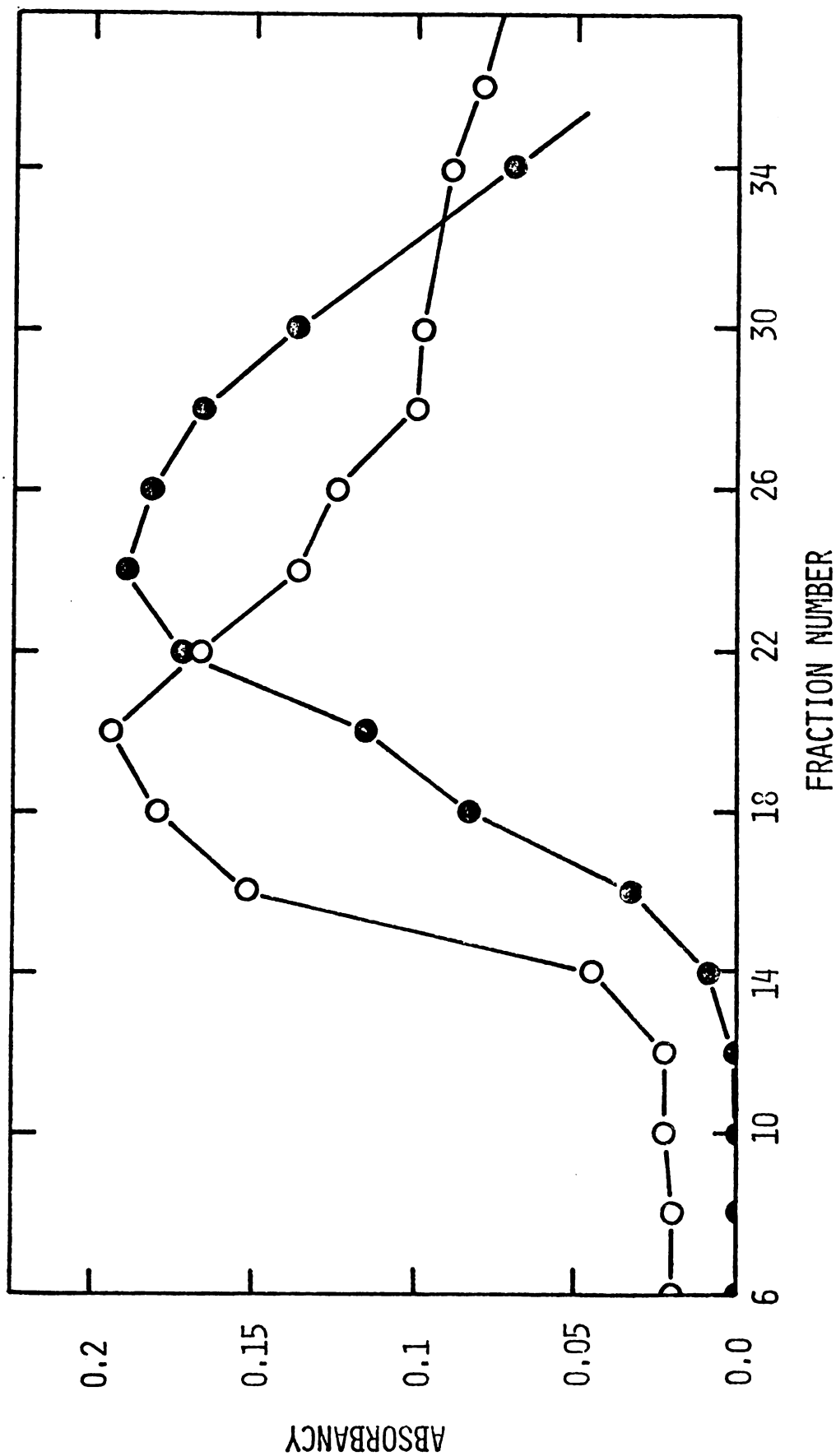
Two more DEAE-cellulose columns were run exactly as above using 12 ml of the dialyzed 45-80% ammonium sulfate-precipitated fraction in each. Ultrafiltration of the fractions which had proline dehydrogenase activity yielded 1.3 ml from one of these columns and 2.3 ml from the other. The three DEAE-cellulose columns yielded proline dehydrogenase preparations with specific activities of 0.111, 0.126, and 0.115 units/mg protein.

The purity of the concentrated fractions was visualized by analytical polyacrylamide disc gel electrophoresis (anionic) by the method of Davis (11). With each gel, a protein stain of 1% amido black in 7% acetic acid revealed a

small band with a relative mobility (R_F) of 0.18, a large band with an R_F of 0.3, and a diffuse, continuous band between R_F values of 0.36 and 0.9. Because of this evidence of impurity in the DEAE-cellulose preparations, another purification step was necessary. The procedure chosen was Sephadex G-200 gel filtration.

Sephadex G-200 gel filtration: After running several small Sephadex test columns with the concentrated DEAE-cellulose fractions, the remaining volumes of these fractions were pooled to a volume of 3.66 ml (about 22 mg protein). A Sephadex G-200 column, 2.5 x 33 cm was poured and equilibrated with 400 ml of 0.25 M Tris-HCl, pH 8.0. Two ml (about 12 mg protein) of the DEAE-cellulose fraction were layered onto the column and eluted with the equilibration buffer, collecting 5 fractions of 5 ml and then 40 fractions of 2 ml with a flow rate of 17 ml/h. The absorbancy of the fractions at 280 nm revealed a protein peak in fraction 20, after about 55 ml of eluate (Fig. 2). (The void volume of the column had been determined to be about 50 ml by the use of Blue Dextran 2000.) The proline dehydrogenase eluted in the trailing edge of the initial protein peak, showing a maximum activity in fraction 24 (Fig. 2). Fractions 22 to 29 were pooled and concentrated by ultrafiltration to a volume of 3.8 ml and frozen in 80 µl aliquots at -18C. Specific activities of concentrated Sephadex G-200 fractions from different columns ranged from 0.319 to 0.337. Table 5 summarizes the partial purification of the proline dehydrogenase.

Fig. 2.-- Sephadex G-200 gel filtration of DEAE-cellulose fraction. Protein (A_{280}), O; proline dehydrogenase (A_{443}/15 min), ●.


ABSORBANCY
0.0
0.05
0.1
0.15
0.2
6
10
14
18
22
26
30
34
FRACTION NUMBER

Table

Fracti

Crude

Dialy

Strep.
sulfa

Dial.
Off a
sulfa

DIAS-0

Sephad
G-200

three

Table 5.--Partial purification of proline dehydrogenase.

Fraction	Vol. (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude	64	3008	9.02	0.0029	100
Dialyzed	78	2375	38.00	0.016	421
Strep. sulfate	172	1892	39.73	0.021	432
Dial. 45-80% amm. sulfate	38	802	21.65	0.027	232
DEAE-cell.*	3.6	30.4	3.54	0.117	39
Sephadex G-200	3.8	1.7	0.56	0.328	7

*Compiled data from the combined active fractions of three columns.

Analytical polyacrylamide disc gel electrophoresis of the Sephadex G-200 fraction: Preliminary studies with the partially purified Sephadex G-200 preparation demonstrated that it contained high levels of glutamate dehydrogenase activity. To determine if the proline dehydrogenase could be separated from the glutamate dehydrogenase by polyacrylamide gel electrophoresis, three gels were run with the Sephadex G—200 fraction (about 72 ug protein on each). One gel was stained with a 1% amido black protein stain in 7% acetic acid, another with an L-proline + NAD^+ stain, and the third with an L-glutamate + NAD^+ stain (11). The L-proline + NAD^+ stain consisted of 0.794 g L-proline, 0.58 ml Tris-HCl, pH 7.5, 5 ml phenazine methosulfate (2.5 mg/ml), 5 ml nitro-blue tetrazolium (0.5 mg/ml), 2.3 ml 0.02 M NAD^+, and 10.12 ml water. The L-glutamate + NAD^+ stain was the same as the L—proline + NAD^+ stain with the exception of 10 mM L-glutamate (final concentration) substituted for L-proline.

The gel stained with amido black showed a minor band with an R_F of 0.18 and a major band with an R_F of 0.3. The gel stained with L-proline + NAD^+ showed a single minor band with an R_F of 0.29; and the gel stained with L-glutamate + NAD^+ showed a major band with an R_F of 0.31 and a continuous diffuse band from the top of the gel to the major band (Fig. 3). These data demonstrate that the proline dehydrogenase and glutamate dehydrogenase migrate very closely together on polyacrylamide gels.

