To the Graduate Council:

I am submitting herewith a thesis written by Yongzhang Luo entitled " Purification and Partial Characterization of Pyridoxal Dehydrogenase from Porcine Liver. " I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this thesis and recommend its acceptance:

Elizabeth E. Howell

John W. Koontz

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

PURIFICATION AND PARTIAL CHARACTERIZATION OF PYRIDOXAL DEHYDROGENASE FROM PORCINE LIVER

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Yongzhang Luo

August 1989

To my parents, Mr. Qingbin Luo (羅 慶 賓) and Ms. Honglan liu (劉 洪 蘭), for their support and understanding

and

To my wife, Yujuan (育 娟), for her love, support, sacrifice, and understanding.

ACKNOLEDGEMENTS

The author wishes to thank Dr. Jorge E. Churchich for his excellent guidance and patience while the author was studying in Dr. Churchich's laboratory. He feels very fortunate to have been able to work with such an excellent scientist. The author also would like to express his sincere appreciation to Dr. Leaf Huang for his helpful suggestions and kind help. He is grateful to his committee members, Dr. John Koontz and Dr. Liz Howell, for their encouragement in the preparation of this thesis.

ABSTRACT

Aldehyde dehydrogenase (ALDH), an NAD-dependent enzyme which oxidizes acetaldehyde to acetate is localized in cytosol, mitochondria and microsomes of mammalian liver cells. The oxidation of aldehyde substrates to carbonic acids is catalyzed by different enzymes. The reaction is highly exergonic and, therefore, irreversible even in dehydrogenase reactions with NAD as electron/hydrogen acceptor. Knowledge of the catabolism of pyridoxal, which is one kind of aromatic aldehyde and one of the derivatives of Vitamin B_6 in mammalian tissues is still incomplete. An aldehyde dehydrogenase which oxidizes pyridoxal to pyridoxic acid has been postulated to be the key enzyme regulating the inactivation rate of Vitamin B_6. Two assay methods, fluorometric and spectrophotometric methods, were developed in the identification of pyridoxal dehydrogenase from porcine liver. It has been detected that this enzyme catalyzes the conversion of PL and NAD to PA and NADH, respectively and the reverse reaction does not occur. PD was purified to 1300 fold from porcine liver and its molecular weight was estimated by gel filtration to be 123,000 daltons. The pH optimum lies between 8.5-9.0. It has been found that PD shows very high specificity for PL and does not oxidize benzaldehyde and salicylaldehyde which are believed to be the common substrates of aldehyde dehydrogenases. Thus, PD is not a general aldehyde dehydrogenase.

The K_m values for PL and NAD were determined to be 2.46 uM and 75.7 uM at PH 7.5, respectively. Inhibition studies show that both NADH and 1,10-phenanthroline are competitive with respect to NAD. 4,7-phenanthroline also shows almost the same inhibition to PD with 1,10-phenanthroline, indicating that there is no zinc ion in the active site of PD. The uncompetitive inhibition pattern of NADH with respect to PL indicates the reaction catalyzed by PD follows an ordered sequential mechanism. The stereochemistry of hydride transfer by PD was shown to be pro-S.

TABLE OF CONTENTS

FIGURES

TABLES

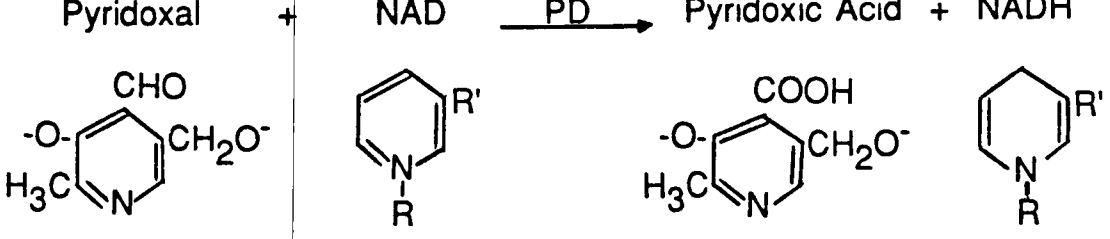
CHAPTER I

INTRODUCTION

Aldehyde dehydrogenase (ALDH) , an NAD-dependent enzyme which oxidizes acetaldehyde to acetate is localized in cytosol, mitochondria and microsomes of mammalian liver cells (1). Both cytosolic and mitochondrial ALDHs have subunit molecular weight of approximately 55,000 and are isolated as tetramers (2). The oxidation of aldehyde substrates to carbonic acids is catalyzed by different enzymes. The reaction is highly exergonic and, therefore, irreversible even in dehydrogenase reactions with NAD as electron / hydrogen acceptor (3). Knowledge of the catabolism of pyridoxal, which is one kind of aromatic aldehyde and one of the derivatives of vitamin B_6 in mammalian tissue is still incomplete. An aldehyde dehydrogenase which oxidizes pyridoxal to pyridoxic acid has been postulated to be the key enzyme regulating the inactivation rate of Vitamin B_6 (4) . A similar enzyme has been detected and partially characterized in bacteria (5) . Attempts to purify this enzyme, pyridoxal dehydrogenase, from mammalian tissues, i.e., brain and liver, have been unsuccesful because the enzyme is endowed with low specific activity. Thus, the low specific activity of pyridoxal dehydrogenase is one of the major problems hindering its identification in mammalian tissues.

To circumvent this problem, a continous fluorometric assay based on the detection of one of the products, 4-pyridoxic acid, in the presence of substrates, pyridoxal and NAD, was developed. Another spectrophotometric assay method based on the absorbance increase at 340 nm due to the formation of NADH was also applied. These methods have been applied to the detection of pyridoxal dehydrogenase isolated from porcine liver by a combination of CM-Sephadex, DEAE-Sephadex, Hydroxylapatite Chromatography and Sepharose-CL-4B gel filtration.

Pyridoxal dehydrogenase from porcine liver is shown to catalyze the conversion of pyridoxal and NAD to pyridoxic acid and NADH, respectively. This reaction appears to be irreversible.



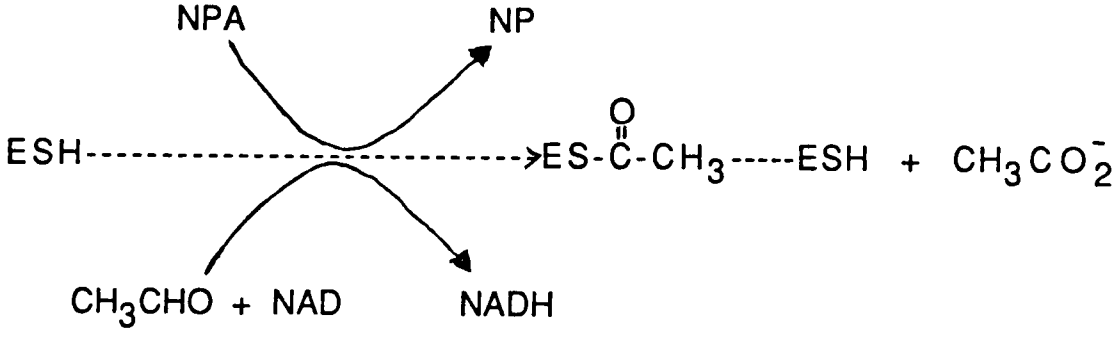
(scheme I)

The molecular weight of pyridoxal dehydrogenase from pig liver estimated by gel filtration is 123,000 daltons. The pH optimum was determined to lie between pH 8.5-9.0. This enzyme has a very high specificity for pyridoxal and does not oxidize benzaldehyde or salicylaldehyde, which are believed to be the common substrates of aldehyde dehydrogenases (6, 7, 8,).

At pH 7.5 the Km values for pyridoxal and NAD are 2.46 uM and 75.7 uM, respectivly. The Km values for pyridoxal and NAD are comparable to the values previously reported for the enzyme

isolated from bacteria (5).

