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PROLINE DEHYDROGENASE FROM CLOSTRIDIUM SPOROGENES:

PURIFICATION AND PARTIAL CHARACTERIZATION

presented by

Daniel J. Monticello

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Master's degree in Microbiology

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PROLINE DEHYDROGENASE FROM *CLOSTRIDIUM SPOROGENES*: PURIFICATION AND PARTIAL CHARACTERIZATION

By

Daniel Joseph Monticello

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1979

ABSTRACT

PROLINE DEHYDROGENASE FROM CLOSTRIDIUM SPOROGENES:
PURIFICATION AND PARTIAL CHARACTERIZATION

By

Daniel Joseph Monticello

A single enzyme catalyzing the L-proline-dependent reduction of NAD^+ and the Δ-pyrroline 5-carboxylic acid (PCA)-dependent oxidation of NADH has been purified from extracts of Clostridium sporogenes. The enzyme has a molecular weight of 217,000, based on calculations from linear sucrose gradient centrifugation data, and is composed of two subunits, each weighing 108,000, based on SDS analytical disc gel electrophoresis. The L-proline-dependent NAD reducing activity of the enzyme was found to be more sensitive to incubation in low ionic strength buffer than the PCA-dependent NADH oxidizing activity of PDH. Proline dehydrogenase is inhibited by glutathione, cysteine, copper sulfate, p-chloromercuribenzoate and adenine nucleotides. Inhibition of the PCA-dependent NADH oxidizing activity of PDH by hydroxylamine was shown to be due to a reaction of the inhibitor with the substrate (PCA) and not to inactivation of the enzyme. The conversion of proline to PCA by PDH is noncompetitively inhibited by L-glutamate (K_i = 0.23 mM at pH 7.4, and 0.65 mM at pH 10.2). PDH activity in the reverse direction (PCA to proline) is not affected by 100 mM L-glutamate.

DEDICATION

To my family and friends, who saw me as I might be, and spent the time to help me there, and Elizabeth, who saw me as I am, and understands as we keep changing.

ACKNOWLEDGMENTS

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

I would also like to express my thanks to Dr. H. L. Sadoff and Dr. R. R. Brubaker for the use of their laboratory facilities.

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INTRODUCTION

The conversion of Δ^1-pyrroline 5-carboxylic acid (PCA) to proline by PCA reductase [L-proline:NAD(P)$^+$ 5-oxidoreductase EC1.5.1.2] has been observed in animal tissue (29, 41, 42) and a number of microorganisms (9, 33). This activity is irreversible, and is believed to function in the biosynthesis of proline from glutamate or ornithine (which is converted to PCA via ornithine-δ-transaminase). In these organisms, proline is oxidized to PCA by an irreversible proline oxidase (4, 12, 18).

Laycock and Costilow (7) observed that crude extracts of cells of Clostridium sporogenes and Clostridium botulinum catalyse the nicotinamide adenine dinucleotide (NAD)-dependent interconversion of L-proline and PCA. Costilow and Cooper (6) demonstrated that NAD-linked proline oxidation and NADH-linked PCA reduction in C. sporogenes is probably catalyzed by the same enzyme. NAD-dependent proline dehydrogenases (PDH) have also been identified in a number of plants (21, 22, 31) and in Chlorella (23). There is some evidence that these enzymes are reversible.

Clostridium sporogenes (26) and some higher plants (24) convert ornithine to proline via Δ^1-pyrroline 2-carboxylic acid (P2CA). Obviously, these organisms do not require PCA reductase for this conversion. It has been suggested that PDH may function

in the oxidation of proline to glutamate in C. sporogenes (6). This is supported by the observation that the oxidation of proline to PCA is inhibited strongly by L-glutamate (Costilow and Cooper, unpublished data).

The principle objectives of this investigation were:

1. Purification of PDH to unequivocally determine if the NAD-linked oxidation of proline and the NADH-linked reduction of PCA are catalyzed by the same protein.
2. Determination of the molecular parameters of the enzyme such as molecular weight, number and size of subunits, and inhibitors.
3. Quantitative determination of the effects of L-glutamate on PDH activity.

LITERATURE REVIEW

The Interconversion of Ornithine, Glutamate and Proline

The structural similarity of the amino acids ornithine, proline and glutamate has provoked much experimentation and speculation as to their possible metabolic interrelationships. The suggestion that animals can convert glutamate to proline was first made by Abderhalden (1) who, in 1912, demonstrated in dogs that protein hydrolysates rich in glutamate but with greatly reduced amounts of proline (which had been alcohol extracted from the hydrolysates) were as nutritionally effective as whole hydrolysates.

As early as 1910, Neubaur and his colleagues (27) presented experimental evidence that natural amino acids were oxidized to their corresponding keto acids and ammonia in rat liver perfusion studies. In 1936, Bernheim, Bernheim and Gillaspie (3) used rat kidneys to examine the oxidation of amino acids. They were able to follow the course of the oxidations manometrically, and to assay for the keto acids formed from various amino acids by precipitation with either phenylhydrazine or sodium bisulfite. Such treatment led to the formation of yellow crystals which were easily visualized. Additional of proline to a purified preparation, followed by addition of bisulfate after the completion of oxidation led to the formation of crystals. However, when proline was added to tissue

slices, no crystals were formed on addition of bisulfite. This led the authors to hypothesize:

> . . . A scheme fitting all these facts would involve the loss of one hydrogen atom from the nitrogen and one from the adjacent carbon to which the carboxy group is not attached. This would leave a double bond which might hydrolyze to the corresponding aldehyde. The purified preparation evidently takes the oxidation no further and this would account for the formation of the bisulfate compound. With the tissue slices, however, this aldehyde could be oxidized to the acid, thus giving glutamic acid.

This hypothesis was supported by the work of Weil-Malherbe and Krebs (42), who observed the _in vitro_ conversion of proline to glutamate in rat tissue in 1935, and by Krebs (14), who demonstrated the oxidation of D-proline to Δ^1-pyrroline-2-carboxylic acid (P2CA) in 1939. These observations were the beginning of an investigation into the interconversion of glutamate and proline which would include species as diverse as rates, fungi, bacteria and plants.

The first experiments demonstrating the _in vivo_ conversion of proline to glutamate were performed by Stetten and Schoenheimer (38) in 1943. Deuterium labeled proline (produced by shaking α-pyridone, an organic precursor of proline, in 99.6 atm of deuterium gas at 100°C for 7 hours) and ^{15}N-proline were used in this study to determine the fate of proline fed to rats. These experiments demonstrated that the nitrogen in proline remained with the molecule in its conversion to glutamate. This was strong experimental support for the hypothesis proposed earlier by Benheim et al. (3). Concurrently, Blanchard et al. demonstrated _in vitro_

that a single amino acid oxidase from rat liver was responsible for the oxidation of at least 11 naturally occurring amino acids.

In 1949, Taggert and Krakaur (40) demonstrated in rabbit kidney preparations that the intermediate between proline and glutamate was Δ^1-pyrroline 5-carboxylic acid (PCA), and that it was in spontaneous equilibrium with glutamic γ-semialdehyde. Lang and Schmid (17) reproduced these results in 1951. The metabolic pathway between proline and glutamate in Escherichia coli was being examined at the same time by Vogel and Davis (41), by means of metabolic studies on mutants. In 1952, they demonstrated that PCA was the intermediate in this pathway, and showed that some proline auxotrophs were able to grow when supplied with P5CA. They obtained similar results in proline auxotrophs of Neurospora crassa.

In 1957, Meister, et al. (25) suggested that P2CA and PCA might be involved in alternate pathways between glutamate and proline. They demonstrated that these compounds were reduced to proline by two different enzymes in Neurospora crassa and Aerobacter aerogenes. In addition, they found an enzyme capable of reducing both Δ^1-piperidine 2-carboxylic acid to pipecolic acid and P2CA to proline, in rat tissue and in Pisum sativum (garden peas).

One of the early problems in the experiments with P5CA was the difficulty of preparing the compound in a pure form. In 1960, Strecker (39) overcame this problem and was able to purify and to some extent characterize the compound. In this work, he notes

that P5CA does not make a very stable addition product with bisulfite, but will react with 2,4-dinitrophenylhydrazine to make a stable compound. Both of these reagents were believed to be attacking the free aldehyde (glutamic γ-semialdehyde) which is in spontaneous equilibrium with PCA.

The Conversion of Ornithine to Proline

Muth and Costilow (26) demonstrated that ornithine is converted to proline in C. sporogenes via α-keto-δ-aminovaleric acid and P2CA intermediates. They utilized uniformly labeled ornithine (^{14}C), also labeled in the δ-amino group with ^{15}N, and demonstrated that this nitrogen atom was conserved in the enzymatic cyclization of ornithine to proline. In addition, they showed that this conversion was catalyzed by a single enzyme, ornithine cyclase (deaminating). C. sporogenes does not convert ornithine to PCA via an ornithine-δ-transaminase pathway. If glutamate is produced from ornithine in this species, the most likely pathway is via proline and PCA, as outlined in Figure 1.

Recently (1979), Mestichelli, et al. demonstrated that ornithine is converted to proline via P2CA in several higher plants (Nicotiana tabacum, Datura stramonium and Lupinus angustifolius). Using tracer methods with ornithine labeled with ^{3}H and ^{14}C, these investigators demonstrated that the conversion to proline takes place with the maintenance of the δ-hydrogen atoms but with the loss of the α-hydrogen atoms. This indicated a route via α-keto-δ-aminovaleric acid and P2CA, and disproved the accepted route via

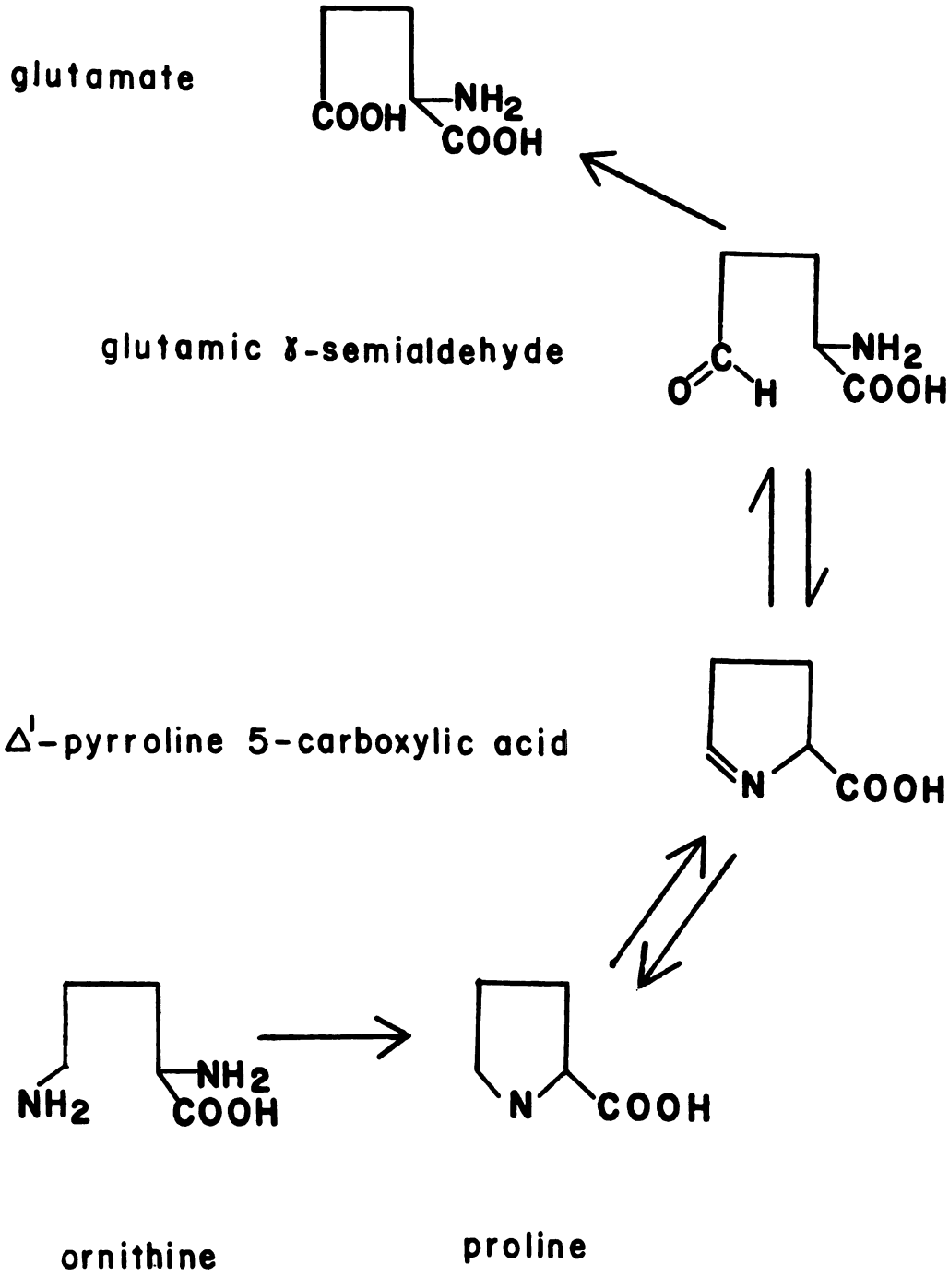


Figure 1.--Postulated relationship of ornithine, proline, and glutamate in Clostridium sporogenes.