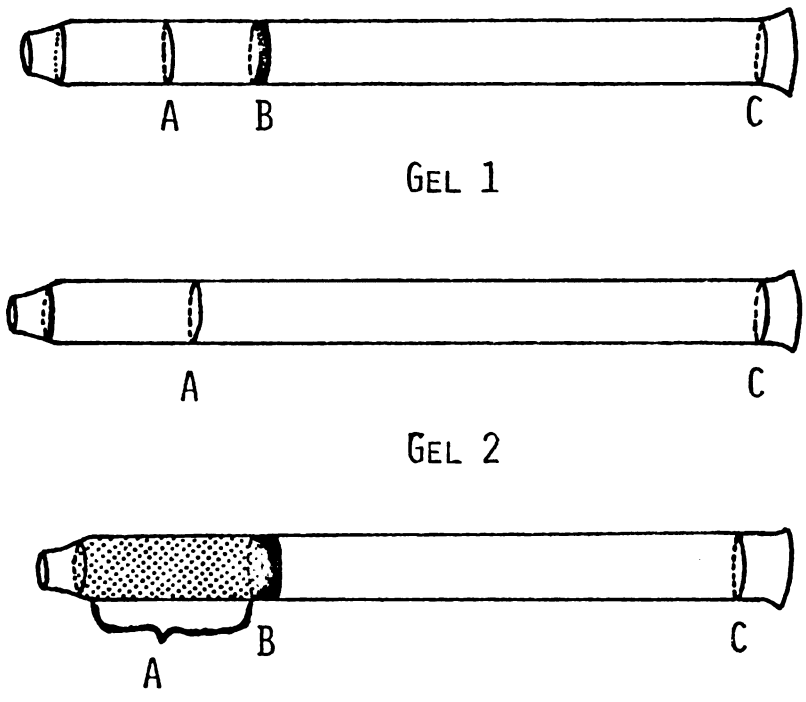


Fig. 3.--Polyacrylamide disc gel electrophoresis of the Sephadex G-200 fraction. Gel 1, stained with amido black; A: R_F = 0.18; B: R_F = 0.3; C: dye front. Gel 2, stained with L-proline + NAD^+; A: R_F = 0.29; C: dye front. Gel 3, stained with L-glutamate + NAD^+; A: R_F = 0.0 -0.3; B: R_F = 0.31; C: dye front.

Separation of proline dehydrogenase and glutamate dehydrogenase by polyacrylamide gel electrophoresis: Although the proline dehydrogenase and glutamate dehydrogenase migrated closely together on polyacrylamide gels, an attempt was made to separate the two enzymes by taking very thin slices of these gels and assaying each for proline and glutamate dehydrogenase activities. Two dialyzed 45-80% ammonium sulfate preparations and the Sephadex G-200 fraction were electrophoresed as before, and the gels were sliced into 3 mm sections. Each slice was put into a 13 mm tube containing 0.2 ml of 0.1 M Tris-HCl, pH 8.0. After equilibration for 2 hours at 4C, the slices were assayed for enzyme activities.

To assay for glutamate dehydrogenase, the rate of reduction of 10 mM NAD^+ was monitored as the increase in absorbancy at 340 nm, using 5 mM L-glutamate as substrate and 50 µl of the Tris buffer used to cover each gel slice as enzyme in a 1 ml total volume reaction mixture. Results showed that glutamate dehydrogenase activity peaked in gel slice no. 5 of the gels used for the ammonium sulfate fractions and in gel slice no. 6 of the gel used for the Sephadex G-200 fraction (Fig. 4). To assay for proline dehydrogenase, the following reactants were placed into each gel slice tube: 0.1 M Tris-HCl, pH 8.0, saturated with o-aminobenzaldehyde; 0.375 M L-proline; and 10 mM NAD^+. Enzyme was the Tris solution covering each gel, bringing each reaction mixture to 1 ml total volume. After equilibration to 38C, L-proline was

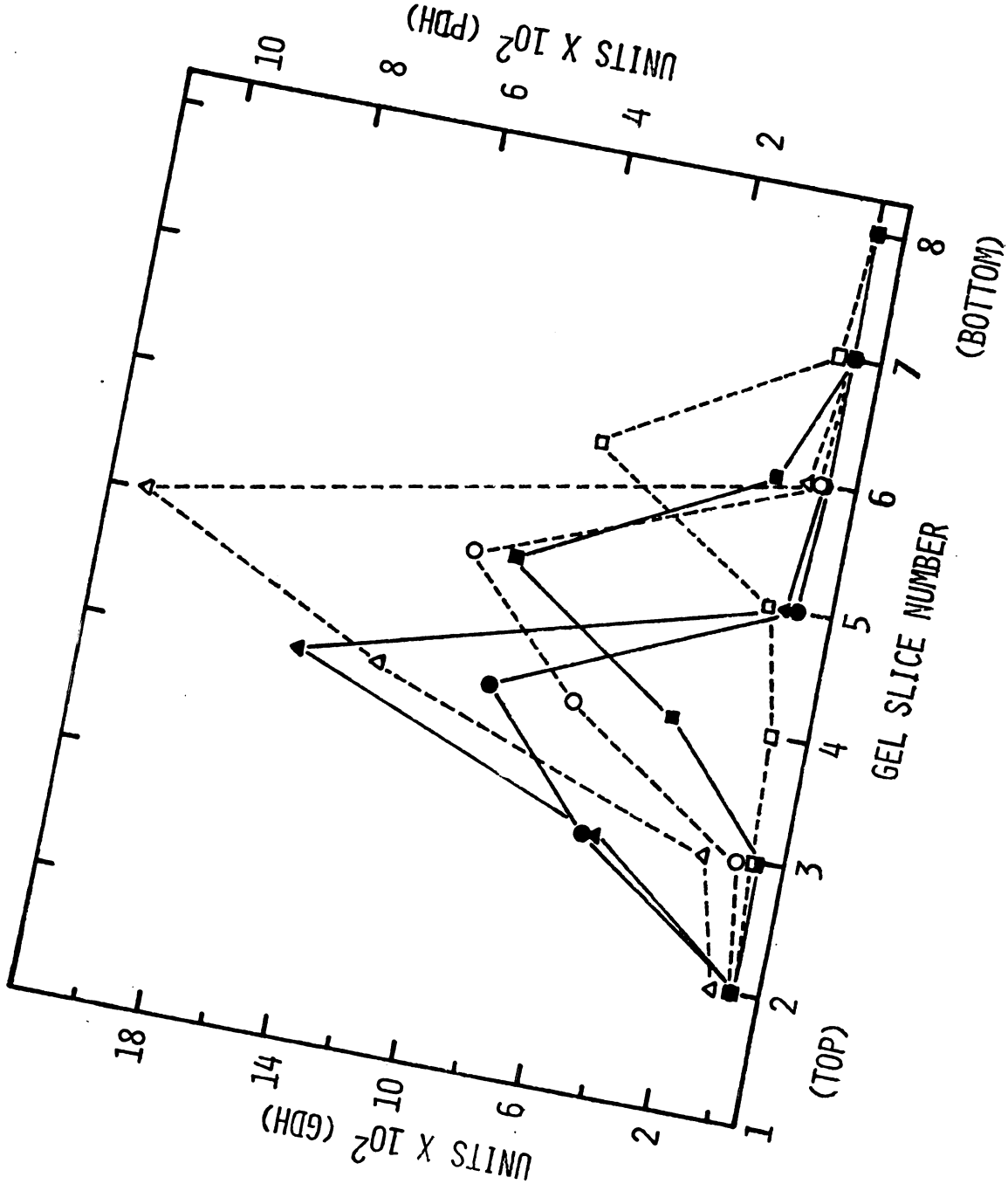
added to start the reactions which were stopped after 1 h with an equal volume of 10% TCA. Proline dehydrogenase activity was measured as absorbancy to 443 nm against a pre-acidified control. The enzyme activity peaked in gel slice no. 4 of the gels with the ammonium sulfate fractions and in slice no. 5 of the gel with the Sephadex G-200 fraction (Fig. 4).

These data are in agreement with the positions of the enzymes on stained gels (Fig. 3) and indicate that the proline dehydrogenase and glutamate dehydrogenase are different enzymes since there was little or no proline dehydrogenase activity in the gel slices which contained the glutamate dehydrogenase peaks.

Separation of proline dehydrogenase and glutamate dehydrogenase by DEAE-cellulose and hydroxylapatite chromatography: A 40-liter culture of C. sporogenes was produced, harvested, and sonicated as described above to yield a crude extract. This was dialyzed, treated with streptomycin sulfate, fractionated with 50-80% ammonium sulfate, and dialyzed. The specific activity of the proline dehydrogenase increased about 10-fold through these procedures, from 0.0028 units/mg protein in the crude extract to 0.024 units/mg in the dialyzed ammonium sulfate fraction. The glutamate dehydrogenase in these fractions was purified 2.3 fold from the crude extract.

A DEAE-cellulose column (2.5 x 42 cm) was then poured and equilibrated with 0.15 M Tris-HCl, pH 7.5, with 10%

Fig. 4.--Separation of glutamate dehydrogenase (GDH) from proline dehydrogenase (PDH) by polyacrylamide disc gel electrophoresis. Reactions for GDH and PDH were as described in text. GDH from 0.4 mg ammonium sulfate fraction, ○; GDH from 0.8 mg ammonium sulfate fraction, △; GDH from Sephadex G-200 fraction, □; ●, ▲, ■, PDH from same respective fractions.