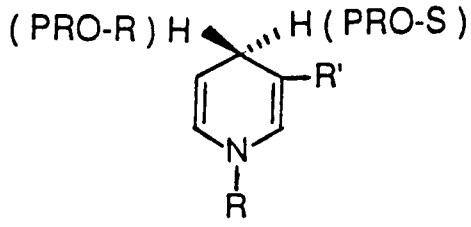
Pyridoxal dehydrogenase from porcine liver, like other ALDHs, can hydrolyze p-nitrophenyl ester to p-nitrophenol. This observation led to the conclusion that ALDH forms covalent intermediates during its catalytic process (1). It has been suggested that a thiohemiacetal is initially formed between ALDH and aldehyde which is oxidized to thioacyl intermediate. The same intermediate can be formed directly by the attack of the cysteine on the nitrophenyl ester (9).



(Scheme II)

ESH represents one subunit of ALDH, NPA is nitrophenyl acetate, and NP is nitrophenol.

Pyridine nucleotide-linked enzymes are well known to transfer hydride stereospecifically to (or from) the pyridine 4-position of the oxidized (or reduced) coenzyme (for a review, see ref. 10). Pro-R stereospecific enzymes transfer hydride to or from the pro-R side (see scheme III), while pro-S stereospecific enzymes transfer hydride at the opposite side. Known pyridine nucleotide-linked enzymes seem to be approximately equally distributed in their stereospecificity (11).



(Scheme III)

In order to determine the stereospecificity of the hydride transfer reactions that involve methylene prochiral centers, it is imperative to carry out the reactions with deuterium- or tritium-labeled compounds (for a review, see Ref. 12). In the case of the reactions of pyridine nucleotide-linked enzymes, C-4 hydrogen of nicotinamide of NAD(P), one of the C-4 methylene hydrogens of dihydronicotinamide of NAD(P)H, or the substrate hydrogen that is going to be transferred to the coenzyme must be replaced with the isotope. Once the isotope-labeled aldehyde substrate is prepared, the stereochemistry of a given enzyme can be determined by carrying out the reaction of the enzyme with the isotopicaly labeled aldehyde substrate in the presence of the enzyme and the oxidized coenzyme. The position of the isotope in the reduced coenzyme can be determined by carrying out another reaction by using a known stereospecific enzyme. The analysis for the presence or absence of the isotope in the resultant oxidized coenzyme directly reveals the stereochemistry of that enzyme (13-16).

The purpose of this research project is twofold: first, to isolate pyridoxal dehydrogenase from porcine liver and determine its catalytic parameters; and second, to investigate the stereochemistry of hydride transfer during catalysis.

CHAPTER II

PURIFICATION OF PYRIDOXAL DEHYDROGENASE

A. Materials and Instrumentation

Fluorescence measurements were made using a Bausch and Lomb dual excitation light source. Fluorescence emission was monitored directly from a Pacific photometric recording photometer, or transferred from the photometer to a Mosley-Autograf X-Y recorder. Spectrophotometric measurements were done by a UV-visible 160 Schimadzu Spectrophotometer. Pyridoxal and NAD were purchased from Sigma. DEAE-Sephadex and CM-Sephadex were purchased from Phamacia. Hydroxylapatite was purchased from Bio-Rad.

B. Procedures

1. Fluorometric enzymatic assay

A 2 ml reaction mixture contained 0.5 mM pyridoxal, 0.5 mM NAD in 0.1 M potassium phosphate buffer containing 1.0 mM 2-mercaptoethanol, pH 7.5. The reaction was initiated by the addition of pyridoxal dehydrogenase at 25^{o}C. The reaction mixture was excited at 360 nm and fluorescence emission was monitored at 430 nm.

2. Spectrophotometric enzymatic Assay

A 1.0 ml reaction mixture contained 0.5 mM pyridoxal, 0.5 mM NAD in 0.1 M potassium phosphate buffer containing 1.0 mM 2-mercaptoethanol, pH 7.5. The reaction was initiated by the addition of pyridoxal dehydrogenase at 25°C. The absorbance increase was monitored at 340 nm.

3. Purification

Porcine livers were obtained from Lays Meat Co.. Purification of the enzyme was started as quickly as possible after the livers were collected from the slaughter house. All the purification procedures were done at 4°C.

A Waring blender was used to prepare a 50% (W/V) homogenate in a solution of 0.1 M potassium phosphate buffer (pH 7.0) containing 1.0 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 X g for 45 minutes. The precipitate was discarded, and the supernatant was treated with $(NH_4)_2SO_4$. The precipitate obtained at 40% saturation was discarded and the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$. After centrifugation at 10,000 X g for one hour, the precipitate was dissolved in 5 mM potassium phosphate (pH 7.0) containing 1.0 mM 2-mercaptoethanol (buffer A) and dialysed against the same buffer at 4°C overnight.

The dialysate was centrifuged again at 10,000 X g for 30 minutes. The supernatant was applied to a CM-Sephadex column (29 X 2.6 cm) which was equilibrated with buffer A. The column

was eluted with the same buffer. Pyridoxal dehydrogenase was not retained by the supporting matrix. The active fractions were combined and then applied to a second column of DEAE-Sephadex (30 X 2.6 cm). The column was eluted with buffer A and the enzyme activity was eluted in the first fraction. The fractions containing enzyme activity were applied to the third column of hydroxylapatite (8 X 1.2 cm) which was equilibrated with buffer A. The column was first washed with 50 ml buffer A and then washed with a linear gradient of 60 ml 5 to 250 mM potassium phosphate (30 X 2 ml). The fractions containing the enzyme activity were combined and dialyzed against buffer A, then centrifuged at 10,000 X g for 30 minutes. The supernatant was applied to the fourth column of Sepharose-CL-4B (85 X 1.5 cm) which was equilibrated previously with buffer A. Pyridoxal dehydrogenase eluted from this column by buffer A was stored at -20^{o}C.

4. Electrophoresis

Native gel was prepared by using the routine 10% polymerized gel according to Hoefer Scientific Instruments manual. Protein visualization was achieved using coomasie brillant blue. Bovine serum albumin (67K), ovalbumin (45K), trypsin inhibitor (28K), and lysozyme (14K) were used as molecular weight markers.

C. Results and Discussion

Two assay methods were used in the identification of pyridoxal dehydrogenase from porcine liver as described above. The spectroscopic properties of the fluorophores, pyridoxal, pyridoxic acid and NADH were investigated in some detail. Figure 1* shows the absorption spectra of PL, PA, and NADH at pH 7.0. The absorption band of NADH is red shifted when compared to the absorption bands of PL and PA. Thus, the spectrophotometric assay, based on changes in absorbance at 340 nm due to the formation of NADH, was used to determine the specific activity of purified preparations of pyridoxal dehydrogenase. Figure 2 shows the emission spectra of the fluorophores recorded at pH 7.0. The emission bands of PL and PA are centered at 380 nm and 425 nm, respectively, wherease the emission band of NADH is known to display a maximum at 460 nm. However, the relative quantum yield of PA is at least 20 fold greater than that of NADH as indicated in Figure 2. Using quining bisulphate as standard of quantum yield of 0.50, the quantum yield of PA was determined to be 0.40 (data not shown). It is obvious from the emission spectra that by selecting excitation wavelenghs of 340-360 nm and emission wavelenghths at 420-440 nm, it is possible to suppress the background fluorescence caused by the substrate pyridoxal. Therefore, the increase in fluorescence intensity at 430 nm

---

*All figures and tables can be found in the appendixes.

(excitation at 360 nm) monitored during the reaction catalysed by pyridoxal dehydrogenase is mainly due to the formation of 4-pyridoxic acid since the contribution of NADH to the total fluorescence is approximately 10%. It was observed that the initial reaction rates were strictly linear and as little as 10 ug quantities of the enzyme could be detected in a five minute assay period.