glutamic γ-semialdehyde and PCA. Enzymes for the conversion of proline to PCA has been found in several higher plants (21, 22, 31). It seems likely that the pathway for the conversion of ornithine to glutamate outlined in Figure 1 may be operational in some higher plants.

PCA Reductase

In 1964, the nature of the regulation of proline biosynthesis in E. coli was further characterized. Baich and Pierson (2) showed that in proline biosynthesis, control is localized in the first reduction step from glutamic acid to glutamic γ-semialdehyde. This reaction is strongly inhibited by small amounts of proline in growing and resting cells. The second reaction, the reduction of PCA (which is in spontaneous equilibrium with glutamic γ-semialdehyde) to proline by PCA reductase (PCAred) proceeds unrestrained in the presence and absence of proline. They isolated a proline excreting mutant, and in this strain the reduction of gluamic acid to glutamic γ-semialdehyde is relatively insensitive to proline.

In 1977, PCA reductase from E. coli was partially purified and characterized by Rossi et al. (33). The enzyme, with an approximate molecular weight of 320,000, was found to have a similar K_m for PCA regardless of whether NADH or NADPH was used as a cofactor. The K_m of the enzyme was much higher for NADH than NADPH (0.23 and 0.03, respectively). They observed that this non-particulate enzyme was not repressed by growth in the presence of proline, but was inhibited by the reaction end products, proline and NADP.

This enzyme is not reversible even at very high substrate concentrations.

Costilow and Cooper (6) identified a PCAred in extracts of C. sporogenes cells which was specific for L-PCA and NADH. The K_m for L-PCA of this enzyme was found to be 0.33 mM at pH 8.0, and 0.2 mM at pH 6.5. Based on a sedimentation coefficient of 10.38 they estimated the molecular weight of the enzyme to be 200,000 daltons. In addition, the PCAred activity copurified extensively with proline dehydrogenase which catalyzes the reverse reaction. This evidence suggested that the activities were located on the same protein moiety. However, a completely purified preparation was not obtained.

Proline Oxidase

In 1962, Johnson and Strecker (12) demonstrated that the oxidation of proline in rat liver required oxygen and cytochrome c. In addition, non-physiological oxidants could serve as electron acceptors. They found that pyridine nucleotides were not required for activity, nor were they reduced in the presence of proline and the liver preparation. They suggested that the proline oxidase was linked to the respiratory chain, and did not require dissociable coenzymes. Soon after this, Peisach and Strecker (29) demonstrated a non-reversible, pyridine nucleotide linked PCAred (Δ^1-pyrroline 5-carboxylic acid specific) was responsible for the reduction of PCA to proline in calf liver extract. Both NAHD and NADPH served as electron donors, although NADPH was the preferred substrate.

These observations added support to the growing belief that the conversion of glutamate to proline is catalyzed by enzymes which differ from those used in the oxidation of proline.

In 1964, Ling and Hendrick examined proline oxidase in the yeast Hansenula subpelliculosa (18). They found two distinct enzymes. One was constitutive, and it converted very small amounts of proline to P2CA. They speculated that this enzyme was associated with the use of proline as a nitrogen source. The second enzyme was inducible with proline, and yielded PCA. They also reported the presence of NAD-linked enzymes in the crude extracts able to oxidize both P2CA and PCA.

In the same year, Frank and Ranhand (11) examined proline catabolism by E. coli. They noted that proline was oxidized in the same fashion as had been previously noted in animal tissue. Proline was oxidized to PCA by a membrane bound oxidase, and the PCA oxidized to glutamate by a pyridine nucleotide linked PCA dehydrogenase. Analysis of mutants demonstrated that the proline oxidase and PCA reductase were unrelated. Mutants with one activity and not the other were readily isolated. However, they also observed that it was impossible to separate the proline oxidase and the PCA dehydrogenase, and suggested that the entire proline oxidase complex may exist as a single physical entity.

DeHauwer et al. (9) in 1964, and Dendinger and Brill (10) in 1970, studying Bacillus subtilis and Salmonella typhmurium respectively, demonstrated that these bacteria had pathways for proline degradation identical to E. coli. Laishley and Bernlohr

(16), in 1968, presented some evidence to suggest that PCA may be the inducer of proline catabolism in B. licheniformis.

The proline oxidase from E. coli was purified and characterized in 1978, by Scarpulla and Soffer (34). They were able to separate the enzyme from PCA dehydrogenase, to which it is tightly coupled in the bacterial membrane (11). They demonstrated that the formation of PCA was more than 99% dependent on the presence of an artificial electron transport system (consisting of phenazine methosulfate and p-iodonitrotetrazolium) in the assay preparation. This indicates that molecular oxygen does not function as a proximate electron acceptor. The authors suggested that the enzyme be termed a dehydrogenase, to distinguish its activity from the amino acid oxidases which are directly linked to molecular oxygen.

In a subsequent paper by these authors in 1979 (35), they examined the role of leucyl-, phenylalanyl-tRNA:protein transferase in the regulation of the membrane bound proline oxidizing enzyme. In mutants lacking the transferase, an increase in the level of proline oxidation was observed. In this investigation, the authors exclude the possibility of modification of the proline oxidase demonstrating that the increased activity is due to more of the enzyme in the mutants than the wild-type cells. They suggest rather than modifying the enzyme by direct covalent modification, the transferase, or an acceptor substrate(s) may perhaps be a regulatory molecule which controls the biosynthesis of proline oxidase (dehydrogenase) at the transcriptional or translational level.

Proline Dehydrogenase

In 1969, Costilow and Laycock (7) reported that C. sporogenes and C. Botunlinum Type A contained a pyridine nucleotide linked PCAred. In addition, they demonstrated for the first time a pyridine nucleotide linked proline oxidation. The oxidation was specifically NAD-dependent and the product was PCA. Subsequently, NAD-linked proline oxidation has been observed in several higher plants and in algae. In 1971, Mazeliis and Fowden found a proline dehydrogenase in peanut seedlings (22). This non-particulate enzyme was specific for L-proline and NAD. The pH optimum of the enzyme was 10.3, and NADP acted as a competitive inhibitor. The product of the oxidation was not identified, but it was not believed to be P2CA or PCA. However, a proline dehydrogenase (PDH) from wheat germ was identified in 1974 by Mazelis and Creveling (21) which converted proline to PCA. This enzyme was reversible. The dehydrogenase activity was specific for L-proline and NAD, and the reductase activity for PCA and NADH. NADP and NADPH were good competitive inhibitors. They estimated the molecular weight of the molecule to be 200,000 daltons.

Shortly after these initial findings, PDH was reported as being present in two other systems. Rena and Splittstoesser (31) demonstrated an NAD-linked proline oxidation, and an NADH-linked PCA reduction in pumpkin cotyledons. Although unable to purify the enzyme, their data suggested that the two activities were catalyzed by the same protein molecule. They noted that the ratio of PDH:PCAred changed during the storage of the enzyme, and that

PCAred activity was inhibited to some extent by sodium bisulfite, and to a much greater extent by hydroxylamine. McNamer and Stewart (23) found PDH activity in Chlorella. The activity had a pH optimum of 10.2, and was specific for L-proline and NAD. They estimated the molceular weight as ". . . greater than 100,000. . . ." The reversibility of the reaction was apparently not studied.

In 1978, Costilow and Cooper reported on the partial purification of PDH from C. sporogenes. They demonstrated that the two activities coelute from diethylaminoethyl (DEAE)-cellulose, hydroxylapatite and Sephadex G-200 columns. Both have identical sedimentation coefficients and isoelectric points and are heat-stabilized by high ionic strength buffer. The activities of both PDH and PCAred could be reduced by 50% when glucose was added to the growth medium. The pH optima for the two activities were very different, pH 10.2 for PDH and 6.5-7.5 for PCAred. A small increase in the pH resulted in large shifts of the reaction equilibrium toward PCA. However, even at pH 8.6 the equilibrium constant for PDH was about 2.5×10^{-5}. The authors proposed that L-proline and L-PCA are interconverted by ". . . either a single enzyme or an enzyme complex . . ." in extracts of C. sporogenes cells.

Some doubt has been cast on the significance of PDH in plants. Boggess et al. (5) in 1978, demonstrated that mitochondria isolated from etiolated shoots of corn, wheat, barley, soybean and mung bean exhibited a proline dependent uptake of oxygen subject to respiratory control and independent of NAD concentration. These authors suggest that PDH may not play a role in proline oxidation

in vivo, based on the facts that (1) ". . . the necessity to assay it at high pH . . .," and (2) . . . that it copurifies with PCA reductase, the NADH-linked proline biosynthetic enzyme that is typically stable and present in relatively high activity. . . ." However, Costilow and Cooper indicate in a paper published at the same time (6) that the high pH optimum of PDH is to be expected if the oxidation of proline and the reduction of PCA are catalyzed by a reversible enzyme.

In organisms which convert ornithine to proline via P2CA, proline dehydrogenase may play an important role in the oxidation of ornithine and proline to glutamate. Until recently, only species of the clostridia (7, 26) had been shown to convert ornithine to proline in this manner. In most organisms, ornithine is thought to be converted to proline via a ornithine-δ-transaminase enzyme (which converts ornithine to PCA) and then a PCA reductase. Such a pathway would not require the presence of PDH for the conversion of ornithine to glutamate. However, Mestichelli et al. (24) have shown that several species of plants convert ornithine to proline via P2CA. In addition, they argue that the evidence supporting the proposal that ornithine is converted to proline via PCA in other plant, animal and microbial systems can be interpreted to show that this conversion results from the loss of the α-amino group from ornithine (yielding P2CA), not the δ-amino group (which yields PCA). Obviously, this would make PDH very important for the conversion of ornithine to glutamate in these systems.

It is clear that despite the large amount of accumulated information on the interconversions of ornithine, proline and glutamate, this system is still not well understood. The times seems ripe for a re-examination of the metabolic relationship among these amino acids.

MATERIALS AND METHODS

Culture and Cultural Methods

Clostridium sporogenes (ATCC 7955, National Canners Association PA 3679) was used in all experiments. Large batches of the cells for enzyme purification were regularly grown in 20 liter glass carboys at 37°C. All growth experiments were conducted utilizing a Coy Manufacturing Co. anaerobic chamber.