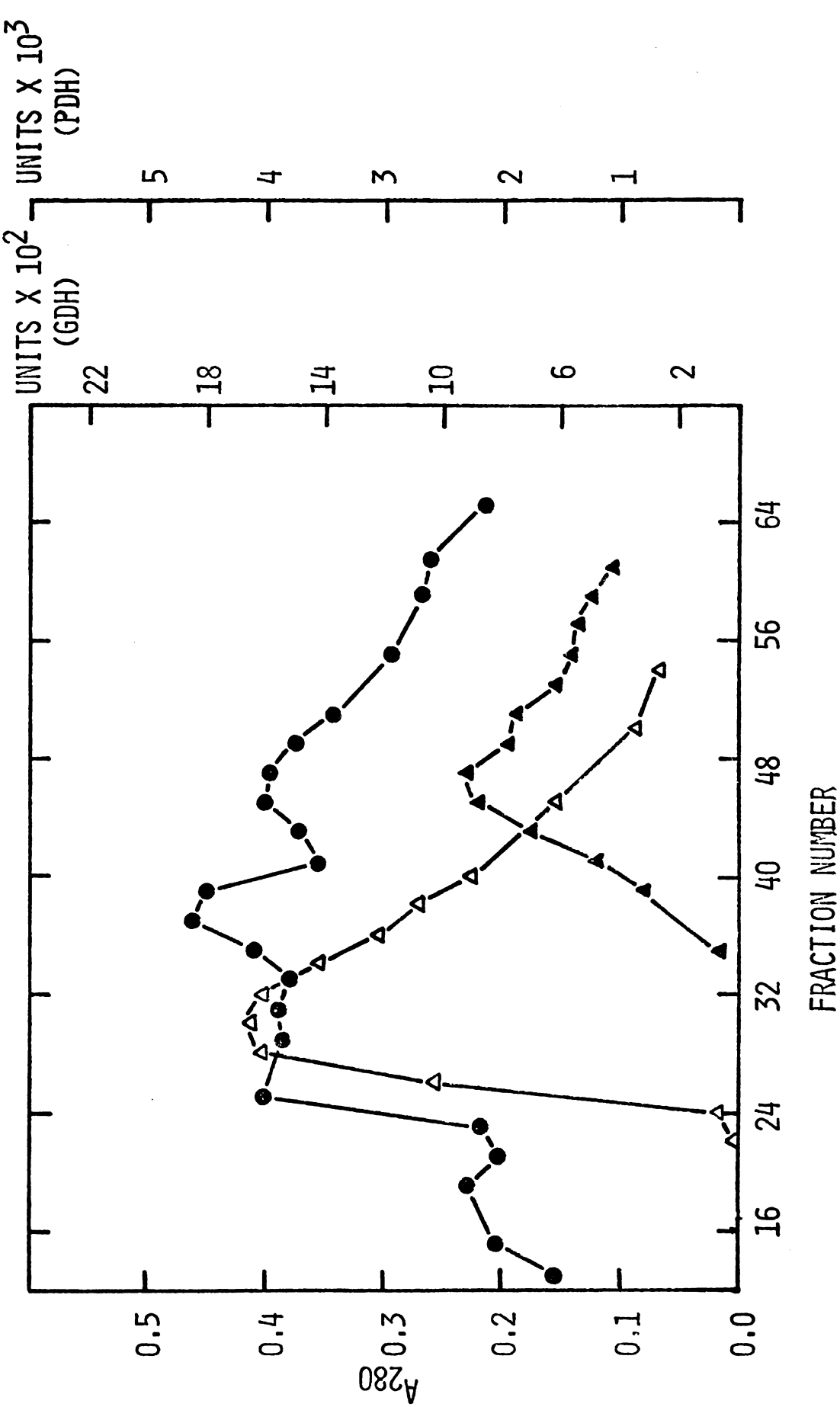

UNITS X 10² (PDH)
10
8
6
4
2
UNITS X 10² (GDH)
18
14
10
6
2
1
2
3
4
5
6
7
8
(TOP)
GEL SLICE NUMBER
(BOTTOM)

glycerol and 2 mM dithiothreitol (DTT). About 1.12 g protein of the dialyzed ammonium sulfate fraction was layered onto the column, and the column was washed with the equilibration buffer until the A_{280} of the eluate was below 0.2. The protein remaining on the column was eluted with a linear gradient of Tris-HCl, pH 7.5, with 10% glycerol and 2 mM DTT, from 0.15 to 0.3 M. Eighty 10-ml fractions were collected with a flow rate of 35 ml/h. The glutamate dehydrogenase peaked 17 fractions before the proline dehydrogenase (Fig. 5). On the basis of these data, fractions 43 to 53 (Fig. 5) were pooled and concentrated by ultrafiltration to yield a proline dehydrogenase preparation with a specific activity of 0.133 units/mg protein. This preparation, however, still contained a significant concentration of glutamate dehydrogenase.

A preliminary run demonstrated that the use of Sephadex G-200 gel filtration was of no benefit in separating the two dehydrogenases. The peak activities of both enzymes eluted together from the column.

A hydroxylapatite column was prepared by suspending anhydrous BIO·RAD HTP hydroxylapatite powder in 20 mM potassium phosphate, pH 7.3, and packing it into a column (1.5 x 30 cm) as specified by the manufacturer. After equilibrating the column with 100 ml of the same buffer, about 12.5 mg protein of the concentrated DEAE-cellulose fraction were layered onto the column and eluted in 5-ml fractions with a

Fig. 5.--Elution of enzymes from DEAE-cellulose. Protein (A_{280}), ●; proline dehydrogenase (PDH) activity in 100 µl samples, measured by the 15 min tube assay, ▲; glutamate dehydrogenase (GDH) activity in 5 µl samples, Δ.


UNITS X 10^3 (PDH)
UNITS X 10^2 (GDH)
A_{280}
FRACTION NUMBER

linear gradient of potassium phosphate, pH 7.3, from 0.02 to 0.2 M (75 ml of each). Twenty-five fractions were collected with a flow rate of 40 ml/h. Six more fractions were eluted with 0.2 M, seven more with 0.3 M, and seven more with 0.4 M potassium phosphate, pH 7.3, for a total of 45 fractions. Three major peaks eluted from the column (Fig. 6). Glutamate dehydrogenase eluted with the initial protein peak and also in the second and third protein peaks. Proline dehydrogenase eluted with the trailing edge of the third protein peak and was nearly free of glutamate dehydrogenase activity (Fig. 6). Fractions 43 and 44 were pooled to give a proline dehydrogenase preparation with a specific activity of 1.29 units/mg protein. Glutamate dehydrogenase activity in this preparation was 0.09 units/mg protein. Table 6 summarizes the purification procedures used to separate proline dehydrogenase and glutamate dehydrogenase.

Characterization of Proline Dehydrogenase

Enzyme stability: The proline dehydrogenase protein appears to be quite stable. The enzyme from each purification step lost no activity for at least two months when stored at -18C. It did gradually lose activity upon repeated thawing and refreezing. Also, as was reported for the pumpkin proline dehydrogenase (32), the *C. sporogenes* enzyme was suprisingly stable to heat up to 50C.

Fig. 6.--Elution of enzymes from hydroxylapatite. Protein (A_{280}), ●; proline dehydrogenase (PDH) in 100 µl samples, measured by the 15 min tube assay, ▲; glutamate dehydrogenase in 5 µl samples, △.