Figure 3 shows the elution profile of PD from the hydroxylapatite column. Two protein peaks were obtained, but only the second peak, which was eluted at approximately 200 mM potassium phosphate, contains the enzyme avtivity. The concentration of protein was determined by Bio-Rad method (18) with bovine serum albumin as standard. Figure 4 shows the elution profile of PD from Sepharose-CL-4B column. Blue dextran, thyroglobulin, gamma-globulin, ovalbumin and myoglobin were used as molecular weight markers. From this profile, the molecular weight of PD was estimated to be 123,000 daltons (17) as shown in Figure 5. Figure 6 shows the native gel electrophoresis of the enzyme from hydroxylapatite column. It looks like the pyridoxal dehydrogenase is highly purified. The highly purified enzyme has a specific activity of 0.68 unit/mg. One unit of activity is defined as the amount of enzyme required to produce one umole of NADH per minute at $25^{o}C$, pH 7.5.

A summary of the purification is shown in Table I. It is known from this table that about 80% of the PD activity was lost during the 40-60% ammonium sulphate treatment. Further

experiment should be done to get maximum recovery of enzyme activity, for example, to check the PD activity from fraction of 0-40% of $(NH_4)_2SO_4$ and fraction of 60-80% of $(NH_4)_2SO_4$. Among the four columns used in the enzyme purification, the hydroxylapatite column was shown to give the greatest extent of purification. To this end, it should be pointed out that more than five affinity columns were used in this purification procedure, but none of these columns worked. The five affinity columns tested are: 1. pyridoxal agarose, 2. NAD-agarose, 3. blue-dextran, 4. ADP-agarose, and 5. pyridoxamine coupled with Affi-gel-15. Further purification should be focused on seaking for a suitable affinity column.

CHAPTER III

PARTIAL CHARACTERIZATION OF PYRIDOXAL DEHYDROGENASE

A. Materials and Instrumentation

Life time measurements of the fluorescence of pyridoxic acid and pyridoxic lactone were done by a combination of Nanosecond Fluorescence Spectrometer ORTEC Model 9200 and Multichannel Analyzer ORTEC Model 6220. Polygram thin layer chromatography plates were from Macherey-NAGEL Co..

B. Procedures

1. Identification of reaction products

a. Synthesis of pyridoxic lactone: Pyridoxic lactone was synthesized by the method of Snell (5). A solution of 10.0 mg 4-pyridoxic acid in 5.0 ml of 1.0 N HCl was boiled at 100^{o}C for 30 minutes and then adjusted to pH 7.0 with KOH. The synthesized pyridoxic lactone was purified by running thin layer chromatography. The developing solvent system used was water : acetone : tert-butanol : triethylamine (20 : 35 : 40 : 5 , V/V).

b. Preparation of the reaction products: An aliquot of PD solution was added to 0.5 mM NAD and 0.5 mM pyridoxal in 0.1 M sodium phosphate, pH 7.5 and incubated at 25^{o}C for 3 hours. The

reaction was monitored at 340 nm until no further increase in absorbance was observed. The reaction mixture was stopped by heating in a boiling water bath for 3 minutes and then centrifuged in a microcentrifuge. The supernatant was applied to thin layer chromatography plates. PL, PA, PCL, and NADH were used as markers. The developing solvent system was water : acetone : tert-butanol : triethylamine (20 : 35 : 40 : 5, V/V).

The spots on thin layer plate were visualized by ultraviolet lamp. Those spots characterized by Rf values identical to authentic PA were scraped from the thin layer plate, dissolved in water and examined by absorbance and fluorescence spectroscopy at different pH values.

2. Irreversable reaction study

The investigation of the irreversable reaction was done by incubation of PD with 0.5 mM PA and 0.5 mM NADH at pH 8.0 to pH 9.0 at $25^{o}C$. The reaction was monitored at 340 nm for several hours.

3. Heat stability

An aliquot of PD solution at pH 7.0 was incubated at different temperatures for at least 30 minutes, then centrifuged. The supernatant was used for an activity assay.

4. pH dependant:

In order to obtain the pH optimum of pyridoxal dehydrogenase, an aliquot of enzyme was assayed at different pHs as described in chapter II.

5. Substrate Specificity

The specificity of PD for NAD was done by substitution of NAD by NADP and APAD , respectively, in the presence of pyridoxal as described under the assay methods in chapter II. The specificity for PL was done by substitution of pyridoxal by PLP, PN, PM, benzaldehyde, p-nitrobenzaldehyde, salicylaldehyde, acetaldehyde, and propionaldehyde as described in the assay methods in chapter II.

6. Inhibition studies

The inhibition of the enzyme by NADH, 1,10-Phenanthroline, and 4,7-Phenanthroline with respect to NAD (or PL) were carried out at fixed concentration of PL (or NAD) as described under the assay methods in chapter II. The concentrations of these inhibitors were varied between 0.02 mM and 0.08 mM.

7. Steady state kinetics

In order to determine the Km value of PL, the concentration of NAD was kept constant (0.5 mM) while the concentration of PL was varied between 1.0 uM and 100.0 uM. For the Km determination of NAD, the concentration of NAD was varied while the concentration of PL was fixed at 0.5 mM as described under the assay methods in chapter II.

8. Esterase assay

The esterase activity was determined by the enzymatic hydrolysis of p-nitrophenyl acetate in 10 mM sodium phosphate buffer, pH 8.0 at 25^{o}C by mixing p-nitrophenyl acetate and PD. The reaction was monitored at 400 nm due to the formation of

p-nitrophenol (19, 20).

C. Results and Discussion

Two products were identified by thin layer chromatography in the reaction mixture containing the substrates PL, NAD, and the enzyme PD. The products, visualized by ultraviolet lamp, exhibited Rf values identical to control samples of authentic PA and NADH as shown in Figure 7. Over a pH range of 1-8, the absorbance and fluorescence properties of one of the products of the reaction catalyzed by PD coincided with those of authentic PA as shown in Table II.

Attempts to observe the reverse reaction by incubation of PD with PA and NADH have thus far failed because no decrease in the absorbance at 340 nm due to the oxidation of NADH was observed. In view of these results, the reaction catalyzed by PD can be described as follows:

$$\text{NAD} + \text{Pyridoxal} \xrightarrow{\text{PD}} \text{NADH} + \text{Pyridoxic acid}$$

The reverse reaction does not occur.

Pyridoxal dehydrogenase is stable at 37^{o}C for at least 2.5 hours (data not shown). When the temperature is raised to 45^{o}C the enzyme is almost completely denatured (figure 8).

Figure 9 shows the pH dependency of the enzyme activity. From this curve, the pH optimum lies between 8.5-9.0. The specific activity at pH 8.5, 25^{o}C is approximately 1.0 umole/min. mg. The pH optimum for PD from porcine liver is almost the same

as that from bacteria, but it should be kept in mind that at pH 9.0 or above, the enzyme from mammalian tissue probably is not stable.

In an attempt to gain more information on the specificity of the reaction catalyzed by the enzyme, the reduction of NAD in the presence of several aldehyde substrates was studied over the pH range of 6.5-9.0, as was the oxidation of pyridoxal. Table III shows the specificity of PD for aldehyde substrates. Maximal catalytic activity is obtained with the substrate pyridoxal. Two other substrates, propionaldehyde and acetaldehyde, yield much lower Vmax values than pyridoxal. P-nitrobenzaldehyde, a synthetic substrate metabolized by several dehydrogenases, is not a substrate of PD. Therefore, pyridoxal dehydrogenase from mammalian tissue has high specificity for pyridoxal. When compared to pyridoxal dehydrogenase partialy purified from Pseudomonus SP (5), the mammalian enzyme is also unable to metabolize PLP, benzaldehyde, and salicylaldehyde in the presence of the cofactor NAD, indicating that this enzyme, like the bacteria enzyme, is not a general aldehyde dehydrogenase.

Table IV shows the specificity for NAD and NAD analogues. It has been found that NAD is the preferred cofactor of PD. NAD-acetyl and NADP as alternate cofactors yield lower enzymatic activity in the presence of pyridoxal. The steady rate of NAD reduction is sensitive to pH and the maximal initial velocity values were observed over the pH range of 8.5-9.0. Most of the initial velocity studies conducted with the enzyme were

performed at pH 8.0 using sodium phosphate buffer.