Growth Media

The media used in this investigation were:

Medium A: 4.0% trypticase, 2 ppm thiamine-hydrochloride and 0.05% sodium thioglycollate, brought to pH 7.4 with sodium hydroxide.

Medium B: Modified Perkins and Tsuji (30) synthetic medium, consisting of salts, vitamins, sodium thioglycollate and 10mM concentrations of L-arginine, glycine, L-histidine, L-leucine, L-lysine, L-serine, L-methionine, L-phenylalanine, L-threonine, L-valine, L-tyrosine, with 0.5 mM concentrations of L-tryptophan and L-cysteine.

Buffers

The buffers employed in these studies were:

Buffer A: 0.15 M tris(hydroxymethyl)aminomethane-chloride buffer, 2 mM dithiothreitol, 10 mM L-glutamate (free base), 10% glycerin, pH 7.4.

Buffer B: 20 mM potassium phosphate buffer, 2 mM dithiothreitol, 10% glycerin, pH 7.4

Buffer C: 0.25 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 7.4.

Buffer D: 0.25 M potassium phosphate buffer, pH 7.4.

Enzyme Assays

Proline dehydrogenase (PDH) was assayed by monitoring the proline-dependent reduction of NAD, or the PCA-dependent oxidation of NADH. Assays for proline oxidation referred to as PDH determinations were conducted at two pH levels. In both assays the rate of reduction of NAD was followed by monitoring the increase in absorbancy at 340 nm (A_{340}). Reaction mixtures of 1 ml at pH 10.2 contained 0.2 M sodium bicarbonate buffer, 10 mM NAD and 50 mM L-proline. At pH 7.4, 0.32 M potassium phosphate buffer was used instead of bicarbonate. In studies of L-glutamate inhibition, 10 mM L-glutamate was added to the reaction mixture prior to the addition of L-proline, to ensure that there was no glutamate dehydrogenase activity in the enzyme preparation. The NADH-linked reduction of PCA by PDH (referred to as PCAred in the text) was assayed by monitoring the loss of absorbancy at 340 nm. Typical reaction mixtures contained 0.32 M potassium phosphate buffer (pH 7.4), 0.75 mM PCA, 0.1 mM NADH and enzyme. Endogenous oxidation of NADH was corrected for when necessary.

Glutamate dehydrogenase (GDH) was assayed in reaction mixtures of 0.25 M potassium phosphate buffer, pH 7.4, 0.1 mM NADH,

110 mM ammonium chloride, 50 mM α-ketoglutarate and enzyme. The α-ketoglutarate-dependent oxidation of NADH was monitored at 340 nm. Catalase activity in the sucrose density gradient experiments was assayed with 20 mM hydrogen peroxide in 50 mM potassium phosphate buffer at pH 7.4. Loss of absorbancy at 240 nm was monitored. Reaction mixtures for alcohol dehydrogenase contained 320 mM ethanal, 10 mM NAD, in 50 mM sodium pyrophosphate buffer, pH 8.8.

For all of the enzymes, one unit of enzyme activity is defined as that amount of enzyme necessary to convert 1 μmole of substrate to 1 μmole of product in 1 minute. Specific activity is defined as units of enzyme per milligram of protein. Protein concentrations were routinely assayed by the method of Kalb and Bernlohr (13) and in some cases the method of Lowry et al. (19). In the assays utilizing NAD and NADH, the millimolar extinction coefficient of reduced nicotinamide adenine dinucleotide employed was 6.22 mM^{-1} cm^{-1}.

Preparation of Δ^1-Pyrroline 5-Carboxylic Acid

DL-PCA was produced by the method of Williams and Frank (43), which involves the peroxidation of δ-hydroxylysine to glutamate-semialdehyde, which is in equilibrium with PCA. Quantitative assays of PCA were performed by measuring the color formed with o-aminobenzaldehyde, using a millimolar extinction coefficient of 2.94 mM^{-1} cm^{-1} at 444nM (43). This assay is based on the formation of yellow dihydroquinolinium salts when cyclic imines react

with o-aminobenzaldehyde. Preparation were stored until use in 1 N hydrochloric acid at -20°C.

Gel Electrophoresis

Native protein disc gel electrophoresis was accomplished by the method of Davies (8). Gels were stained for protein using Coomassie blue G, and destained in 7% acetic acid. PDH activity in the gels was detected by incubating the gel at 37°C in 23 ml of 25 mM tris-chloride buffer (pH 7.5) containing 300 mM L-proline, 2 mM NAD, 12 mg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium. GDH activity in the gel was located in a similar fashion, replacing proline with 10 MM L-glutamate.

Subunit molecular weight was determined by the sodium dodecyl sulfate (SDS) disc gel electrophoresis method of Laemmli (15). Samples of the purified protein were mixed with buffer containing 3% SDS and 5% 2-mercaptoethanol, and subsequently heated at 90°C for 15 minutes. Samples were layered onto 7% acrylamide gels (10 cm in length) with a 1 cm 3% acrylamide stacking gel. The electrode buffer consisted of 0.1% SDS in 25 mM tris-193 mM glycine buffer, pH 8.3. A current of 1 ma per tube was applied through the gels until the dye (bromphenol blue) front just entered the lower gel, at which time the current was increased to 2 ma per gel.

The gels were immersed overnight in a 10% trichloroacetic acid, 33% methanol solution, to fix the protein and extract the SDS. Protein bands were developed with Coomassie blue G, and destained in 7% acetic acid.

Sucrose Density Centrifugation

A value for the molecular weight of PDH was obtained using linear sucrose gradients (6 to 30%) by the method of Martin and Ames (20). Samples of the purified enzyme (2 μg) were combined with alcohol dehydrogenase (yeast) and catalase (bovine liver) standards in 50 mM potassium phosphate buffer (pH 7.4) with a total volume of 150 μl, and layered on the gradient. The gradient was centrifuged in an SW50.1 rotor in a Spinco model L ultracentrifuge (Beckman-Spinco) at 35,000 rpm for 10.5 h. Two-drop fractions were collected (43 total fractions), and assayed for the enzyme activities.

Column Chromotography

In preliminary experiments, a variety of chromatographic systems were analyzed to determine their utility in purifying the enzyme, and in the separation of PDH from traces of GDH activity. The methods used in the final preparation are described below.

DEAE-Cellulose Chromatography

Diethylaminoethyl(DEAE)-cellulose (Cellex D, Biorad Laboratories) was equilibrated with buffer A as per the manufacturer's instructions, and used to prepare a 3.0 x 45 cm column. A sample previously equilibrated by dialysis against buffer A was layered onto the column. The column was washed with buffer A, and the effluent monitored at 280 nm with an Isco model UA-2 absorbance recorder and optical unit. After an initial absorbance peak was observed and the recorder returned to baseline, a linear gradient

was developed from 0.15 to 0.30 M tris-chloride, pH 7.4 (250 ml resevoirs). Concentrations of DTT, glutamate and glycerin were as in buffer A. In preliminary experiments NAD or L-proline were substituted for L-glutamate in the elution buffers. Fractions of 3 ml were collected, with a flow rate of 40 ml/h, and assayed for PCAred and GDH. Fractions with a PCAred:GDH ratio of greater than one were pooled, and concentrated to a final volume of 5 ml by ultrafiltration through a Diaflo PM-10 (Amicon Corp.) membrane using nitrogen as the pressurizing gas.

Sephadex G-10 Desalting Column

A 1 x 20 cm column of Sephadex G-10 (Pharmacia Fine Chemicals) column was employed to quickly desalt enzyme preparations, and equilibrate the protein in a new buffer system. Samples of 5 ml applied to the column eluted off in 7 ml, after a void volume of 8 ml.

Hydroxylapatite Column Chromatography

A 1.5 x 30 cm hydroxylapatite column (Biogel HTP, Biorad Labs) was prepared and poured as per the manufacturer's instructions, and equilibrated with buffer B. Enzyme preparations equilibrated into this buffer with a Sephedex G-10 column were applied to the column and washed with a sufficient volume of the starting buffer to return the A_{280} recorder to baseline. The enzyme was eluted with a linear gradient from 0.02 to 0.30 M potassium phosphate buffer, pH 7.4 (150 ml of each). Concentrations of DTT and glycerin

were as in buffer B. Fractions of 2.5 ml were collected with a flow rate of 30 ml/h, and assayed for PCAred and GDH. Fractions containing more than 10% of the PCAred activity observed on the peak fraction were pooled and concentrated through a Diaflo PM-10 membrane to a final volume of 5 ml.

Biogel A-5M Column Chromatography

A 2.5 x 45 cm column of Biogel A-5M (Biorad Labs) was equilibrated with buffer C. An elution volume of 50 ml was determined for blue dextran (molecular weight 2,000,000). A 5 ml sample was applied to the column, and after 80 ml had eluted off, 0.5 ml fractions were collected (flow rate of 10 ml/h), and assayed for PCAred and GDH. Fractions with PCAred activity and no detectable GDH activity were pooled and concentrated to a final volume of 5 ml.

Sephadex G-200 Column Chromatography

A 2.5 x 45 cm column Sephadex G-200 (Pharmacia) was equilibrated overnight in buffer C. A void volume of 70 ml was determined with blue dextran. A 5 ml sample was layered on the moist column bed, and after it was moved into the column, and washed in with small volumes of buffer, a head pressure of 10 cm was applied. Fractions with PCAred activity were pooled, concentrated to a volume of 5 ml, and stored in 0.5 ml aliquots at -21°.

Chemicals

o-Aminobenzaldehyde, γ-hydroxylysine (a mixture of hydroxy-DL-lysine and allohydroxy-lysine), NAD and NADH were obtained from Sigma Chemical Co. These and all other chemicals used were of the highest standards of purity available.

RESULTS

Purification of the Enzyme

A summary of the effectiveness of the purufication procedure is given in Table 1. Whenever possible, all enzyme preparations were maintained in high ionic strength buffer and held at refrigerator temperatures. Procedures used in individual steps are outlined below.

Growth of Cells

For the batchwise purification of PDH, 18 liters of medium A in a 20 liter glass carboy were inoculated with 2 liters of exponentially growing C. sporogenes previously cultured in an anaerobic chamber (see Materials and Methods). The carboy was equipped with air locks and stirred slowly with a magnetic stirrer at 37°C. After 8-10 hours, the cells were harvested with a Sharples continuous flow centrifuge, model AS-10. The collected cells were suspended in buffer D, 10 ml per gram wet weight of cells.

Preparation of Crude Extracts

Although sonication was used in some early experiments, crude extracts were usually prepared with a French press, which was found to be more effective. Cell debris was removed by centrifugation at 20,000 x g for 20 minutes.

TABLE 1.--Purification scheme for proline dehydrogenase.*

Step	Preparation	Total Protein	Specific Activities			Activity Ratio		
			PDH	PCAred	GDH	PDH	PCAred	GDH
1	Crude extract	1200 mg	0.01	0.30	9.6	1	30	960
2	Streptomycin sulfate	1330 mg	0.01	0.24	10.9	1	24	1090
3	Ammonium sulfate	137 mg	0.035	1.09	0.83	1	28	23
4	DEAE-cellulose	10.1 mg	0.20	6.4	5.4	1	32	27
5	Heated, 65 -5 min	1.1 mg	1.46	33.6	51.2	1	23	35
6	Hydroxylapatite	0.6 mg	0.96	96.0	7.8	1	100	7.8
7	Biogel A-5M	0.41 mg	1.12	103	0	1	92	0
8	Sephadex G-200	0.25 mg	1.8	174	0	1	96	0

*PDH was assayed at pH 7.4 (37°C). The reverse reaction of PDH (PCAred) and GDH were also assayed at pH 7.4. See Materials and Methods for experimental details.