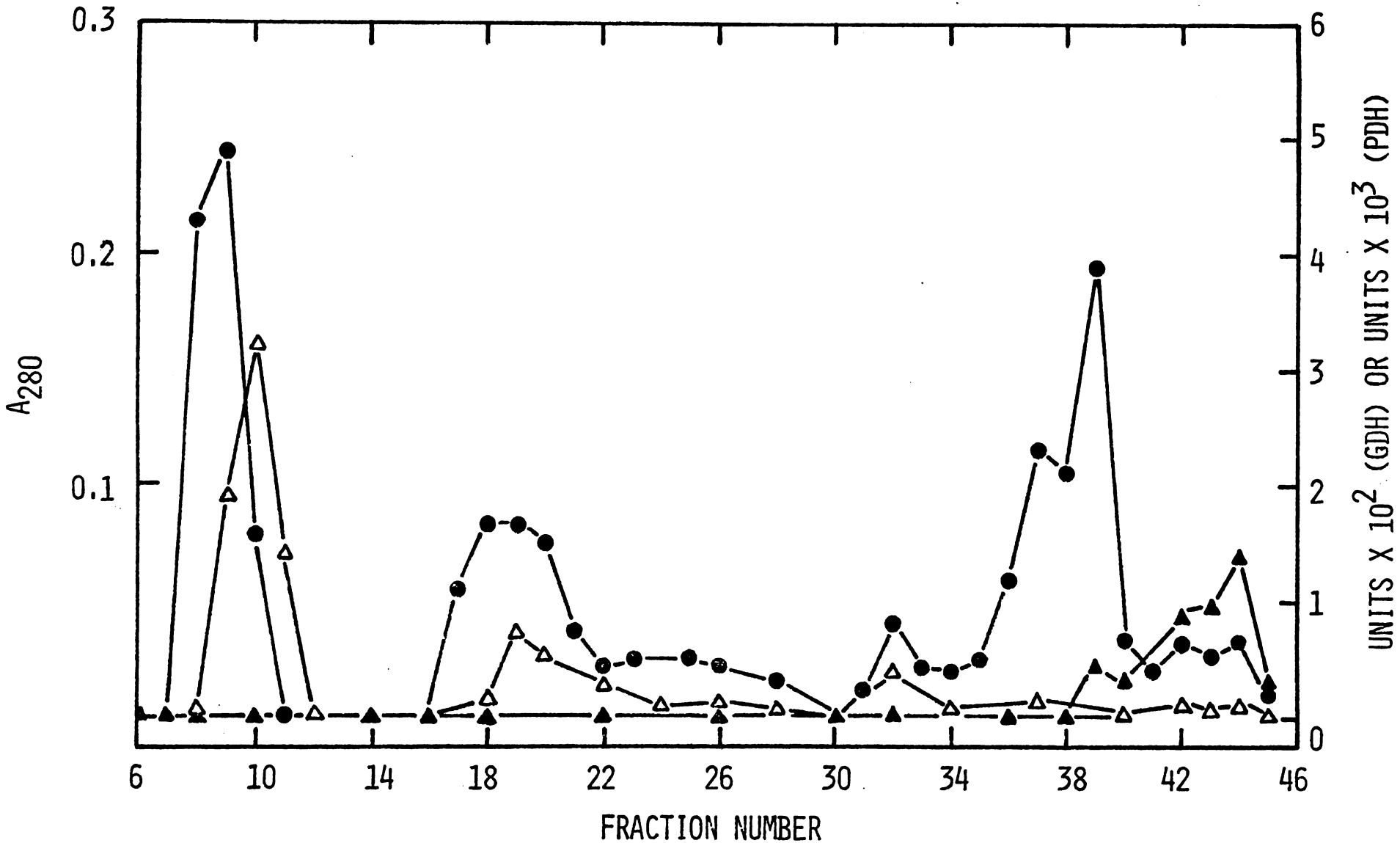

0.3
0.2
0.1
A_{280}
6
5
4
3
2
1
0
UNITS X 10^2 (GDH) OR UNITS X 10^3 (PDH)
6
10
14
18
22
26
30
34
38
42
46
FRACTION NUMBER

Table 6.--Partial purification of proline dehydrogenase II.

Fraction	Vol. (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude	49	3136	9.09	0.0029	100
Strep. sulfate	180	2700	56.7	0.021	623
Dial. 45-80% amm. sulfate	42	1134	27.2	0.024	299
DEAE-cell.	11	28	3.7	0.133	41
Hydroxyl-apatite	10	0.17	0.22	1.29	2.4

Relationship between enzyme concentration and measured activity: To show the relationship between enzyme concentration and enzyme activity, proline dehydrogenase reactions were conducted with a Sephadex G-200 fraction, monitoring the rate of reduction of NAD^+ at 340 nm using various concentrations of protein. The rate of reduction was directly dependent upon the concentration of protein in the reaction mixture over the range tested. (Fig. 7).

Effect of pH on enzyme activity: The effect of pH on proline dehydrogenase activity was determined by conducting assays for the enzyme using 0.1 M concentrations of four buffer systems: (a) potassium phosphate; (b) Tris-HCl; (c) sodium carbonate-bicarbonate; and (d) glycine-sodium hydroxide. Enzyme activity was measured as the rate of reduction of NAD^+ at 340 nm. Maximal activity of proline dehydrogenase occurred at pH 10 (Fig. 8). At the pH routinely used to assay for proline dehydrogenase, pH 8.0, only about 20% maximal activity was observed. At this pH, however, there was strong P5CA reductase activity which may have masked proline dehydrogenase activity. At pH 10, there was no P5CA reductase activity, possibly allowing greater expression of proline dehydrogenase activity. Therefore, a pH of about 10 would be the optimal pH for assaying proline dehydrogenase *in vitro*, but this pH may or may not be the optimal pH *in vivo* for the conversion of L-proline to P5CA.

The reason that a pH of 8.0 was routinely used for

Fig. 7.--Correlation of enzyme concentration and measured activity. Proline dehydrogenase activity is given in units measured by the NAD^+ reduction assay; enzyme was a Sephadex G-200 fraction.

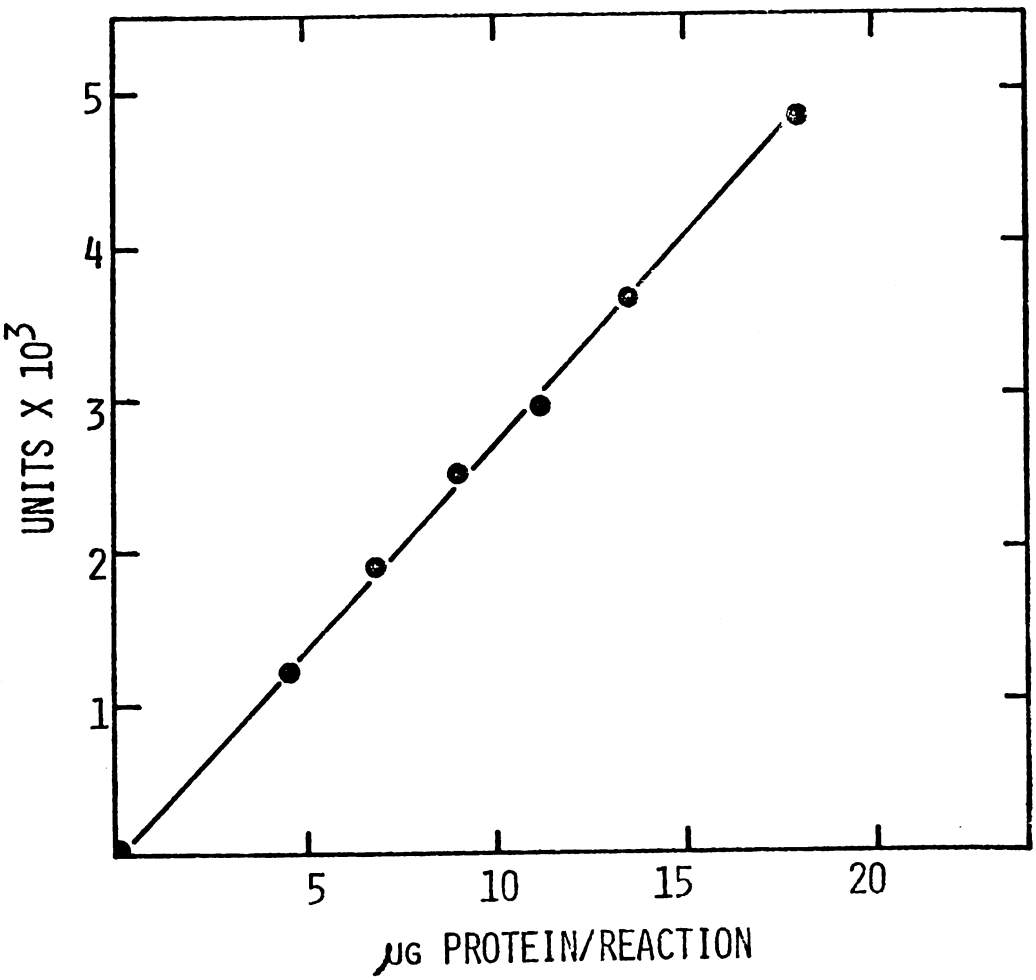
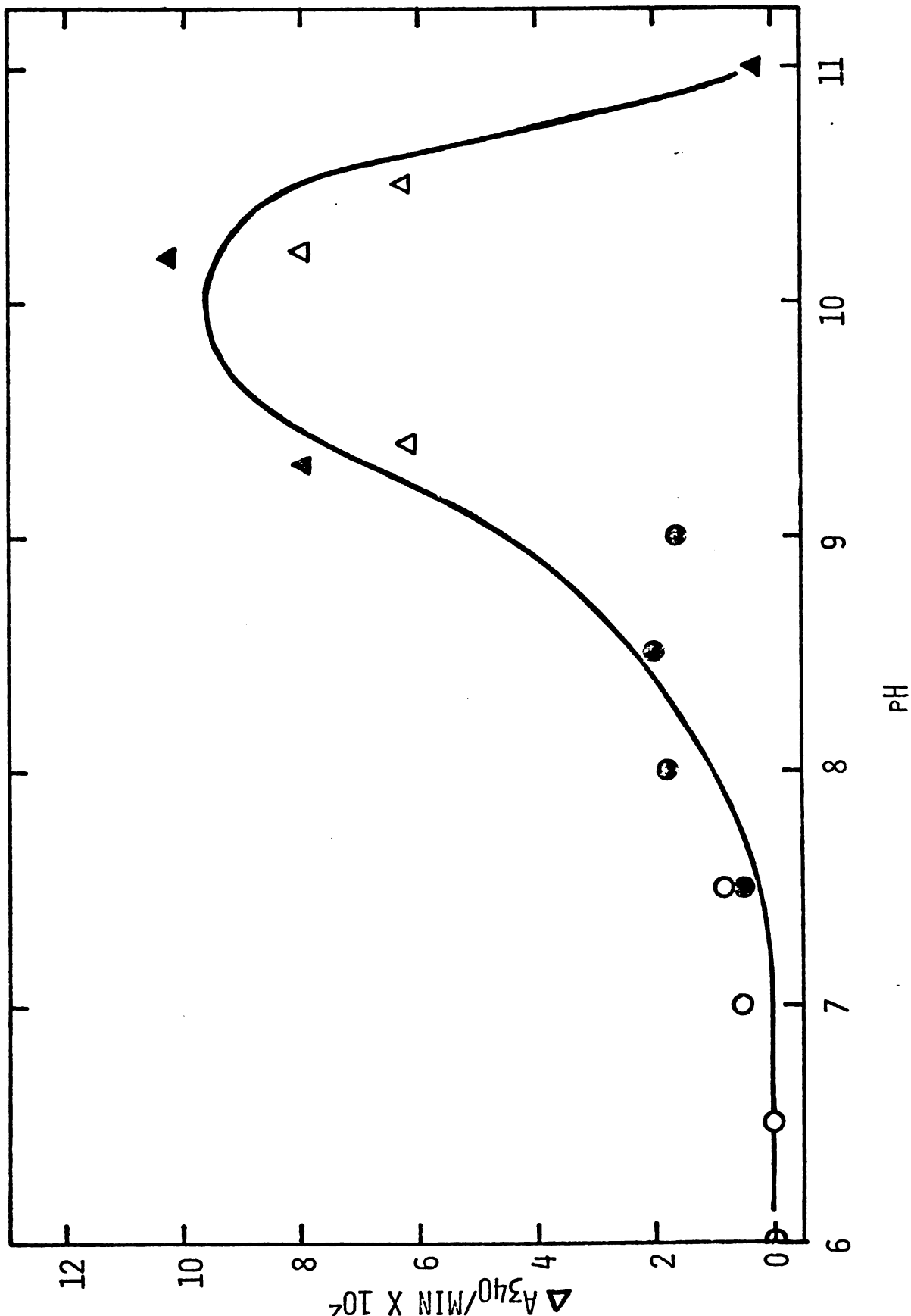