Two methods were used in the determination of the catalytic parameter Km: a fluorometric method that measures the rate of formation of PA, and a spectrophotometric method which monitors the rate of formation of NADH at 340 nm. Figure 10 shows a typical double reciprocal plot obtained with the fluorometric method when the concentration of PL was varied between 1.0 uM and 100.0 uM at a fixed concentration of NAD (0.5 mM). Similar plots obtained at fixed concentrations of PL and varied concentrations of NAD were used to determine the Km values of NAD. The results are included in Table V. The Km value for PL is comparable to the value previously reported for the enzyme isolated from bacteria.

Double reciprocal plots of intial velocity versus NAD or PL concentrations gave rise to an intersective pattern of straight lines (figure not shown), which is consistent with a sequential mechanism for the addition of substrates. The inhibition studies of NADH and 1,10-phenanthroline indicate that both NADH and 1,10-phenanthroline are competitive with respect to NAD when the concentration of PL was fixed (Figure 11 and 12). 4,7-phenanthroline, which is not a zinc chelator, also shows almost the same inhibition of PD (data not shown), indicating that there is no zinc ion in the active site of PD. On the other hand, the inhibition pattern of NADH with respect to PL when the concentration of NAD was fixed shows uncompetitive inhibition (Figure 13). It indicates that the addition of substrates follows a

sequential ordered mechanism.

All aldehyde dehydrogenases studied have been found to catalyze also the hydrolysis of p-nitrophenyl acetate in the presence or in the absence of NAD (21) and a linear correlation is found for both esterase and dehydrogenase activity: inhibition or inactivation of dehydrogenase activity is accompanied by simultaneous loss of esterase activity. Table VI shows the multi-function of pyridoxal dehydrogenase, i.e., it also has esterase activity. In contrast to horse liver aldehyde dehydrogenase (6, 19), NAD and NADH exhibit inhibition of the esterase activity and NADH shows more potent inhibition than NAD. With most enzymes the esterase activity is generally regarded as supportive evidence for the acyl-intermediate reaction mechanism for aldehyde dehydrogenase (1). Therefore, it may be that pyridoxal dehydrogenase possesses a similar reaction mechanism.

CHAPTER IV

STEREOCHEMISTRY OF HYDRIDE TRANSFER

A. Materials and Instrumentation

NaB^3H_4 , alkaline phosphatase, glutamate dehydrogenase, malic dehydrogenase, and yeast alcohol dehydrogenase were purchased from Sigma. Scinitallation counting was done using a Beckman LS-230 liquid scintillation system.

B. Procedures

1. Enzymatic synthesis of [4-^{3}H]-pyridoxal-5-phosphate

An incubation mixture containing 20 umole pyridoxal-5-phosphate, 40 umole NaB^3H_4 (16.3 mCi), 50 umole tris-HCl (pH 7.6) in a final volume of 5 ml was allowed to react at 25^{o}C for 15 minutes in the dark. The reaction was stopped by addition of 1.0 N HCl and brought to pH 7.6 by addition of 1.0 M tris buffer. Complete reduction of PLP to PNP was verified by absorption spectroscopy. The mixture (5.0 ml) containing 1000 units of PNP oxidase was incubated for 8.0 hours in the dark with gentle shaking. At the end of the reaction, conversion of PNP to PLP as catalysed by oxidase was detected by absorbance measurements at 390 nm. The reaction mixture was then applied

to a w-aminohexyl-sepharose column (20 X 1.0 cm) equilibrated with 0.02 M ammonium bicarbonate. After standing in the dark for 2.0 hours at 25°C, the column was successively washed with 100 ml of ammonium bicarbonate, 200 ml of water and 100 ml of 0.5 M acetic acid. [4-^{3}H]-pyridoxal-5-phosphate, eluted with 0.5 M acetic acid, was lyophilized in the dark and stored at -25°C. The specific activity is 4.0 mCi/mmole.

2. Preparation of [4-^{3}H]-NADH

A reaction mixture containing 1.0 umole of [4-^{3}H]-PLP (4.0 uCi) and 2.0 units of alkaline phosphatase in a total volume of 1.0 ml of 0.02 M ammonium carbonate (pH 8.6) was incubated at 25°C for 30 minutes. The solution was lyophilized and dissolved in 1.0 ml of 20 mM potassium phosphate (pH 7.5) containing 2.0 umole NAD and 100 units of pyridoxal dehydrogenase. The mixture was allowed to incubate at 25°C until no further increase in absorbance at 340 nm was observed. At the end of the reaction , the mixture was applied to a column packed with DEAE-Sephadex (40 X 1.0 cm) equilibrated with 10 mM ammonium bicarbonate, pH 7.8. Then eluted the column with a gradient made up of 200 ml of 0.0l M and 0.5 M ammonium bicarbonate (the radioactive NADH was eluted at approximately 0.15 M ammonium bicarbonate). The radioactivity of the fractions containing [4-^{3}H]-NADH were pooled and lyophilized for further experiment.

3. Stereochemistry of hydride transfer:

a. Thin layer chromatography: Reaction mixtures containing 0.1 umole of [4-^{3}H]-NADH, 0.2 umole of oxaloacetate, 2.0 units

malic dehydrogenase (pro-R) in a total volume of 0.2 ml of 20 mM potassium phosphate (pH 7.5) and 0.1 umole [4-^{3}H]-NADH, 0.2 umole a-ketoglutarate, 2.0 umole NH_4Cl, 3.0 units of glutamate dehydrogenase (pro-S) were incubated for 2.0 hours at 25^{o}C. A control containing 0.1 umole of [4-^{3}H]-NADH in 0.2 ml of 20.0 mM potassium phosphate, pH 7.5 was run in parallel. At the end of the reaction, aliquots (20 ul) withdrawn from the incubation mixtures and reference compounds were applied to thin layer chromatography plate (polygram, gel 400) and developed with 0.5 M LiCl. NADH and glutamate were used as markers. NAD was observed as a quenched spot on the chromatogram under the excitation of ultraviolet lamp. In addition, glutamate was visualized by spraying with ninhydrin followed by heating with heat gun. The spots on the thin layer plate were scraped into counting vials and radioactivity was determined by scintillation counting.

b. Ion exchange chromatography: Reaction mixtures containing 0.8 ml of 0.1 mM [4-^{3}H]-NADH, 0.01 M NH_4Cl, 1.0 mM a-ketoglutarate, and 20.0 units of glutamate dehydrogenase (pro-S) in ammonium bicarbonate buffer, pH 7.3 and 0.8 ml of 0.1 mM [4-^{3}H]-NADH, 0.01 M acetaldehyde, and 20.0 units of yeast alcohol dehydrogenase in ammonium bicarbonate buffer, pH 7.8 were preincubated for 2.0 hours at 25^{o}C. A control containing 0.1 mM [4-^{3}H]-NADH in 0.8 ml of ammonium bicarbonate buffer, pH 7.6 was done in parallel. At the end of the reactions, the reaction

mixtures were boiled at 100°C for 3.0 minutes followed by centrifugation. Then 2.0 mg NAD was added to each of the supernatants as a marker (2.0 mg glutamate was also added to the supernatant of glutamate dehydrogenase mixture). The supernatant of each of the samples was applied to a Dowex-1 X 8 column (27 X 1.0 cm, formate form) which was equilibrated previously with 0.1 M formic acid. The column was then eluted with a linear gradient of 150 ml 0.1-1.5 M formic acid. The fractions containing radioactivity were tested by scintillation counting. The fractions containing NAD and glutamate were identified by their spectra in the region of 200-400 nm.

C. Results and Discussion

The conversion of radioactivity from PL to NADH is shown in Figure 14. The first peak contains PL while the second peak contains NADH which was eluted at approximately 0.15 M ammonium bicarbonate.