Streptomycin Sulfate Precipitation

An equal volume of 5% streptomycin sulfate in buffer D was slowly combined with the crude extract. This solution was stirred slowly overnight at 4°C and centrifuged at 20,000 x g for 20 minutes, to remove nucleic acids.

Ammonium Sulfate Fractionation

In crude extracts the ratio of GDH to PDH is approximately 1000 to 1. GDH from C. sporogenes has been found to elute over a very broad range of ionic strengths in a variety of chromatographic systems (6), and, in the later steps of attempted purifications, constituted the major contaminating protein. Ammonium sulfate precipitation was found to be a very effective means of lowering the GDH:PDH ratio. Even though a very high percentage of the total PDH activity was lost as a result of the procedure used, this was necessary for the final removal of GDH in subsequent steps of the purification.

The streptomycin sulfate treated solution was brought to 70% saturation with ammonium sulfate added over several hours, and stirred slowly overnight at 4°C. Following centrifugation at 20,000 for 20 minutes, the supernatant solution was brought to 80% saturation, stirred overnight and centrifuged. The pellet derived from this treatment was suspended in 50 ml of 70% saturated buffer D, stirred for eight hours and centrifuged at 20,000 x g for 20 minutes. Of the 13.3 units of PDH activity used in this purification step,

only 4.8 units (36%) were recovered. However, of the 14,500 units of GDH in the streptomycin sulfate preparation, only 113 units (less than 0.8%) remained after this step. This step effectively reduced the GDH:PDH specific activity ratio from 1090:1 to 23:1.

DEAE-Cellulose Column

A number of preliminary experiments with different elution buffers demonstrated that under most conditions GDH and PDH co-elute. The addition of 10 mM L-glutamate to the elution buffer greatly improved the separation. Figure 2 shows the results of an early experiment with an extract still having a high GDH:PDH ratio. The addition of the glutamate resulted in the elution of both GDH and PDH earlier in the gradient, and in the partial separation of the two activities. Figure 3 shows the elution profile obtained from the DEAE-cellulose column used in the final purification. The primary objective of this column was not the removal of GDH, but to remove much of the other protein contamination. Consequently, the ratio of GDH:PDH (Table 1) was not greatly affected by this step. It was not possible to eliminate the tailing of the GDH peak into the PDH peak.

Heat Treatment

PDH is quite stable to heating at 65°C for 5 min when dissolved in a high ionic strength buffer. The concentrated preparation from the DEAE-cellulose column was heated to 65°C by immersion in a water bath. The sample was agitated rapidly. After 5 min. the sample was immersed in an ice water bath for 5 min, and then

Figure 2.--Elution profiles from preliminary experiments with DEAE-cellulose columns. The elution buffer in A was tris-chloride buffer with DTT and glycerin. An identical elution system was used in B with the addition of 10 mM L-glutamate. Approximately 30 mg of protein from a preparation from step 3 (Table 1) was used in both columns. Fractions of 4 ml were collected. Symbols: closed circles--proline dehydrogenase (assayed by PCAred activity); open circles--glutamate dehydrogenase. See Materials and Methods for experimental details and enzyme assays.

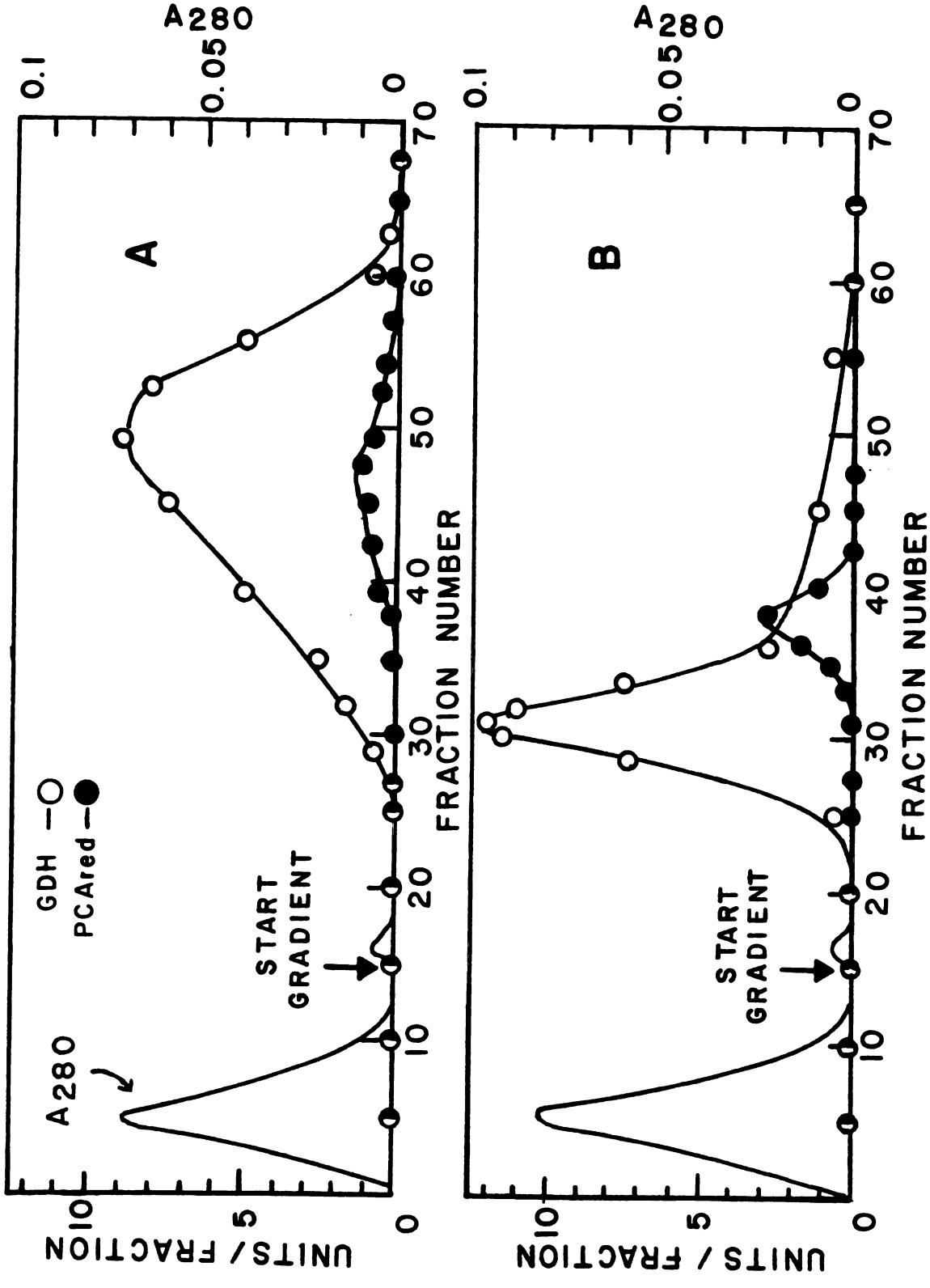
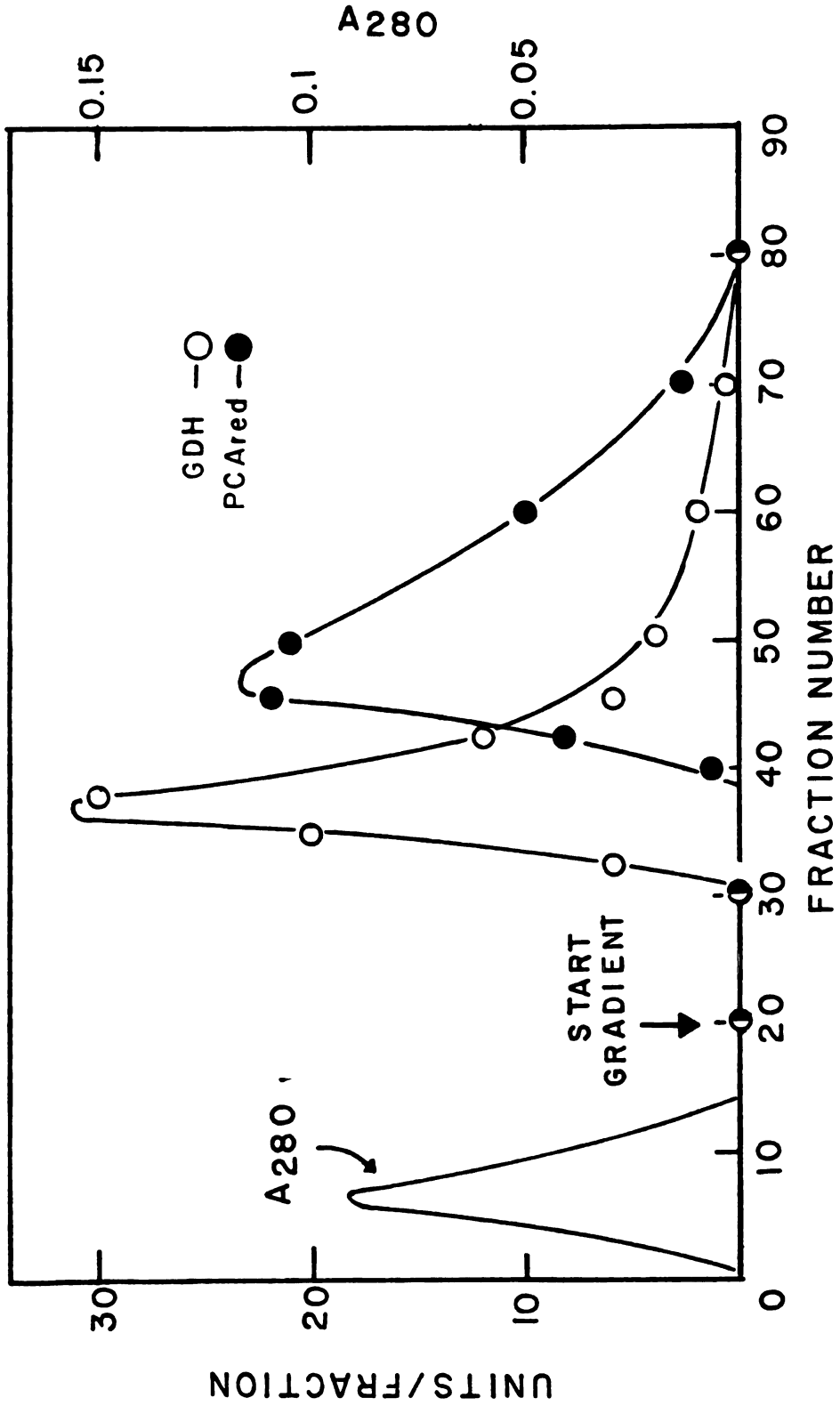

A
B
A280
0.1
0.05
0
70
60
50
40
30
20
10
0
GDH —○
PCAred —●
START GRADIENT
FRACTION NUMBER
UNITS / FRACTION
10
5
0

Figure 3.--Elution profiles of PDH activity and GDH activity from the DEAE-cellulose column used in the final purification scheme. Fractions of 7 ml were eluted with a linear gradient of tris-chloride buffer with DTT, glycerin and 10 mM L-glutamate. 135 mg of protein from step 3 (Table 1) were applied to the column. Symbols: closed circles--proline dehydrogenase (PDH), assayed by monitoring the PCA-dependent oxidation of NADH (PCAred activity); open circles--glutamate dehydrogenase (GDH). See Materials and Methods for experimental details, and Table 1 for recoveries.


A280
0.15
0.1
0.05
GDH
PCAred
START
GRADIENT
A280
UNITS/FRACTION
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90

centrifuged at 20,000 x g for 20 min. GDH is also stable under these conditions, consequently, little change in the GDH:PDH ratio (from 27:1 to 35:1) resulted.