UNITS X 10^3
5
4
3
2
1
5
10
15
20
µG PROTEIN/REACTION

Fig. 8.--Effect of pH on proline dehydrogenase activity. Proline dehydrogenase was measured by the NAD^+ reduction assay, using 9 µg protein of a Sephadex G-200 fraction as enzyme and 100 mM concentrations of 4 buffer systems. Potassium phosphate, ○; Tris-HCl, ●; sodium carbonate-bicarbonate, ▲; glycine-sodium hydroxide, △.

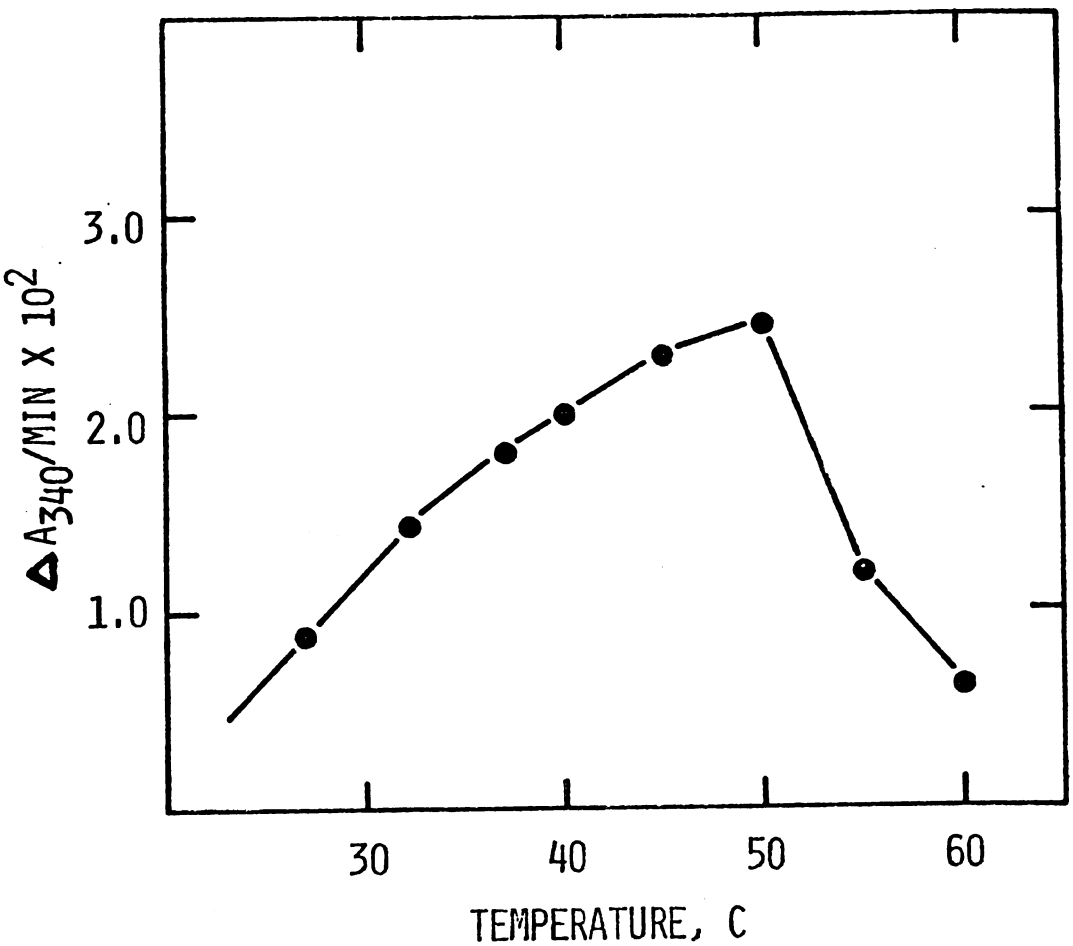

Δ A340/MIN X 10²
0
2
4
6
8
10
12
6
7
8
9
10
11
PH

proline dehydrogenase assays during this investigation was that initial studies of pH with Tris buffer indicated that a pH of 8.0-8.5 was more optimal than 9.0. After the work was essentially complete, references (21, 31, 32) were discovered regarding proline dehydrogenase in eucaryotic cells which led to the reevaluation of the pH optimum for the C. sporogenes enzyme.

Effect of temperature on enzyme activity: To measure the activity of proline dehydrogenase over a range of temperatures, the rate of NAD^+ reduction was monitored at 340 nm using a Sephadex G-200 preparation as enzyme. The activity of the enzyme increased to a maximum at 50C then dropped off sharply above this temperature due to rapid inactivation of the enzyme (Fig. 9). At 37C, the proline dehydrogenase showed only about 72% of maximal activity, but this temperature was used throughout to avoid any possible inactivation of the enzyme by heat.

Response of enzyme activity to oxygen: Experiments to determine whether or not the in vitro enzymatic conversion of L-proline to P5CA was sensitive to oxygen were conducted by adding reducing agents to the proline dehydrogenase reaction mixtures and measuring the activity of the enzyme colorimetrically as the rate of increase in absorbancy at 443 nm. Reactions were run with 10 mM 2-mercaptoethanol, with 1 mM DTT, and with both of these, before and after flushing each reaction mixture with argon. In none of these reactions did the enzyme activity vary from the controls (without reducing

Fig. 9.--Effect of temperature on proline dehydrogenase activity. Proline dehydrogenase was measured by the NAD^+ reduction assay, using 9 µg protein of a Sephadex G-200 fraction as enzyme.