Figure 15 shows the distribution of radioactivity among reactants and products. These results indicate that part of the tritium in NADH was transferred to glutamate by glutamate dehydrogenase. Because glutamate dehydrogenase is pro-S specific (22), the tritium on NADH must therefore be on the pro-S side (or B-side). This means pyridoxal dehydrogenase transferred tritium from radiolabeled pyridoxal to the pro-S side of NADH. Further evidence was given by malic dehydrogenase catalysis. The

latter is known to be pro-R specific (22). The preliminary results indicate that pyridoxal dehydrogenase is pro-S specific.

Figure 16 also shows that most tritium was transferred to glutamate by glutamate dehydrogenase while Figure 17 tells that the tritium on NADH is not on the pro-R side. The smaller peaks in both Figure 17 and 18 indicate that small amount of hot pyridoxal was present in the hot NADH fractions. These results indicate clearly that the stereochemistry of hydride transfer of pyridoxal dehydrogenase is pro-S.

Further experiments on the stereochemistry of hydride transfer of pyridoxal dehydrogenase can be done with Nuclear Magnetic Resonance method by using deuterated pyridoxal (for a review, see ref. 12).

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Weiner, H. (1979), in Majchyowicz E. Nobel (eds), " Pharmacology of Ethanol, " Vol. I, New York, PP. 107.

2. Kunliang Guan and Henry Weiner (1987), in " Enzymology and Molecular Biology of Carbonyl Metabolism ", Alan R. Liss, Inc., PP. 15-24.

3. Christophy Woenckhaus, Elke Bieber and Reinhard Jeck (1987), in " Enzymology and Molecular Biology of Carbonyl Metabolism ", Alan R. Liss, Inc. , PP. 53-65.

4. Stanulovic, M. Jeremic, V. Leskova, C. V and Chayrins (1976), Enzyme, 21, PP. 357-369.

5. Richard W. Burg and Esmond E. Snell (1969), J. Biol. Chem. 244, 2585-2589.

6. Rhoda I. Feldman and Henry Weiner (1972), J. Biol. Chem. 247, 260-266.

7. Bendicht Wermuth (1981), J. Biol. Chem. 256, 1206-1213.

8. Schauenstein, E. Esterbauer, H. and Zollner, H. (1977), in " Aldehyde in Biological Systems ", Pion Limited, London, PP. 126-129.

9. Guang-chou Tu and Henry Weiner (1987), in " Enzymology and Molecular Biology of Carbonyl Metabolism ", Alan R. Liss, Inc., PP. 67-75.

10. Popjak, G. (1970), Enzyme, Vol. 2, PP. 115.

11. Timkovich, R. et al. (1987), J. Biol. Chem. 262, 10911-10913.

12. You, K. (1984/1985), in " CRC Critical Reveiws in

Biochemistry ", Vol. 17, Issue 4, PP. 358-365.

13. Schopfer, L. M. and Massey, V. (1979), J. Biol. Chem. 254, 10634.

14. Arnold, L. J., Jr. and you, K. et al. (1976), Biochemistry, 15, 4844.

15. Oppenheimor, N. J., Arnold, L. J., Jr., and Kaplan, N. O. (1971), Proc. Natl. Acad. Sci. USA, 68, 3200.

16. Viola, R. E., Cook, P. F., and Cleland, W. W. (1979), Anal. Biochem. 96, 334.

17. CRC handbook of biochemistry, (Sobert, H. A., ed.) Chemical Rubber Company, Cleveland, Ohio (1968).

18. Bio-Rad Protein Assay Mannul.

19. Rhoda I. Feldman and Henry Weiner (1972), J. Biol. Chem. 247, 267-272.

20. Kezdy, F. J., and Bender, M. L. (1962), Biochemistry, 1, 1097.

21. Martti Koivusalo and Pekka Rautoma (1987), in " Enzymology and Molecular Biology of Carbonyl Metabolism ", Alan R. Liss, Inc., PP. 135-147.

22. Alan Fersht in " Enzyme Structure and Mechanism ", 2nd, PP. 391.

APPENDIXES

APPENDIX A

LIST OF ABBREVIATIONS

ALDH = Aldehyde Dehydrogenase

APAD = 3-Acetylpyridine Adenine Dinucleotide

NAD = Nicotinamide Adenine Dinucleotide, Oxidized Form

NADH = Nicotinamide Adenine Dinuleotide, Reduced Form

NADP = Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form

PA = Pyridoxic Acid

PCL = Pyridoxic Lactone

PD = Pyridoxal Dehydrogenase

PL = Pyridoxal

PLP = Pyridoxal-5-phosphate

PM = Pyridoxamine

PN = Pyridoxine

PNP = Pyridoxine-5-phosphate

APPENDIX B

FIGURES

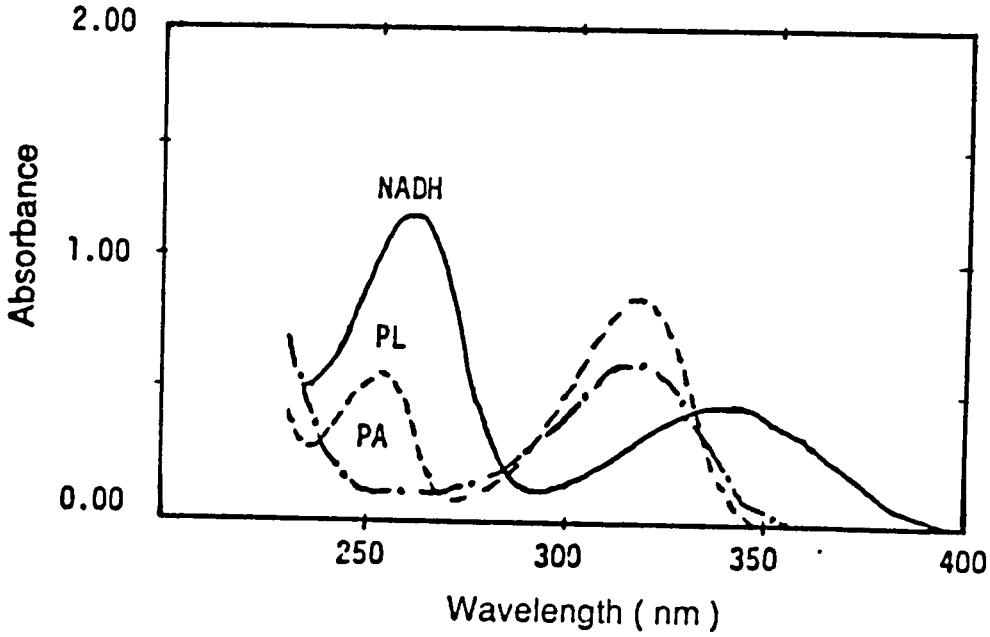


Figure 1. Absorption spectra of PL, PA, and NADH at pH 7.0.

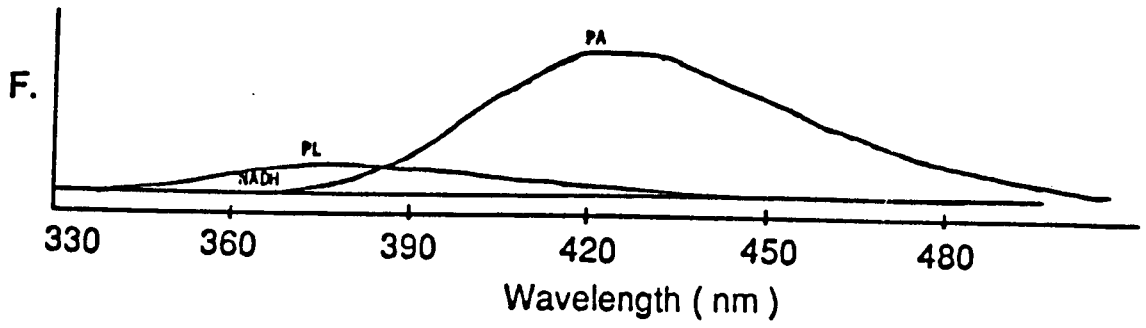


Figure 2. Emission spectra of PL, PA, and NADH at pH 7.0. PL was excited at 315 nm. PA was excited at 360 nm. NADH was excited at 330 nm.