Hydroxylapatite Column

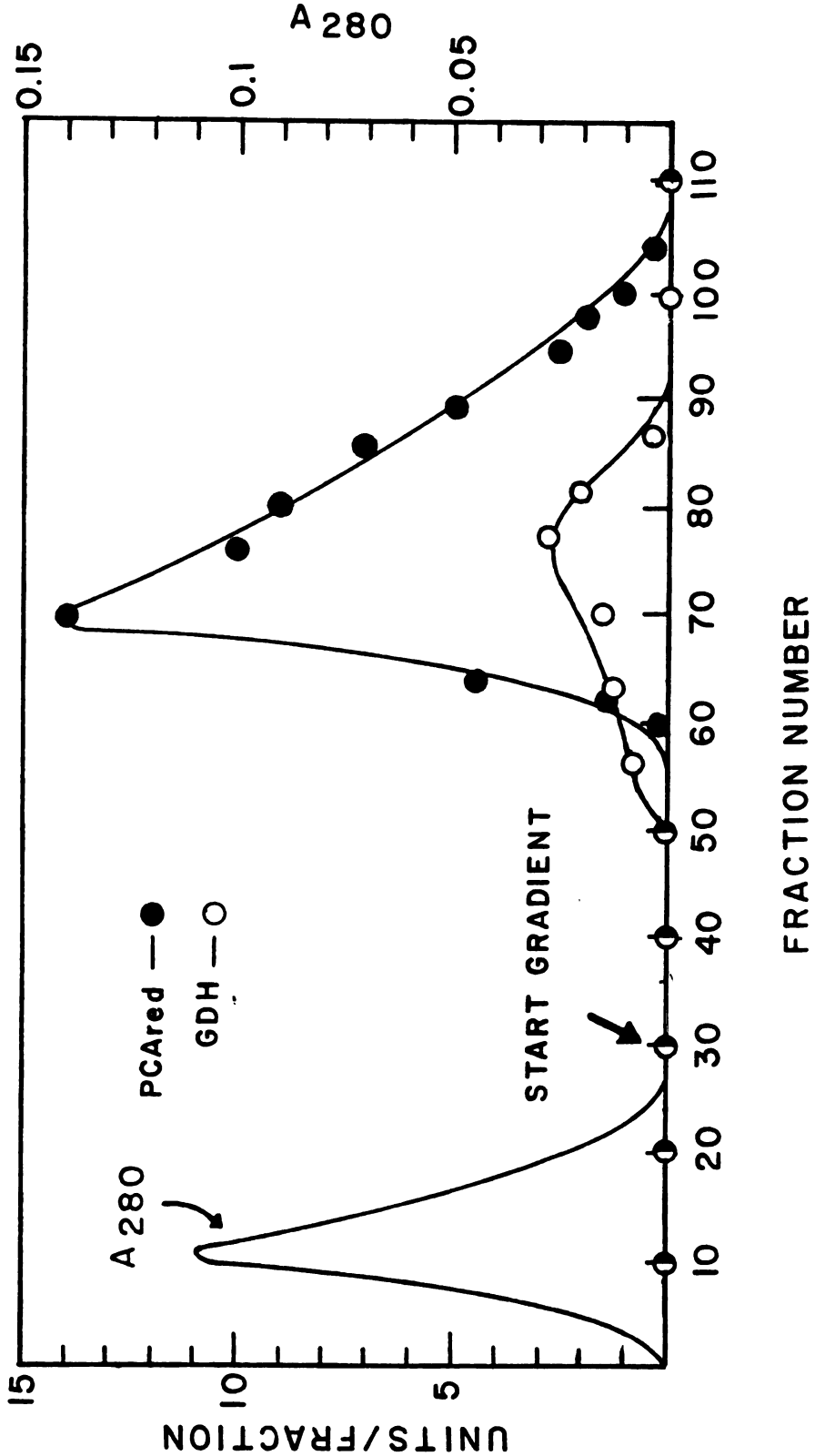
Although not evident in the elution profile (Figure 4) a significant amount of the GDH activity is separated from PDH activity in this purification step (Table 1). In most experiments, traces of GDH were found in all but the last fractions. GDH could be eliminated from some of the PDH by pooling the fractions with PDH activity, concentrating, and repeating the entire chromatographic procedure. A small amount of PDH free from GDH contamination was obtained in this manner.

This purification step results in a large change in the PDH:PCAred ratio (Table 1), from 1:23 to 1:100. This change was observed consistently, and the ratio did not change significantly in the following purification steps. It appears that the proline-oxidizing activity of PDH is more sensitive to low ionic strength than the PCA-reducing activity. The change in the ratio is not reversible with high ionic strength buffers, or with incubation for 30 minutes at 20°C with proline, NAD, NADH, or PCA.

Biogel A-5M Column

Passage of the concentrated enzyme preparation through a Biogel A-5M column resulted in the complete removal of contaminating GDH activity. Analytical disc gel electrophoresis demonstrated that the only proteins remaining had mobilities in the gel much

Figure 4.--Elution profile from hydroxylapitite column chromotography. Fractions of 3 ml were eluted from the column with a linear gradient of potassium phosphate buffer with DTT and glycerin. 1 mg of protein from step 5 (Table 1) was applied to the column. Symbols: closed circles--proline dehydrogenase (assayed by monitoring PCAred activity); open circles--glutamate dehydrogenase (GDH). See Materials and Methods for experimental details, and Table 1 for recoveries.


A 280
0.15
0.1
0.05
110
100
90
80
70
60
50
40
30
20
10
FRACTION NUMBER
UNITS/FRACTION
15
10
5
A 280
PCAred —●
GDH —○
START GRADIENT

higher than the mobility of PDH. These contaminants had Rf values in excess of 0.7, while the Rf of PDH in these gels was found to be 0.3.

Sephadex G-200 Column

This gel filtration column was employed to remove the proteins which migrated much faster than PDH. This proved very effective and resulted in electrophoretically pure PDH (see Figure 5). All fractions from the sephadex column which contained PCAred activity also contained PDH activity.

Characterization of the Enzyme

Molecular Weight of the Native Enzyme

Calculations based on the sedimentation patterns observed with sucrose density contrifugation (Figure 6; Martin and Ames, 20) yield a sedimentation coefficient of 10.2S (using a value of 7.0S for alcohol dehydrogenase, and a sedimentation coefficient of 10.1S for catalase). Costilow and Cooper (6) previously reported a value of 10.3S for this enzyme in a 5-20% sucrose gradient. Comparison with the sedimentation values for catalase (approximate molecular weight 240,000) and alcohol dehydrogenase (approximate molecular weight 150,000) indicates a molecular weight of about 217,000 for PDH.

Molecular Weight of Subunits

SDS gel electrophoresis of the pure enzyme yields one protein band, with an Rf of 0.23. Comparisons with four protein

Figure 5.--Gel scan of the purified enzyme (step 8, Table 1) in a 7% analytical polyacrylamide gel taken at a wave length of 600 nm. See Materials and Methods for experimental details.

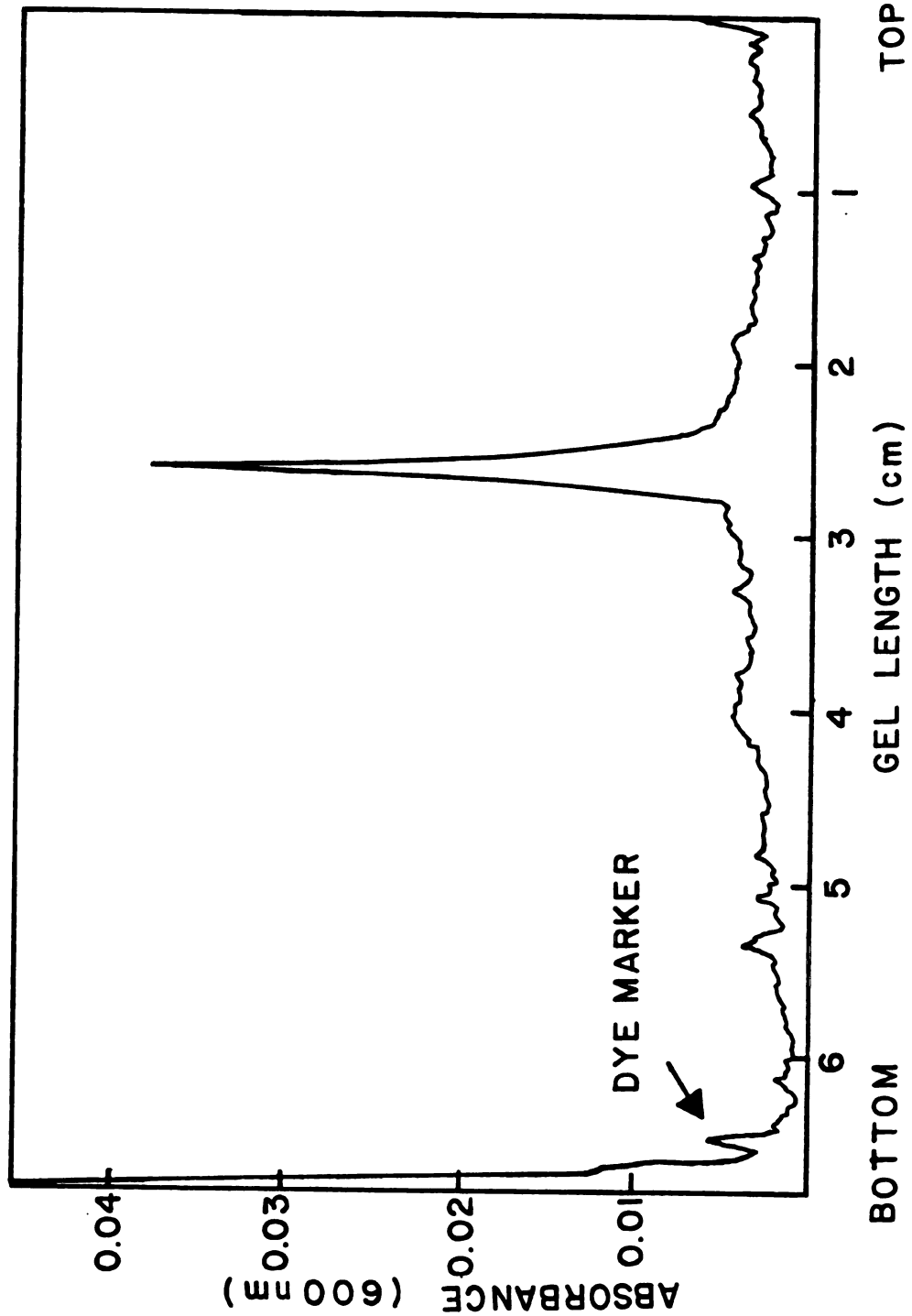
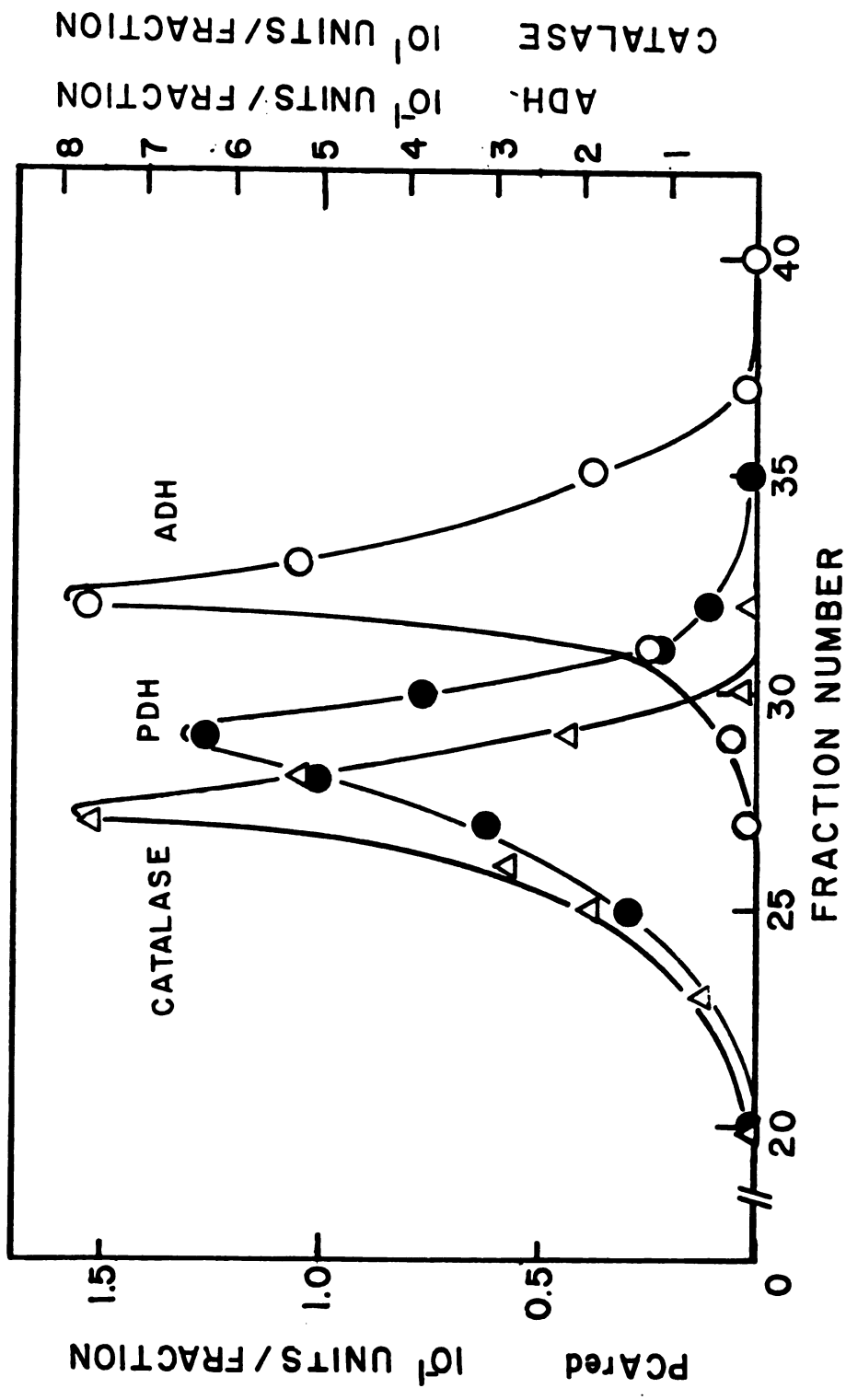