3.0
2.0
1.0
ΔA340/MIN X 10²
30
40
50
60
TEMPERATURE, C

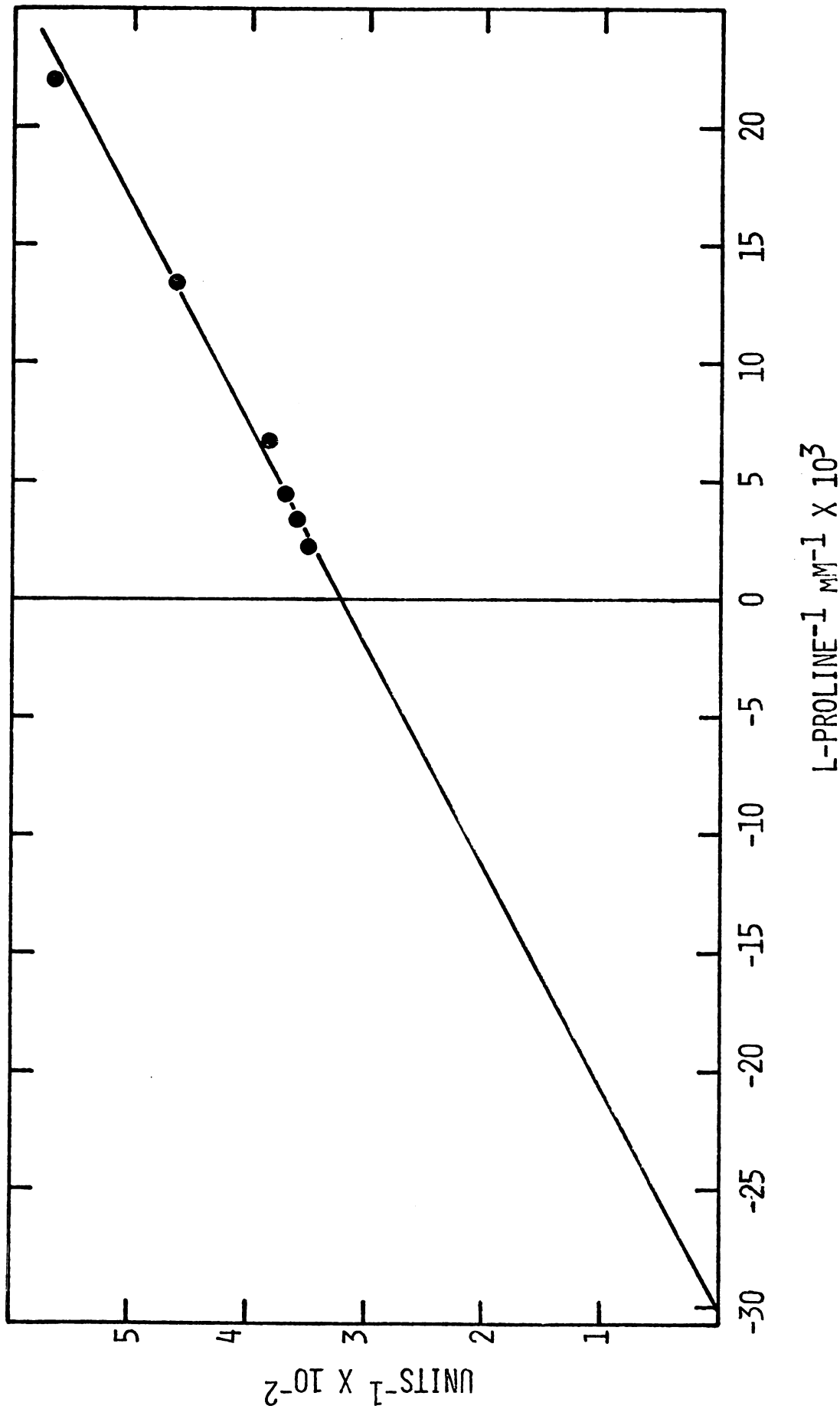
agents and in an air atmosphere). From these data, it appears that oxygen has no rapid effect on the enzyme.

Affinity of proline dehydrogenase for L-proline: The activity of proline dehydrogenase with varying concentrations of L-proline was measured as the rate of reduction of NAD^+ at 340 nm using a Sephadex G-200 fraction as enzyme. The apparent Michaelis-Menton constant (K_m) for L-proline was determined to be 33 mM by plotting the reciprocal of enzyme activity against the reciprocal of L-proline concentration (Fig. 10). At pH 8.0, however, there is strong P5CA reductase activity which may have counteracted the proline dehydrogenase activity. Therefore, this may not be a reasonable estimate of the actual affinity of the enzyme for L-proline.

Further reactions were conducted with 450 mM concentrations of L-alanine, L-aspartate, DL-valine, DL-norvaline, L-leucine, and D-proline. None of these amino acids could act as substrate for proline dehydrogenase. In each case, there was no reduction of NAD^+ observed.

Affinity of proline dehydrogenase for NAD^+: The activity of proline dehydrogenase with varying concentrations of NAD^+ was measured as the rate of reduction of the NAD^+ at 340 nm using a Sephadex G-200 fraction as enzyme. The apparent K_m for NAD^+ was determined to be 1.2 mM from a plot of the reciprocal of enzyme activity against the reciprocal of NAD^+ concentration (Fig. 11). Again, due to possible interference of proline dehydrogenase activity by the highly

Fig. 10.--Lineweaver-Burk plot for L-proline. Proline dehydrogenase was measured by the NAD^+ reduction assay, using 9 µg of a Sephadex G-200 fraction as enzyme.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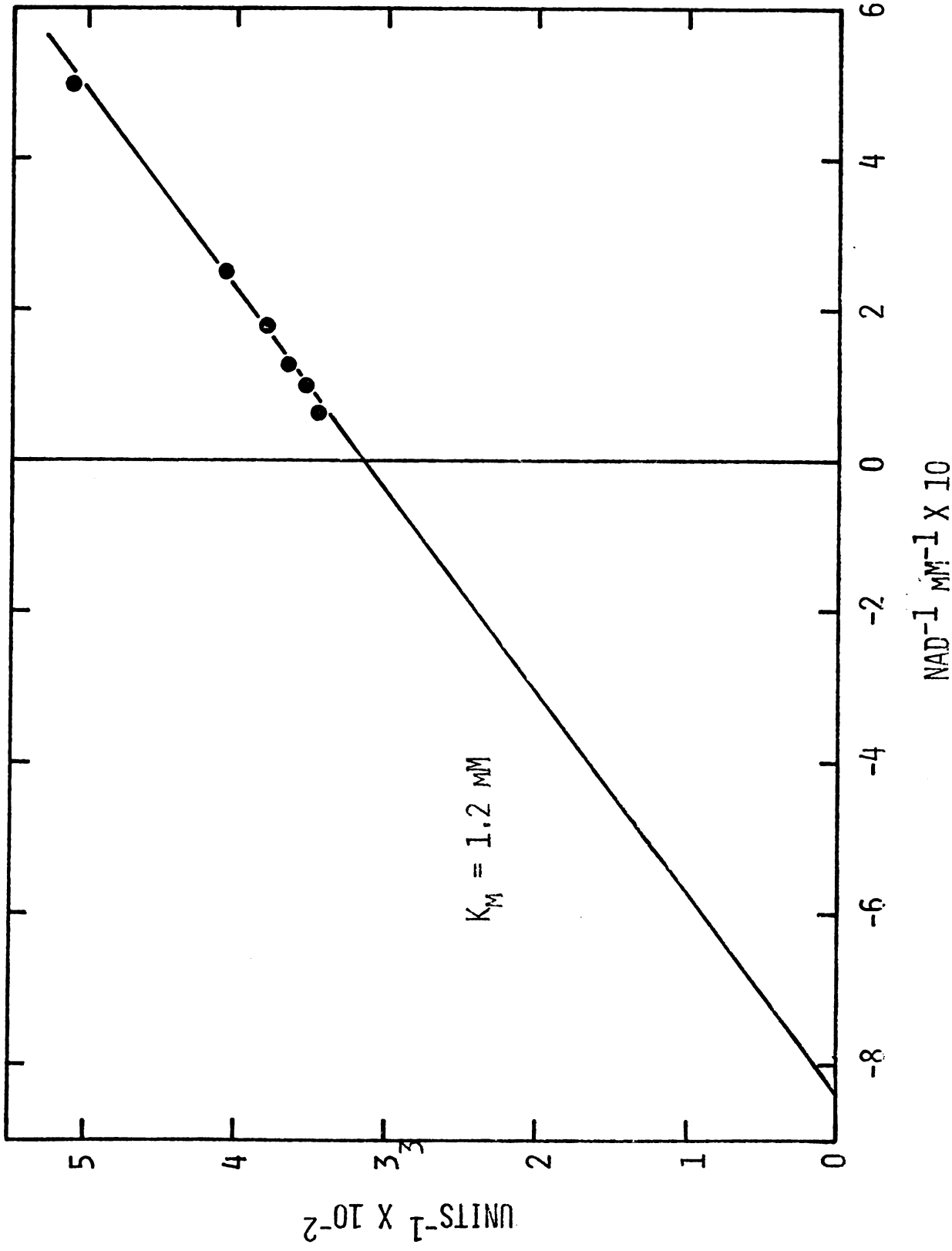
L-PROLINE^{-1} $_{M}M^{-1}$ X 10^{3}

Fig. 11.--Lineweaver-Burk plot for NAD^+. Proline dehydrogenase was measured by the NAD^+ reduction assay, using 9 µg of a Sephadex G-200 fraction as enzyme.