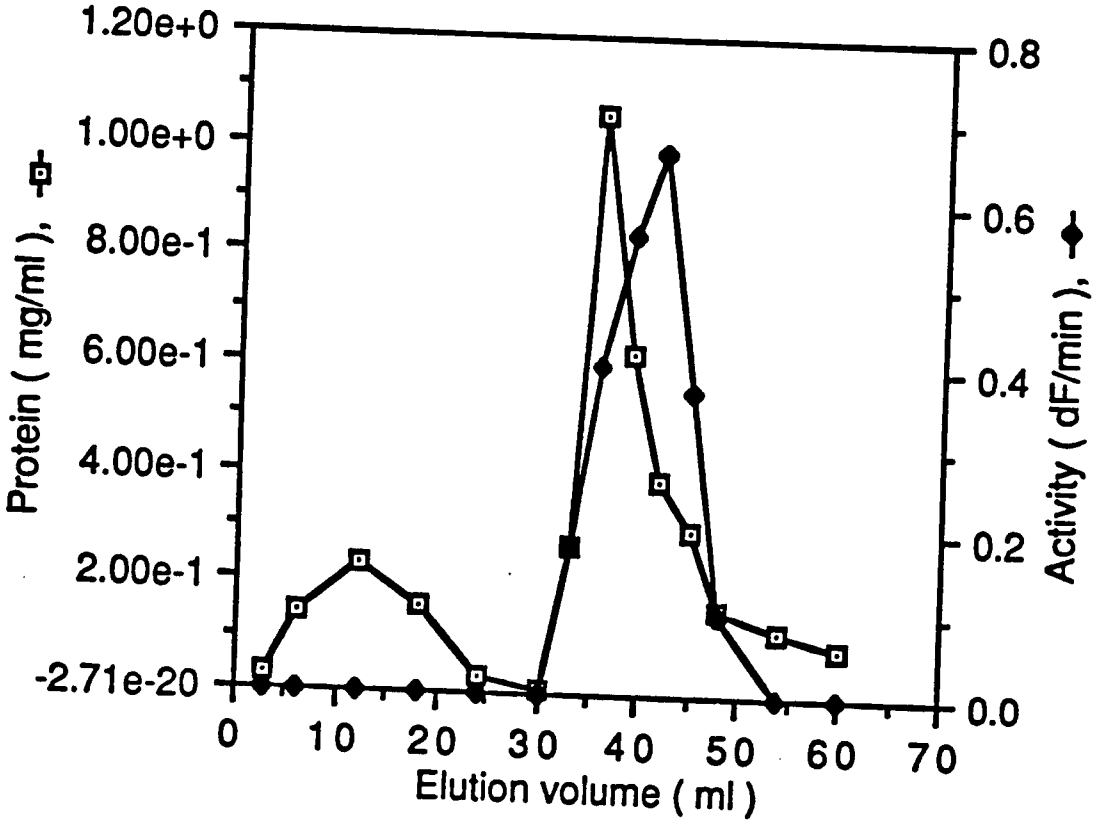


Figure 3. Elution profile of PD from hydroxylapatite column (8 X 1.2 cm).

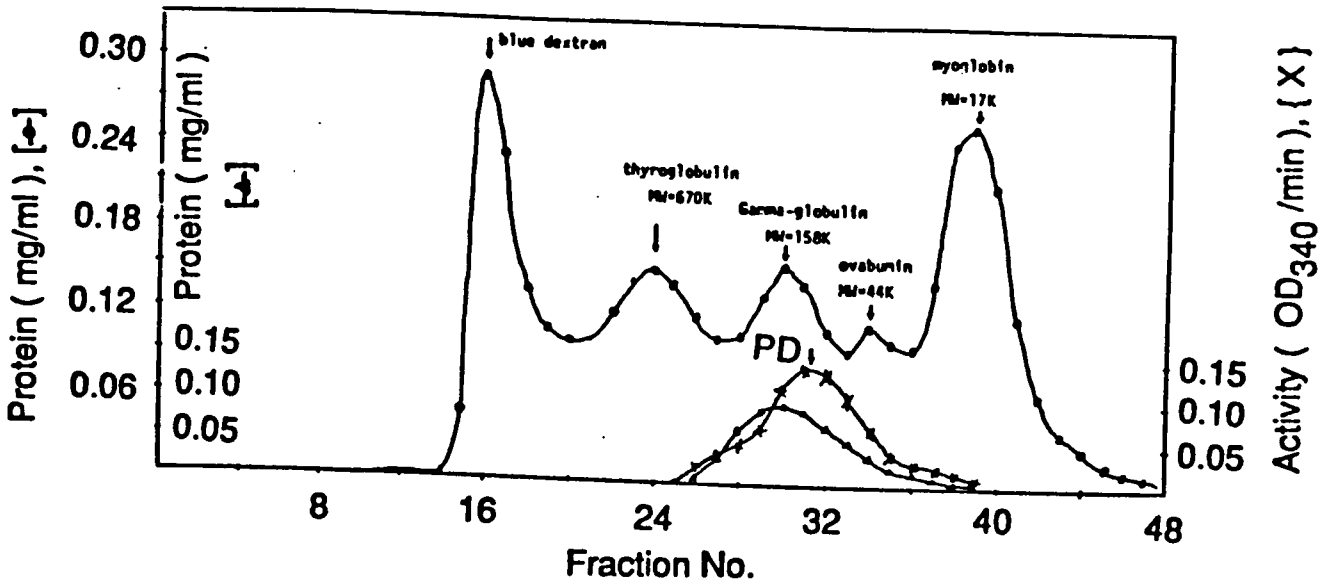


Figure 4. Elution profile of PD from Sepharose-CL-4B gel filtration column (85 X 1.5 cm). The column was calibrated by blue dextran, thyroglobulin (670K), gamma-globulin (158K), ovalbumin (44K), and myoglobin (17k).