ABSORBANCE (600 nm)
0.04
0.03
0.02
0.01
DYE MARKER
BOTTOM
6
5
4
3
2
1
TOP
GEL LENGTH (cm)

Figure 6.--Profile of catalase, proline dehydrogenase, and alcohol dehydrogenase activity in 2 drop fractions after sucrose density centrifugation. PDH was assayed by measuring the PCA-dependent oxidation of NADH (PCAred activity). See Materials and Methods for experimental details.


ADH
PDH
CATALASE
FRACTION NUMBER
PCArea 10¹ UNITS / FRACTION
ADH 10⁻¹ UNITS / FRACTION
CATALASE 10¹ UNITS / FRACTION

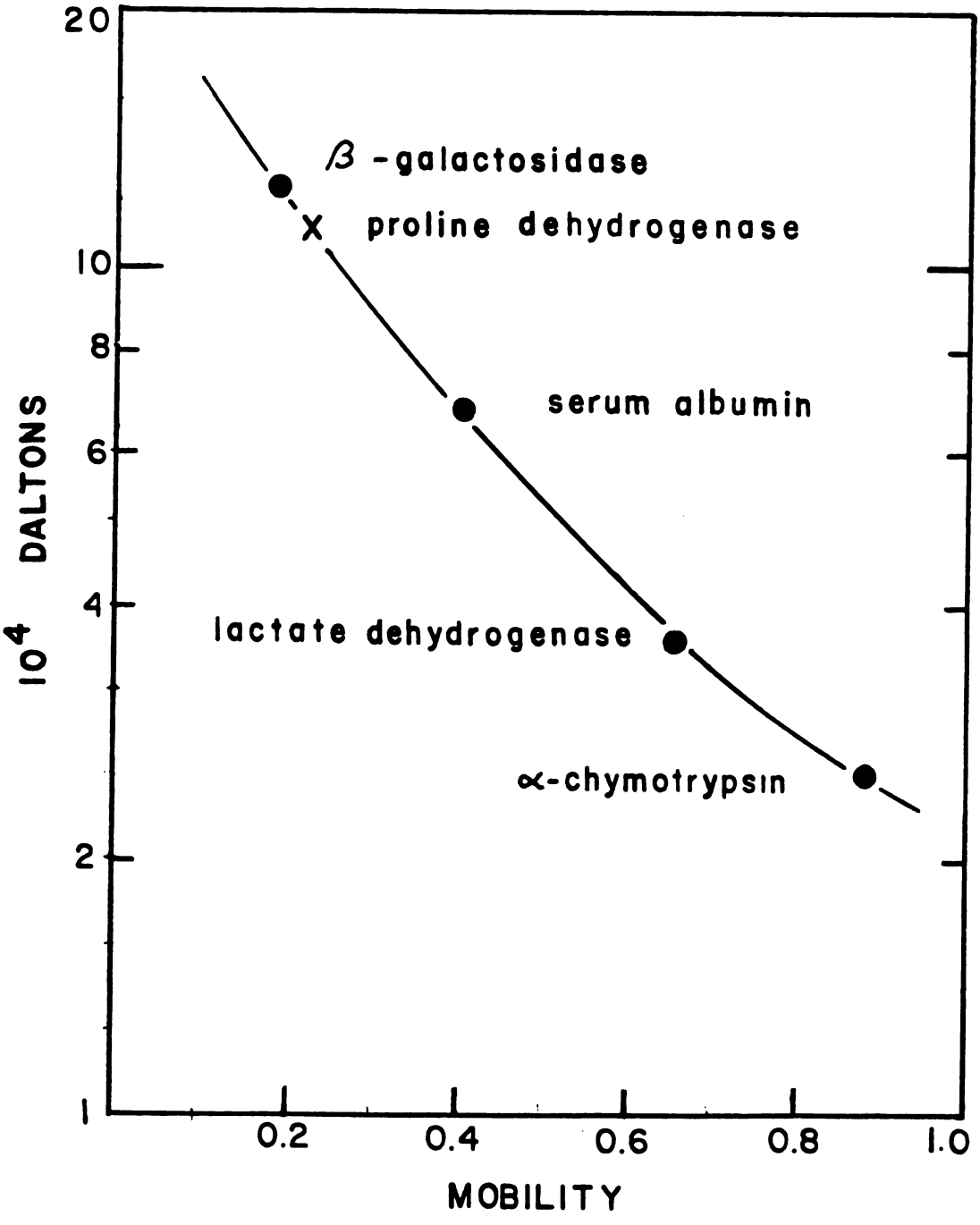
standards (Figure 7), β-galactosidase (Rf 0.18), bovine serum albumin (Rf 0.41), lactate dehydrogenase (Rf 0.66) and α-chymotrypsin (Rf 0.88) indicate that PDH consists of two subunits of the same size. The subunit molecular weight is approximately 108,000.

Stability

Storage at 4°C.--Overnight storage of the partially purified enzyme (prior to step 4, Table 1) at 4°C results in little or no change in the specific activities of PDH and PCAred. However, after the enzyme had passed through a DEAE-cellulose column, the activities became increasing labile. In early experiments, it was observed that overnight dialysis of the enzyme preparation against 20 mM potassium phosphate buffer, pH 7.4, often resulted in the loss of much of the enzyme activity. After such treatment, the specific activity of PDH (measured by PCAred) dropped about 50%, while the PDH activity was reduced by as much as 80%. As a result of these experiments, dialysis of the enzyme after step 4 in the purification was discontinued, and replaced by desalting with a Sephadex G-10 column at 4°C. No reduction of the specific activities were observed resulting from passage through such a column. However, the large increase in the PDH:PCAred ratio observed after hydroxylapatite chromatography probably resulted from the exposure to low ionic strength buffer used initially in the gradient (0.02 M potassium phosphate buffer).

Storage at -21°C.--An enzyme preparation (step 5, Table 1), stored in 0.25 M potassium phosphate buffer, pH 7.4, lost 50% of its activity after storage at -21°C for 13 months. Preparations

Fugure 7.--Comparison of the mobility of proline dehydrogenase and four protein standards on sodium dodecyl sulfate (SDS) analytical disc gels. See Materials and Methods for experimental details.


20
10
8
6
4
2
1
10^4 DALTONS
β-galactosidase
proline dehydrogenase
serum albumin
lactate dehydrogenase
α-chymotrypsin
0.2
0.4
0.6
0.8
1.0
MOBILITY

from steps 1, 2, 3, and 4 (Table 1) were unaffected by this treatment, while losses in PDH activity of between 20 and 40% were typical in most preparations from steps 5 and 6. The purified enzyme preparation has not been examined.

Effect of freezing and thawing.--A highly purified enzyme preparation (step 7, Table 1) stored in 0.25 M tris-chloride buffer (pH 7.4) was assayed for enzyme activity, frozen, stored for 12 hours at -21°C, thawed and assayed for PCAred activity. This treatment resulted in the loss of about 20% of the enzyme activity (Figure 8).

Inhibitory Effects of Various Compounds

The inhibition of the forward and reverse reactions of PDH by various compounds was studied using a partially purified preparation from step 5 (Table 1). Table 2 shows the effects of these compounds on PDH and PCAred activity. Glutathione, cysteine and copper sulfate inhibited PDH in both the forward and reverse directions. PCAred activity was inhibited slightly more than PDH activity. Both activities were inhibited to about the same extent by para-chloromecuribenzoate (PCMB). Adenine nucleotides inhibited PDH activity more than the reverse activity, PCA red.