UNITS^{-1} X 10^{-2}
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0
-2
-4
-6
-8
NAD^{-1} mM^{-1} X 10
K_M = 1.2 mM

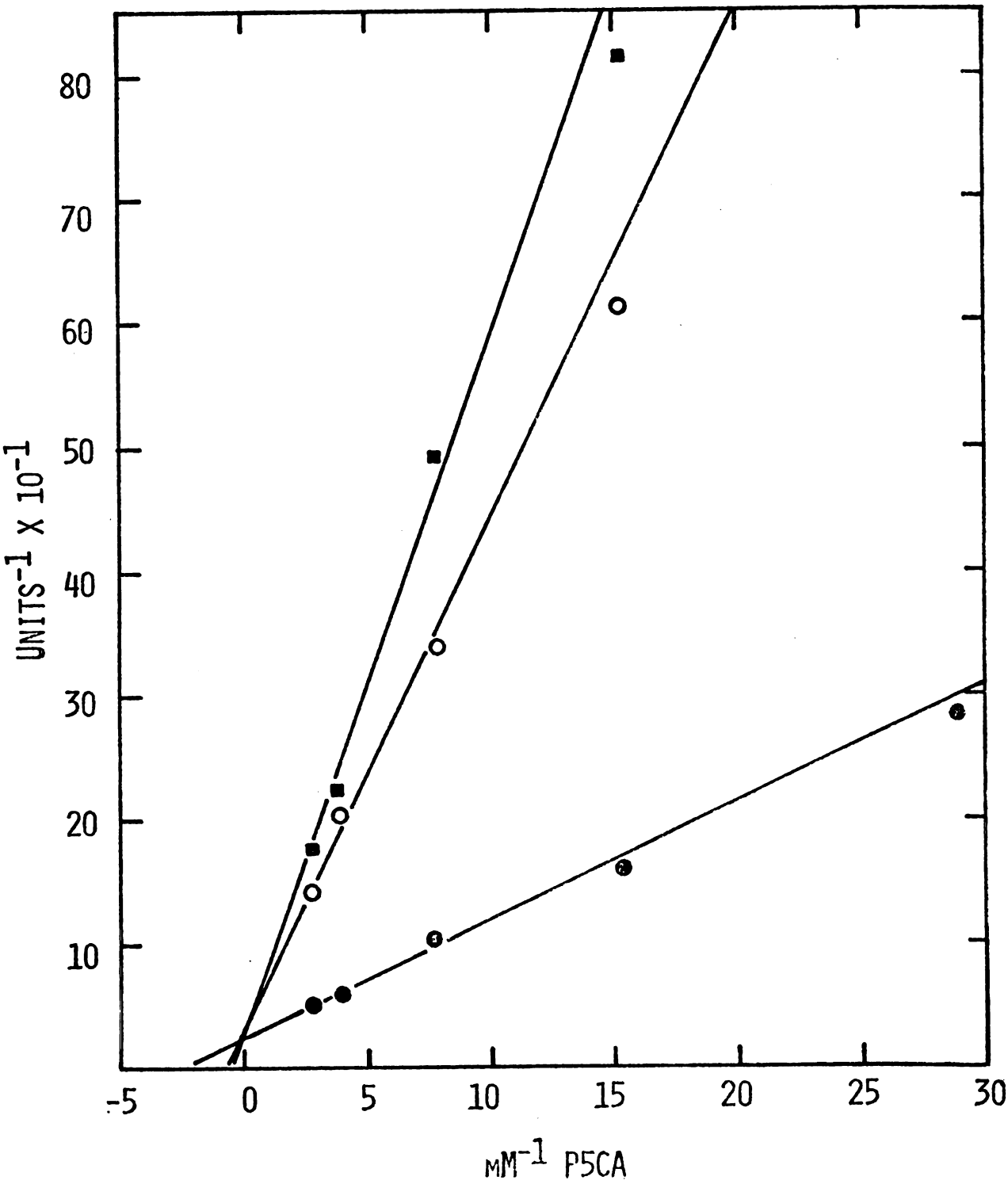
active P5CA reductase at pH 8.0, this K_m for NAD^+ may not be valid.

Reactions similar to those described above were conducted substituting $NADP^+$ for NAD^+ in the reaction mixtures. There was no evidence of any reduction of the $NADP^+$ when used at concentrations from 7.5 to 21 mM. Therefore, the enzyme is quite specific for NAD^+.

Affinity of proline dehydrogenase for Δ^1-pyrroline 5-carboxylic acid: According to an earlier report by Costilow and Laycock (9), the proline dehydrogenase was thought to catalyze the reversible interconversion of L-proline and P5CA, with the equilibrium of the reaction greatly in favor of proline. To test the affinity of the most purified proline dehydrogenase fraction from hydroxylapatite for P5CA, reactions were conducted as described in MATERIALS AND METHODS, measuring the P5CA-dependent oxidation of NADH as the rate of loss in absorbancy at 340 nm. For these reactions, P5CA was prepared from L-proline, using rat liver L-proline oxidase according to the method of Strecker (42). The partially purified enzyme showed a very great affinity for P5CA. A Lineweaver-Burk plot of the data indicated that the apparent K_m for P5CA was 0.5 mM at pH 8.0 (Fig. 12).

To determine the possible competition of P5CA and L-proline for a single active site on the proline dehydrogenase molecule, the same reactions used to determine the K_m for P5CA were conducted first with 225 mM L-proline and then with 300 mM L-proline added to each reaction mixture. A

Fig. 12.--Lineweaver-Burk plot for P5CA. P5CA reductase was measured by the rate of oxidation of NADH at 340 nm. Control (no L-proline), ●; 225 mM L-proline added to the reaction mixture, ○; 300 mM L-proline added to the reaction mixture, ■.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mM^{-1} P5CA

Lineweaver-Burk plot of these data along with the control data (Fig. 12) indicates that the P5CA reductase will bind proline at high concentrations.

Inhibition of proline dehydrogenase by L-glutamate: Since studies with ^{14}C-labeled L-proline and P5CA showed that proline dehydrogenase probably functions as part of an enzyme system to convert L-proline to glutamate, enzyme from each purification step was tested for possible feedback inhibition by L-glutamate. Enzyme activity in each fraction was measured as the rate of increase in absorbancy at 443 nm in the presence of o-aminobenzaldehyde with 0, 1, or 10 mM L-glutamate added to the proline dehydrogenase assay reaction mixture. Results showed at least 60% inhibition of proline dehydrogenase activity in the presence of 1 mM L-glutamate and as high as 82% inhibition in the most purified fraction from hydroxylapatite chromatography (Table 7). There was complete inhibition of activity in all preparations tested by 10 mM L-glutamate. This inhibition could not be reversed by increasing the concentration of NAD^+ in the reaction mixtures to 20 mM or 30 mM, indicating that the inhibition observed was not merely a result of competition for NAD^+ between proline dehydrogenase and the glutamate dehydrogenase present in each purification fraction. Two other compounds structurally similar to glutamate, α-ketoglutarate and L-aspartate, failed to inhibit proline dehydrogenase. Also, L-glutamate and α-ketoglutarate

Table 7.--Inhibition of proline dehydrogenase by L-glutamate.*

Fraction	mM L-glutamate	% Inhibition**
Crude	1	60
	10	100
Strep. sulfate	1	63
	10	100
Dial. 45-80% amm. sulfate	1	80
	10	100
DEAE-cell.	1	73
	10	100
Hydroxyl-apatite	1	82
	10	100

*Proline dehydrogenase activity was measured as the rate of increase in absorbancy at 443 nm. Reaction mixtures contained 450 mM L-proline, 10 mM NAD^+, and sodium glutamate as above, with between 0.2 and 0.009 mg protein per reaction.

**Inhibition was the same at NAD^+ concentrations of 10 mM, 20 mM, and 30 mM.

failed to inhibit the reduction of P5CA to proline, indicating that a separate enzyme may be involved in this reaction.

DISCUSSION

The NAD^+-dependent L-proline dehydrogenase from *Clostridium sporogenes* is the first enzyme of its kind reported to exist in procaryotic cells. All of the enzymes found thus far which oxidize L-proline to P5CA in procaryotes and animals have been oxidases and could not function in the absence of molecular oxygen. Very recently, however, NAD^+-dependent proline dehydrogenases have been found in pumpkin cotyledons (31, 32) and in *Chlorella* (21). There is very high P5CA reductase activity present in these cells, as there is also in *C. sporogenes*.