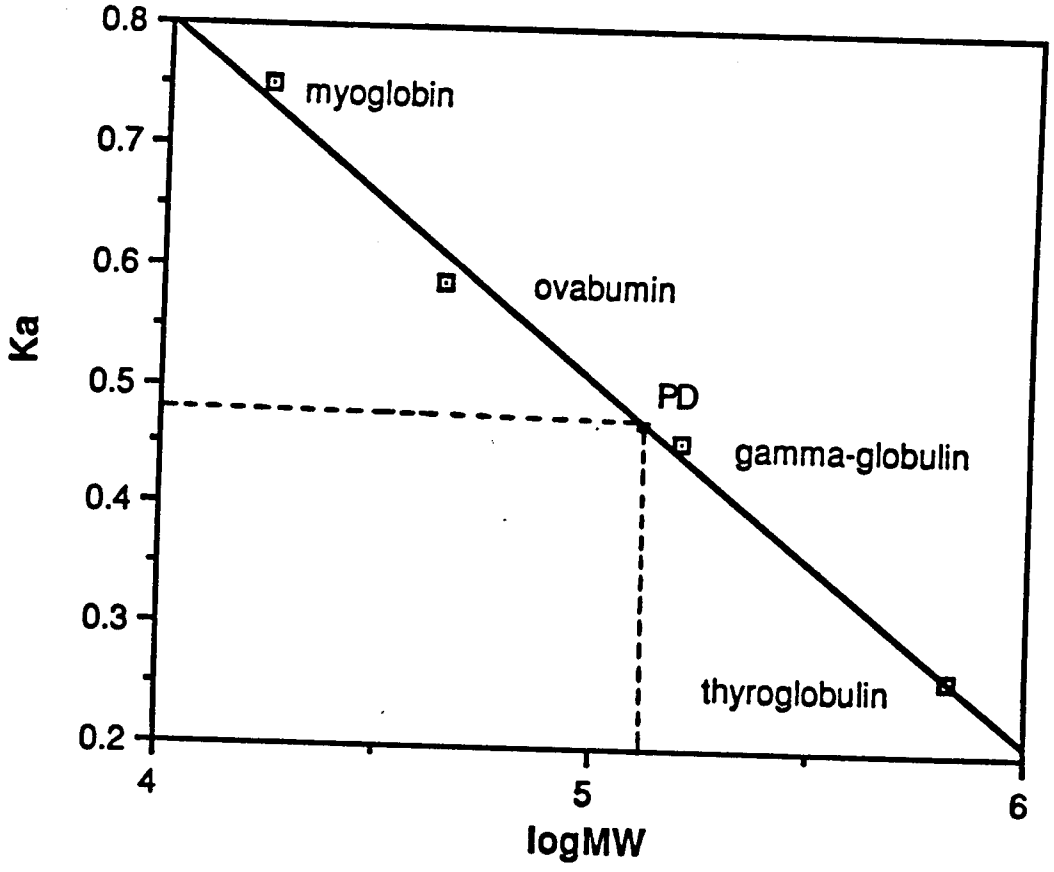


Figure 5. Estimation of molecular weight of PD by Sepharose-CL-4B gel filtration column (85 X 1.5 cm).

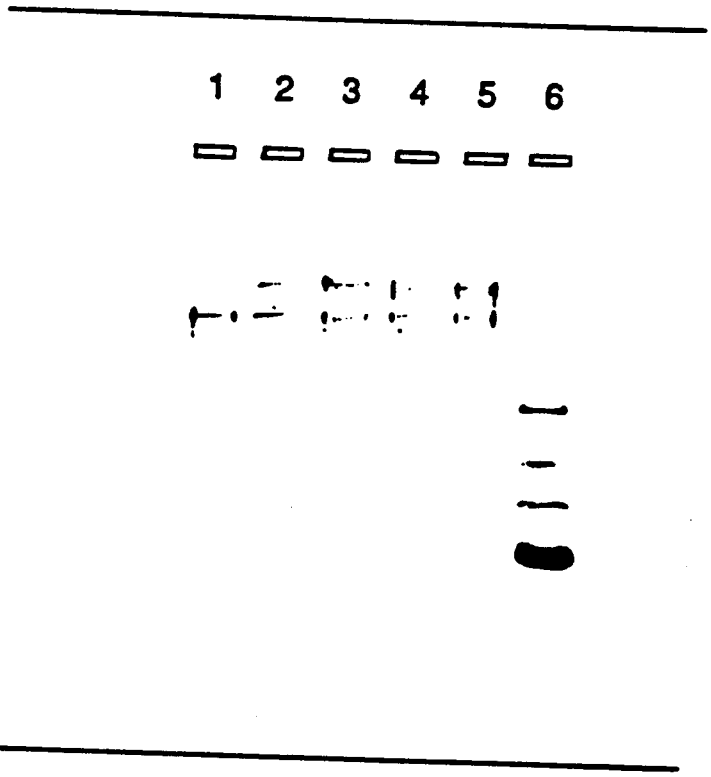


Figure 6. Electrophoresis of native gel. Lane 1 to lane 5 were from different fractions of PD after hydroxylapatite column. Protein standards (bovine serum albumin, overbumin, trypsin inhibitor, and lysozyme) were run in parallel in lane 6.

Compound	Rf
PA	0.78
PL	0.64
PCL	0.56
NADH	0.61

0 PL | 0 PA | 0 PCL | 0 NADH | 0 Assay mix.

Figure 7. Thin layer chromatography of PL, PA, PCL, and NADH. Developing solvent system: water : acetone : tert-butanol : triethylamine=20 : 35 : 40 : 5, V/V. Assay mix. contained PL, NAD, and PD.

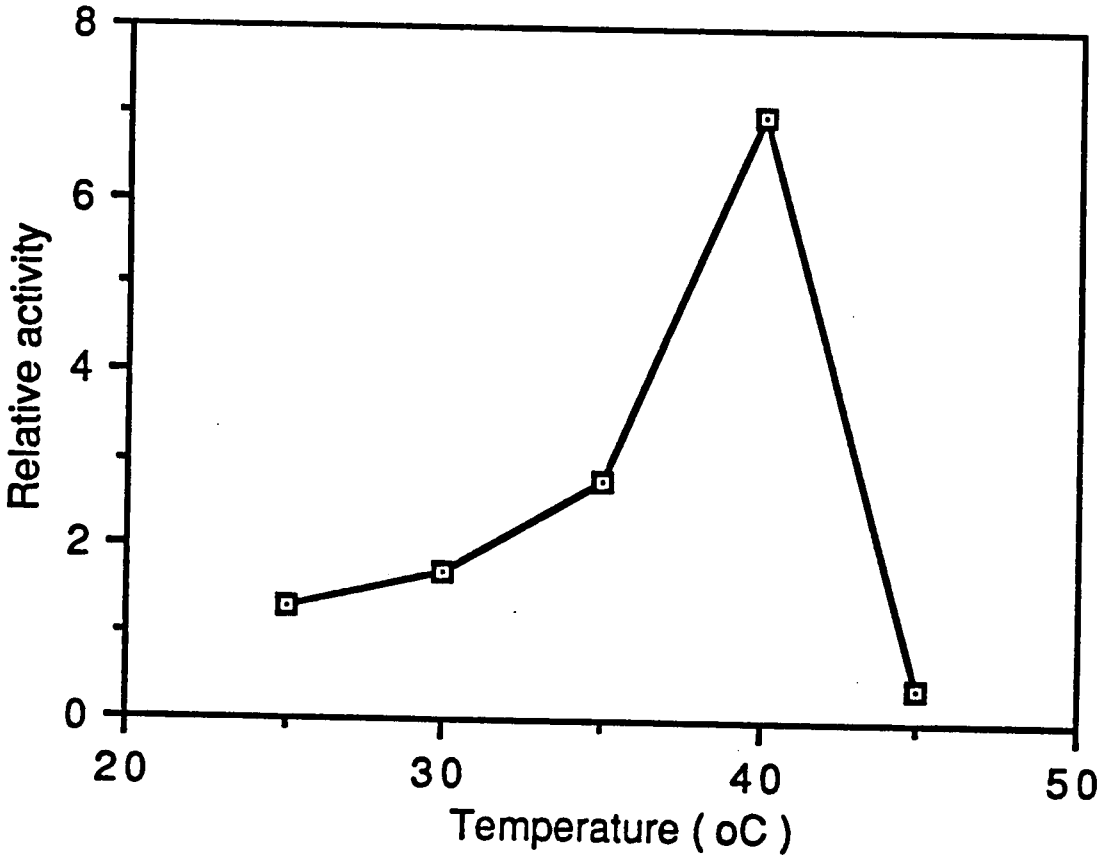


Figure 8. Heat stability of PD. Results were obtained when PD was preincubated at different temperatures for 30 minutes before aasay.