Since hydroxylamine completely inhibited the NADH dependent reduction of PCA, while having no effect on the reverse reaction (PDH activity), experiments were performed to determine if this effect was actually due to the binding of the hydroxylamine to the

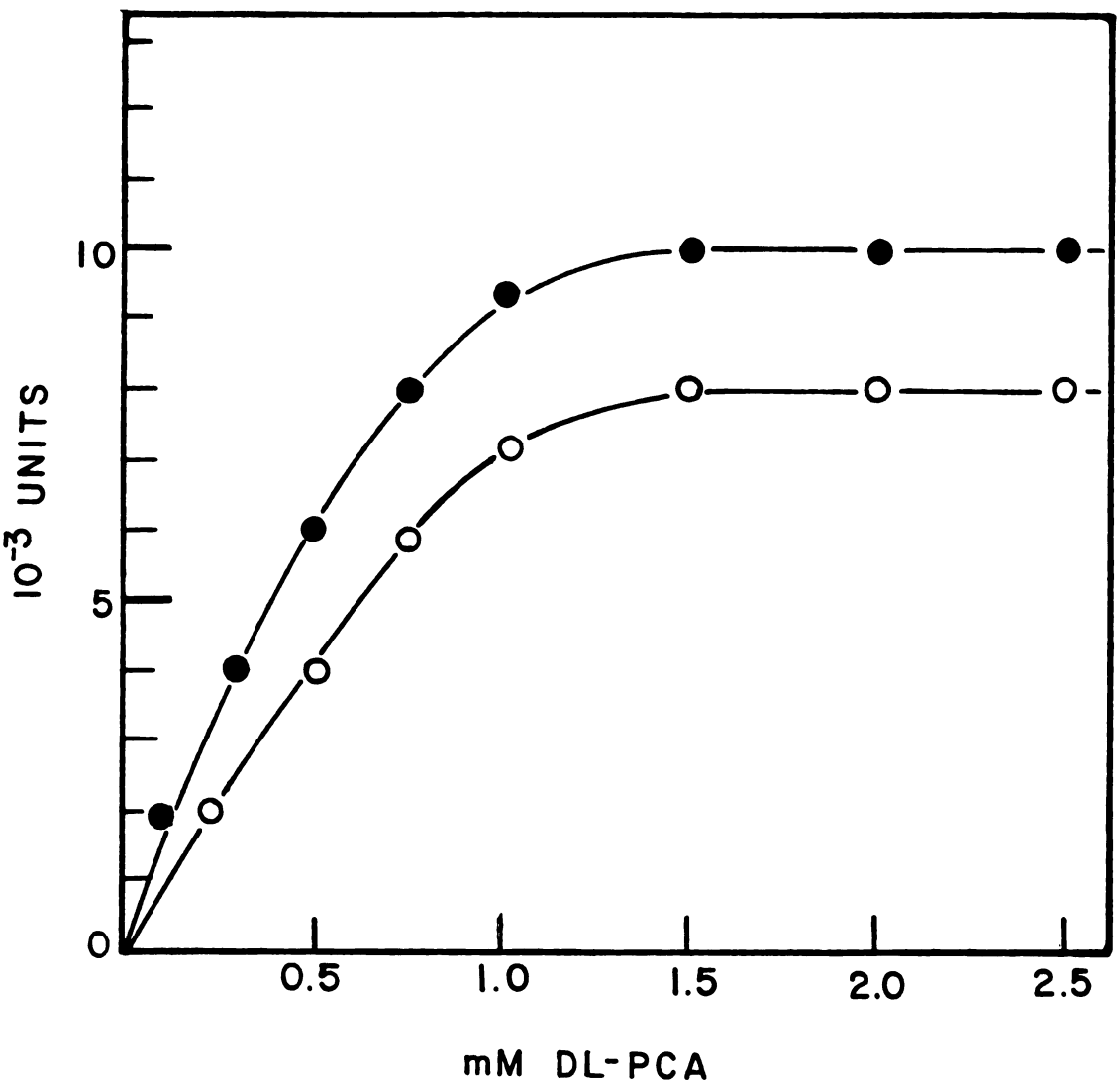


Figure 8.--Effects of freezing and thawing on PCAred activity. Symbols: closed circles--PCAred (activity per 50 µl, before freezing); open circles--PCAred (activity after thawing of 50 µl).

TABLE 2.--Inhibition of proline dehydrogenase and PCA reductase activities.

Compound	Concentration	Relative Activity	
		PDH	PCAred
None	0	100	100
Glutathione	5 mM	33	16
Cysteine	5 mM	38	21
$CuSO_4$	2.5 mM	29	0
p-chloromercuribenzoate	0.5 mM	57	65
Hydroxylamine	0.5 mM	96	0
AMP	2.5 mM	42	78
ADP	2.5 mM	36	50
ATP	7.5 mM	25	65

active site of the enzyme. Enzyme preparations from step 5 (Table 1) were incubated at 4°C for 20 minutes or 30°C for 5 minutes in the presence of 1 mM hydroxylamine. A .05 ml sample of each preparation was passed through a small Sephadex G-10 column (Pasteur pipet) to separate the enzyme from the unreacted hydroxylamine. The enzyme eluted from these columns was assayed in the usual fashion. No significant difference in the PCA dependent oxidation of NADH was observed between the hydroxylamine treated samples and untreated controls. Obviously, the NH_2OH did not bind tightly to the enzyme. Table 3 shows the effect of increasing PCA concentrations in the reaction mixture on the inhibition of the PCA-dependent oxidation of NADH by NH_2OH. An enzyme preparation from step 5 (Table 1) was employed. Concentrations of PCA greater than 1 mM eliminated the inhibition by 0.5 mM NH_2OH. It is quite possible that the inhibition observed results from a reaction of hydroxylamine with glutamic γ-semialdehyde which is in equilibrium with PCA.

Kinetic Studies

The apparent Michaelis constants of PDH for L-proline and PCA were determined using a partially purified enzyme preparation (step 5, Table 1, followed by a second hydroxylapatite column). This preparation was free from GDH contamination. Substrate saturation data was analyzed with Eddie-Schattard plots (36) (Figure 8). The negative reciprocal of the slope is equal to the K_m. The K_m values obtained were 1.5 mM L-proline at pH 10.2, and 110 mM

TABLE 3.--Effect of PCA concentration on inhibition by hydroxylamine.*

PCA	% Inhibition
0.27 mM	77
0.54 mM	51
1.08 mM	6

*The rate of PCA-dependent NADH oxidation by an enzyme preparation from step 5 (Table 1) before and after the addition of hydroxylamine (0.5 mM) was monitored by observing the loss in absorbancy at 340 nm.

L-proline at pH 7.4. The pH optimum for the L-proline-dependent reduction of NAD is 10.2.

The K_m of the enzyme for DL-PCA at pH 7.4 was 0.32 mM with 0.1 mM NADH. The PCA preparations used in these studies contained the D and L stereoisomers in approximately equal amounts. Consequently, the K_m for L-PCA might be expected to be half of that observed for the DL-PCA, or approximately 0.16 mM. This value agrees well with the reported values of the K_m's for L-PCA (6) of 0.2 mM and 0.33 mM at pH 6.5 and 8.0, respectively.

Effect of L-Glutamate on PDH

Proline dehydrogenase is very sensitive to low concentrations of L-glutamate. The initial velocity of L-proline-dependent NAD reduction was assayed with an enzyme preparation free from GDH (step 5, Table 1, followed by a second hydroxylapatite column). The concentrations of the substrate (L-proline) and the inhibitor (L-glutamate), along with the pH, were varied. Figure 9 shows Eddie-Schattard plots of the data at pH 10.2 and 7.4. These plots indicate that the K_m of the enzyme (that is, the negative reciprocal of the slope) remains constant with increasing concentrations of L-glutamate, while the V_{max} values decrease. Such kinetics are typical of non-competitive inhibition. Dixon plots (36) of the inhibition data (Figure 10) yield K_i values of 0.65 mM L-glutamate at pH 10.2 and 0.23 mM at pH 7.4. These data indicate that L-glutamate is more than two times as inhibitory at pH 7.4 than pH 10.2.

Figure 9.--Eddie-Schattard plots of L-glutamate inhibition data. Symbols in A (L-glutamate inhibition of PDH at pH 10.2): open circles--0 mM glutamate; triangles--0.5 mM glutamate; closed circles--1.0 mM glutamate. Symbols in B (PDH inhibition at pH 7.4): triangles--0 mM glutamate; closed circles--0.5 mM glutamate. See the text for experimental details.

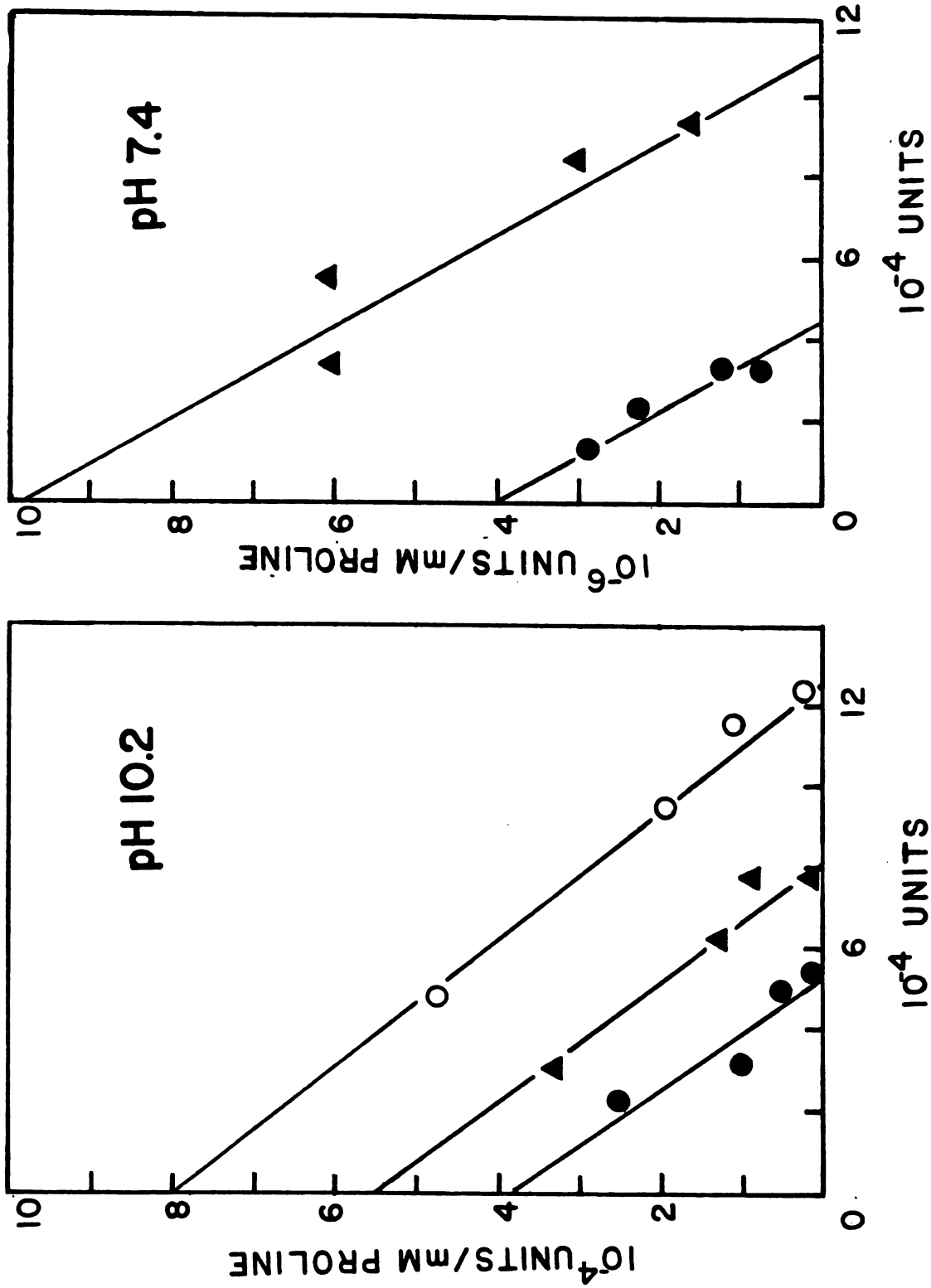
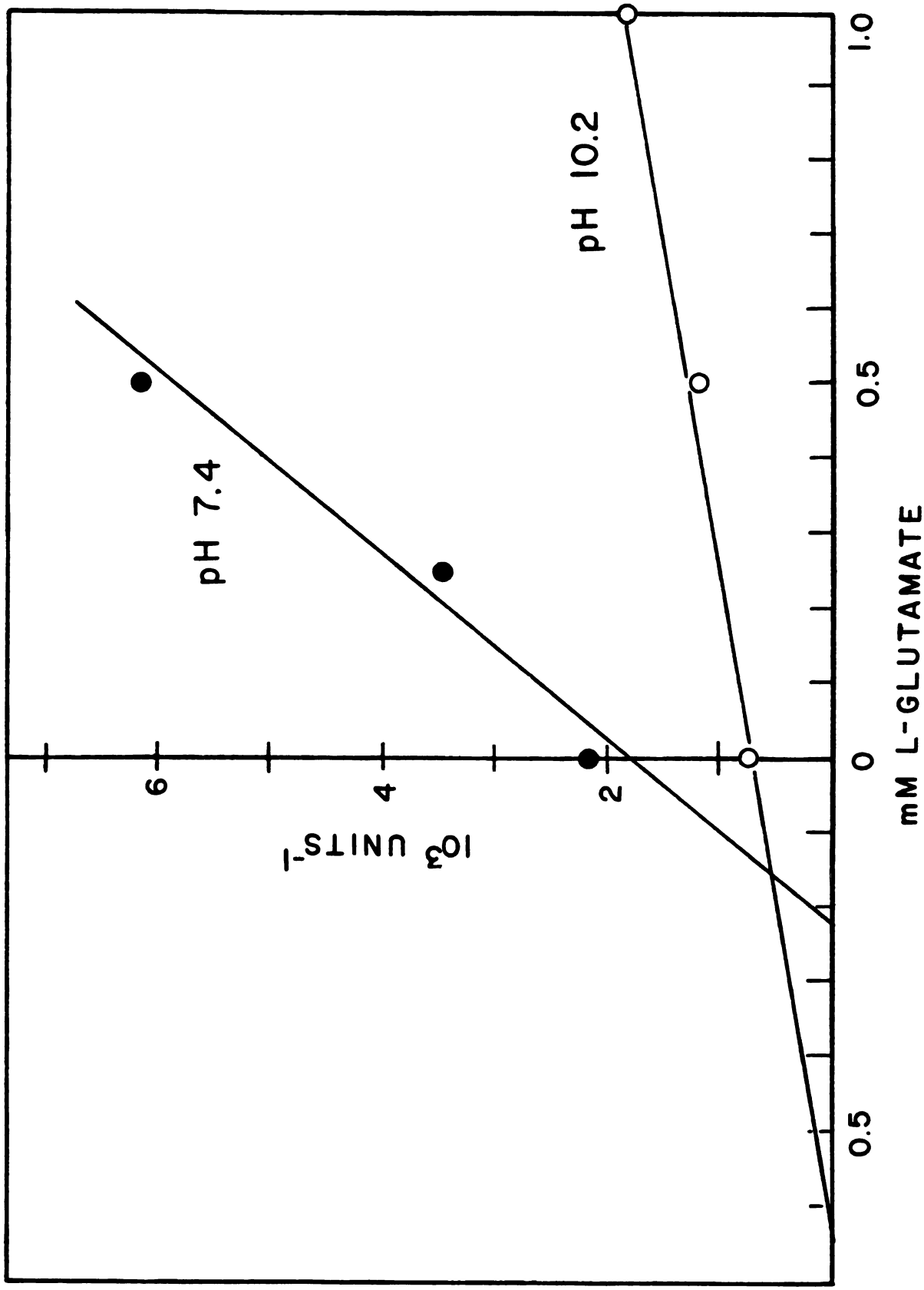