The question of whether or not P5CA reductase activity and proline dehydrogenase activity occur on the same protein(s) is still unresolved. The proline dehydrogenase was first described by Costilow and Laycock (9, 10) in *C. botulinum* and *C. sporogenes* as a reversible P5CA reductase (E.C.1.5.1.2), with the equilibrium far in the direction of proline. Evidence from this study shows that high concentrations of L-proline competitively inhibit the reduction of P5CA to proline, indicating that P5CA reductase and proline dehydrogenase activities may occur at the same site on a single protein. Also, preliminary evidence shows that proline dehydrogenase and P5CA reductase purify together. The ratios of their activities remain relatively constant with each purification step. This was also demonstrated

with the activities from pumpkin tissue (32). On the other hand, L-glutamate significantly inhibits proline dehydrogenase activity from C. sporogenes, but even high concentrations of glutamate do not affect P5CA reductase activity. Efforts to determine the kinetics of inhibition by glutamate have been frustrated so far due to the inability to completely separate glutamate dehydrogenase from proline dehydrogenase preparations. Preliminary evidence, however, indicates that the inhibition is non-competitive and cannot be reversed by very high levels of L-proline. If this is the case, glutamate may function by altering a single protein to inhibit the binding of proline but not P5CA. While there is no reason that this could not occur with a single enzyme, the existence of two different proteins seems a more reasonable explanation.

Rena and Splittstoesser (32) offer some evidence that NAD^+-dependent proline dehydrogenase and NAD(P)H-dependent P5CA reductase activities occur on the same protein molecule in the pumpkin Cucurbita moschata. The ratio of P5CA reductase to proline dehydrogenase remained nearly constant, between 1.4 and 1.6, over several purification steps. Both enzymes were also sensitive to inhibition by some heavy metals, though not to the same extent. The most interesting data reported, however, was the effect of pH on enzyme activity. In pumpkin extracts, P5CA reductase showed maximal activity at pH 6.5, and the maximum ac-

tivity of proline dehydrogenase occurred at pH 10, with no P5CA reductase activity at this pH. Results presented herein with C. sporogenes extracts also demonstrated that proline dehydrogenase activity was greatest at pH 10, and that no P5CA reductase activity was detectable at this pH. With both organisms, P5CA reductase activity appears to be far greater than proline dehydrogenase activity at pH 8.0. There is no evidence as yet with either organism as to the pH at which these reactions occur in vivo. It may be that proline dehydrogenase activity is being masked at pH 8 due to the rapid reduction of the reaction product, P5CA, back to proline. This phenomenon may account for the unusually high apparent K_m (33 mM) indicated by the present data for the proline dehydrogenase substrate, L-proline, at pH 8.0. High proline levels did inhibit P5CA reductase and thus would appear to increase rates of proline dehydrogenase activity. The problem of pH remains unresolved at this time. In any event, these data shed serious doubt on the hypothesis that a single reversible enzyme is responsible for the proline dehydrogenase and P5CA reductase activities in C. sporogenes cells.

Although the data are not conclusive, the present evidence from studies with ^{14}C-labeled proline indicates that proline dehydrogenase is involved in the dissimilation of L-proline in C. sporogenes. The conversion of ^{14}C-L-proline and ^{14}C-P5CA to ^{14}C-glutamate demonstrates the

existence of a P5CA dehydrogenase as well as a proline dehydrogenase in cell extracts. Since no significant conversion of ^{14}C-L-glutamate to ^{14}C-proline could be demonstrated in these cells, P5CA reductase evidently is not involved in an enzyme pathway from glutamate to proline. Neither is it involved in the conversion of ornithine to proline in C. sporogenes (10).

Based on the data accumulated to date, proline dehydrogenase may be involved in an overall scheme to convert arginine to glutamate in C. sporogenes, shown diagrammatically in Fig. 13. C. sporogenes requires arginine for growth in a synthetic medium (Costilow, unpublished data), while neither proline nor glutamate are required. It is also known that arginine is converted to ornithine via the arginine dihydrolase system in Clostridium (23), and that ornithine is primarily catabolized to proline by the enzyme ornithine cyclase (deaminating) (10). The intermediate involved in this conversion is Δ^1-pyrroline 2-carboxylic acid. Most of the proline is then reduced to σ-aminovaleric acid. Ornithine may also be oxidized to a very limited extent in C. sporogenes to alanine, acetate, ammonia, and carbon dioxide, in a reaction coupled with the reduction of proline to σ-aminovaleric acid. This activity is minimal compared to ornithine cyclase activity.

Data presented herein demonstrated a highly significant conversion of ^{14}C-ornithine to glutamate and some

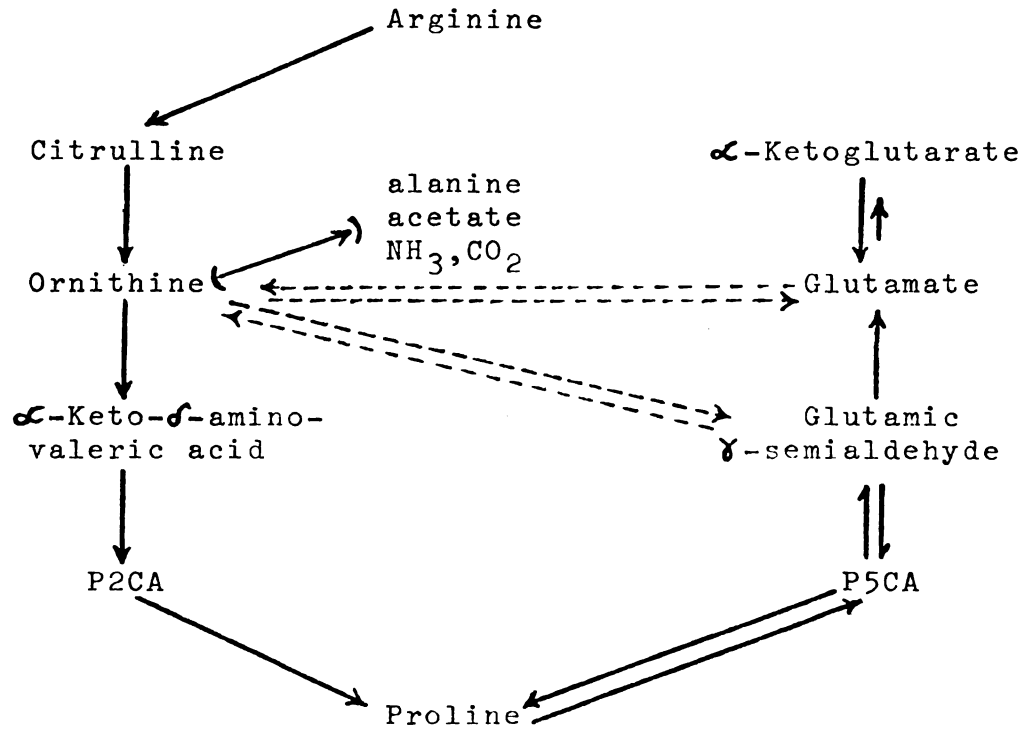

Fig. 13.--Postulated relationships of arginine, ornithine, proline, and glutamate in C. sporogenes. Dashed arrows represent reactions not known to occur in this organism.

conversion of ^{14}C-proline to glutamate. No significant label from ^{14}C-glutamate was found in either proline or in the basic amino acids. Previous reports indicate that ornithine cannot be directly converted to glutamate (10, 23) nor to glutamic γ-semialdehyde by an ornithine δ-transaminase reaction (10). Therefore, it appears likely that glutamate was primarily derived from the conversion of proline. This is the most probable physiological role of proline dehydrogenase in C. sporogenes.

Glutamate dehydrogenase, present in high levels in species of Clostridium (49), was shown to copurify to a great extent with proline dehydrogenase in C. sporogenes. However, the most highly purified fraction obtained from hydroxylapatite chromatography, contained only low levels of glutamate dehydrogenase, showing that these two enzyme activities were catalyzed by different proteins in C. sporogenes. Non-specific glutamate dehydrogenases have been identified in other organisms (43, 49), but L-proline has never been shown to be a suitable substrate for these enzymes.

The proline dehydrogenase activity in cell extracts of C. sporogenes is strongly inhibited by very low levels of L-glutamate (1 mM). This inhibition was shown to occur in fractions from all stages of purification. Doubling and tripling the already high (10 mM) concentrations of NAD^+ in the reaction mixtures could not reverse this inhibition.

This indicates that the inhibition observed was not merely due to the glutamate dehydrogenase reaction competing with the proline dehydrogenase for the available NAD^+. Studies on the precise mechanism of inhibition will require more highly purified proline dehydrogenase preparations. If indeed the primary role of the proline dehydrogenase is in the biosynthesis of glutamate, the glutamate inhibition observed may well be of the feedback type.

Another control of proline dehydrogenase in C. sporogenes appears to be catabolite repression by D-glucose. Simmons and Costilow (34) have shown that the enzymes of the Emden-Meyerhof-Parnass pathway of glucose metabolism are induced by D-glucose. Also, Stern and Bambers (35) demonstrated that in Clostridium kluyveri, α-ketoglutarate can be formed through a partial tricarboxylic acid cycle. If this is the preferred pathway to form glutamate in C. sporogenes, one might expect glucose to repress the enzymes required for alternate mechanisms of synthesizing glutamate, which would include the catabolism of L-proline to glutamate. This is exactly what appears to occur in Klebsiella aerogenes (7, 27, 28). In this organism, glucose represses the enzymes required for the formation of glutamate from histidine and proline.

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