pH 7.4
10^{-6} UNITS/mM PROLINE
10^{-4} UNITS
0
2
4
6
8
10
12
pH 10.2
10^{-4} UNITS/mM PROLINE
10^{-4} UNITS
0
2
4
6
8
10
12

Figure 10.--Dixon plot of the reciprocal of the V_{max} versus concentrations of inhibitor (L-glutamate) at pH 7.4 and pH 10.2. The K_i values calculated from this plot for glutamate inhibition of PDH are 0.65 mM at pH 10.2 and 0.23 mM at pH 7.4.


pH 10.2
pH 7.4
10^3 UNITS^{-1}
6
4
2
0
0.5
1.0
0.5
mM L-GLUTAMATE

The reverse reaction by PDH, the PCA-dependent oxidation of NADH, is not affected by high concentrations of L-glutamate. Initial velocity versus substrate concentration plots show identical hyperbolic curves in the presence of 25, 50, and 100 mM L-glutamate. Apparent K_m and V_{max} values are not changed in the presence of these high levels of glutamate.

Induction of PDH by L-Proline

Earlier reports from this laboratory (6) suggested that PDH might be at least partially inducible. Experiments were conducted to examine the specific activities of PDH in cells grown in complete and synthetic media, with varying concentrations of L-proline. In the standard trypticase medium (medium A, see Materials and Methods) no significant increase in the specific activity of PDH was observed as the levels of L-proline were increased (Table 4). In medium B (the synthetic medium), relatively high concentrations of L-proline did increase the levels of PDH. The specific activity of PDH in medium B was elevated to that observed in medium A by the addition of 40 mM L-proline to the growth medium. Further increases in the proline concentration in medium B did not result in an increase in the specific activity of proline dehydrogenase.

TABLE 4.--The effect of L-proline concentration in the growth medium on the specific activity of proline dehydrogenase in crude extracts of C. sporogenes.

Growth Medium	% L-proline	Specific Activity
A	0	0.027
A	0.10	0.028
A	0.5	0.024
A	1.0	0.025
B	0	0.008
B	0.10	0.006
B	0.25	0.011
B	0.50	0.024

DISCUSSION

The results of this investigation demonstrate that the proline dehydrogenase of C. sporogenes reversible catalyzes the interconversion of L-proline and PCA. Following purification of the enzyme, only one protein band was seen on native disc gels and sodium dodecyl sulfate (SDS) disc gels. The enzyme has a sedimentation coefficient of 10.2 based on sucrose density gradient centrifugation, which corresponds to a molecular weight for a globular protein of 217,000. Based on the results of SDS gel electrophoresis, it appears that the native enzyme may exist as a dimer of two identical subunits each with a molecular weight of approximately 108,000. The molecular weight of the membrane-bound proline dehydrogenase from E. coli was estimated at 200,000 to 260,000, and it appears to be a dimer (35). PCA reductase purified from E. coli had an estimated molecular weight of 320,000 (33). Non-particulate proline dehydrogenases from plant tissues also have molecular weights greater than 100,000 (21, 23, 32).

The results reported in this paper support the findings of Costilow and Cooper (6) who indicated that the proline dehydrogenase and PCA reductase activities of C. sporogenes were probably catalyzed by the same protein. The proline oxidase found in most animals and some microorganisms is not reversible (4, 18, 9, 10,

16), nor is the PCA reductase in these systems (29, 33). There is some evidence to suggest that NAD-dependent proline oxidation and NADH-dependent PCA reduction may be catalysed by the same enzyme in wheat germ (21) and pumpkin cotyledons (31), however no other NAD-linked proline dehydrogenase has been purified.

A significant change in the ratio of PDH activity to PCAred activity was seen after hydroxylapatite chromatography. The ratio of PDH:PCAred changed from 1:30 to 1:100. The same three-fold change in the ratio was observed by Costilow and Cooper (6) following dialysis in low ionic strength buffer. Assaying at pH 10.2 for PDH (which is the optimum pH for the assay), instead of pH 7.4 as in this study, they observed a change in the PDH:PCAred ratio from 1:10 in crude extracts to 1:30. Thus, about a 3X change in the ratio was noted in both instances. Obviously, PDH activity is more sensitive to low ionic strength buffer than the reduction of PCA. It is also more sensitive to heating at 65°C (6), and to freezing, than PCAred activity. Apparently, some conformational changes greatly affect the catalysis in one direction but not in the other. During storage of pumpkin extract in low ionic strength buffer, PCAred activity was lost more rapidly than PDH activity (32).

The NADH-dependent reduction of PCA by PDH (PACred activity) is more sensitive to $CuSO_4$ than PDH activity, while PDH activity is more sensitive to inhibition by adenine nucleotides. The inhibition by AMP, ADP, and ATP is probably the result of competition with NAD and NADH for the adenosine moiety binding site on the enzymes. This enzyme was observed to bind to an AMP-affinity column, which

supports this proposal. Adenine nucleotides also inhibit PDH activity in pumpkin extracts (32). Glutathione, cysteine and para-chloromercuribenzoate inhibition is usually associated with effects on the exposed thiol groups of the enzyme. Glutathione and cysteine are reported to have a stimulatory effect on thiol enzymes, presumably by keeping the thiol groups reduced. In experiments with PDH and PCAred activities from C. sporogenes, no stimulation was observed; in contrast, marked inhibition was found. Para-chloromercuribenzoate also inhibits PDH and PCAred, such that 0.5 mM of the compound reduces both activities by about 40%. Similar results were obtained in pumpkin cotyledon extracts (32). While these results are suggestive, they do not constitute sufficient proof to state that thiol groups are actually involved in the active site of either the PDH or the PCAred activities.

Inhibition of PCAred by hydroxylamine (NH_2OH) has been observed in this investigation, and in several other systems. Rena and Spittstoesser (32) found that PCAred from pumpkin cotyledons was 87% inhibited by 1.0 mM hydroxylamine, while PDH in the same preparations was inhibited only 7%. PCAred in liver (12) was found to be 100% inhibited in 3.3 mM hydroxylamine. These reports, however, do not consider the possibility that the hydroxylamine reacts with PCA, and not the enzyme.

Incubation for 30 minutes in hydroxylamine, and subsequent removal of free hydroxylamine, resulted in an enzyme preparation unchanged in its ability to oxidize NADH in the presence of PCA. Since PCA exists in equilibrium with glutamic γ-semialdehyde, it

is possible that the inhibition by hydroxylamine may be due to a reaction with the free aldehyde of glutamic γ-semialdehyde, and not with a carbonyl group in the active site of the enzyme as had been suggested (32). Such a reaction would have the appearance of affecting the enzyme, since the rate of oxidation of NADH would be reduced or stopped due to the inaccessibility of the substrate, PCA. This possibility is supported by the data in Table 3 showing that the inhibitory effects of hydroxylamine on PCAred activity is eliminated with increasing concentrations of PCA.

The regulation of proline dehydrogenase by glutamate has not been reported in any other system. Studies here of PDH from C. sporogenes show that 0.23 mM L-glutamate (in the standard assay at pH 7.4) is sufficient to reduce PDH activity by 50%. The affinity of the enzyme for glutamate (K_i = 0.23 mM) at pH 7.4 is much greater than for the substrate, L-proline (K_m = 110 mMO. The degree of inhibition by glutamate is dependent on pH, since 0.65 mM L-glutamate is required to produce 50% inhibition at pH 10.2. This may reflect the difference in the affinity of PDH for L-proline at the two pH's, since the apparent K_m for L-proline is 110 mM at pH 7.4, and 1.5 mM at pH 10.2. The reverse activity of PDH (the NADH-dependent reduction of PCA) is not affected by glutamate. These data suggest that the conversion of proline to PCA in C. sporogenes is regulated by cellular glutamate pools, while the reduction of PCA is not regulated in this manner.

It is not clear why PDH activity is more labile than PCAred activity, and why the activities respond differently to

inhibitors. However, the non-competitive inhibition of PDH activity by glutamate (but not PCAred activity), together with the different effects of inhibitors suggests that the active sites of the PDH and PCAred activities are dissimilar, at least to some extent. In light of this, it is not unusual that the activities display different stabilities, despite the fact that they are catalyzed by the same protein.

The role of proline dehydrogenase in C. sporogenes has not been determined. In some organisms, PCA reductase activity is important in the biosynthesis of proline from ornithine and glutamate (2, 29, 41). C. sporogenes can convert ornithine to proline by ornithine cyclase (deaminating) (26). This reaction does not involve a free intermediate but Δ^1-pyrroline 2-carboxylic acid is believed to be formed during the reaction, since the alpha amino group of ornithine is removed. Some plants have also been shown to convert ornithine to proline via a Δ^1-pyrroline 2-carboxylic acid intermediate (24). The mechanism of this conversion is unknown. The NAD-dependent proline dehydrogenase in plants is believed to be involved in the catabolism of proline to glutamate (32). This may also be the case in C. sporogenes, since PDH can be induced to some extent by proline and can be regulated by small concentrations of glutamate.

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