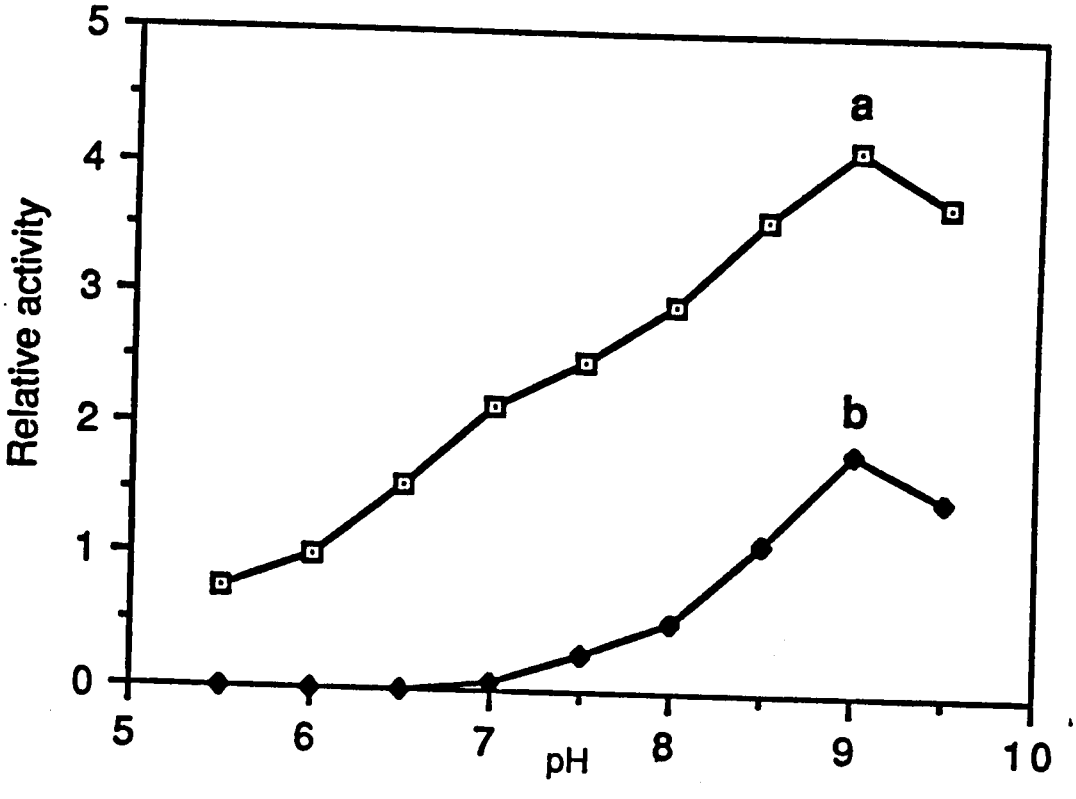


Figure 9. Effect of pH on the reaction rate of PD. a. When PL was aldehyde substrate. b. When propionaldehyde was substrate.

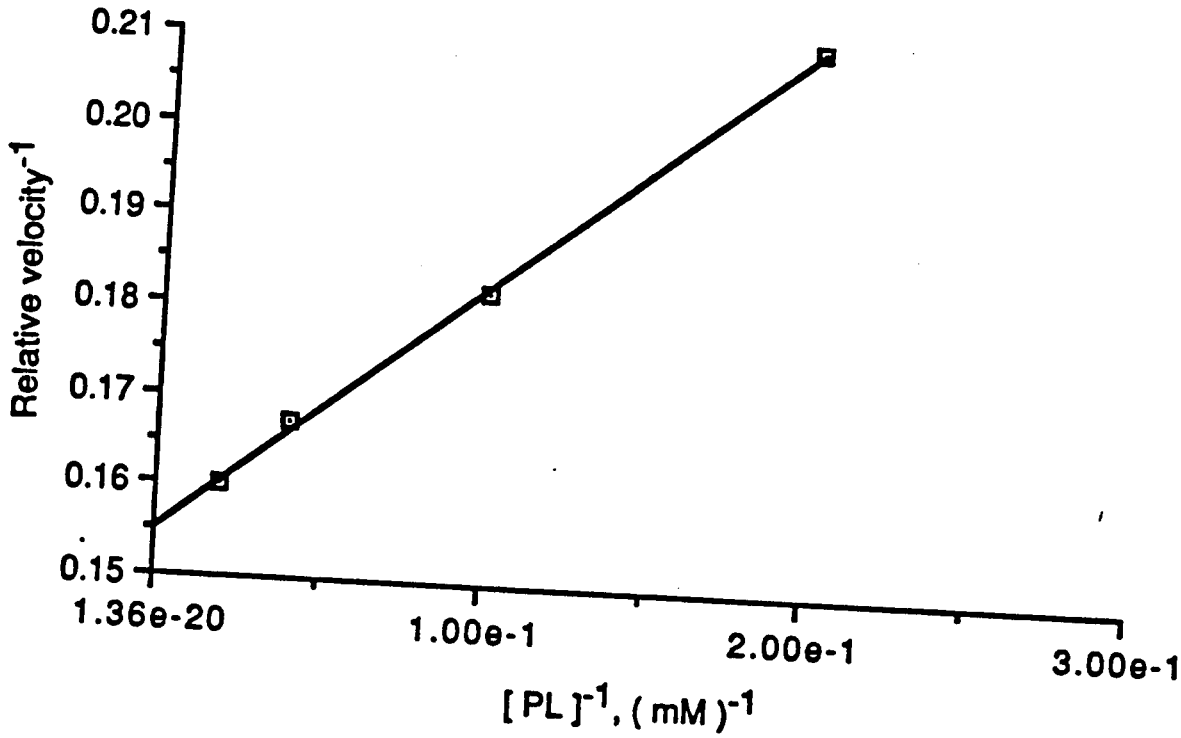


Figure 10. Lineweaver-Burk plot. Results were obtained by fluorometric method when the concentration of PL was varied between 1.0 uM and 100 uM at fixed concentration of NAD (0.5 mM) at pH 8.0.

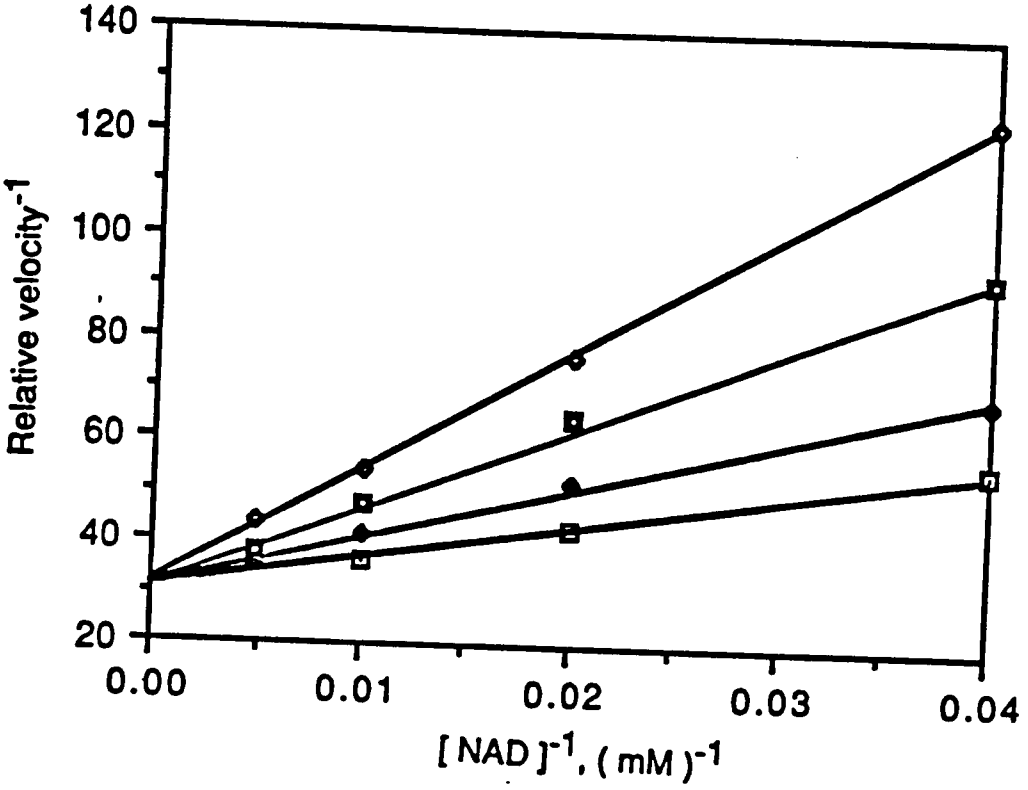


Figure 11. Inhibition of PD activity by NADH at fixed concentration of PL (0.5 mM). Results were obtained at concentrations of NADH of 0.08 mM (◆), 0.04 mM (■), 0.02 mM (◆), and 0 (◘) at pH 8.0.

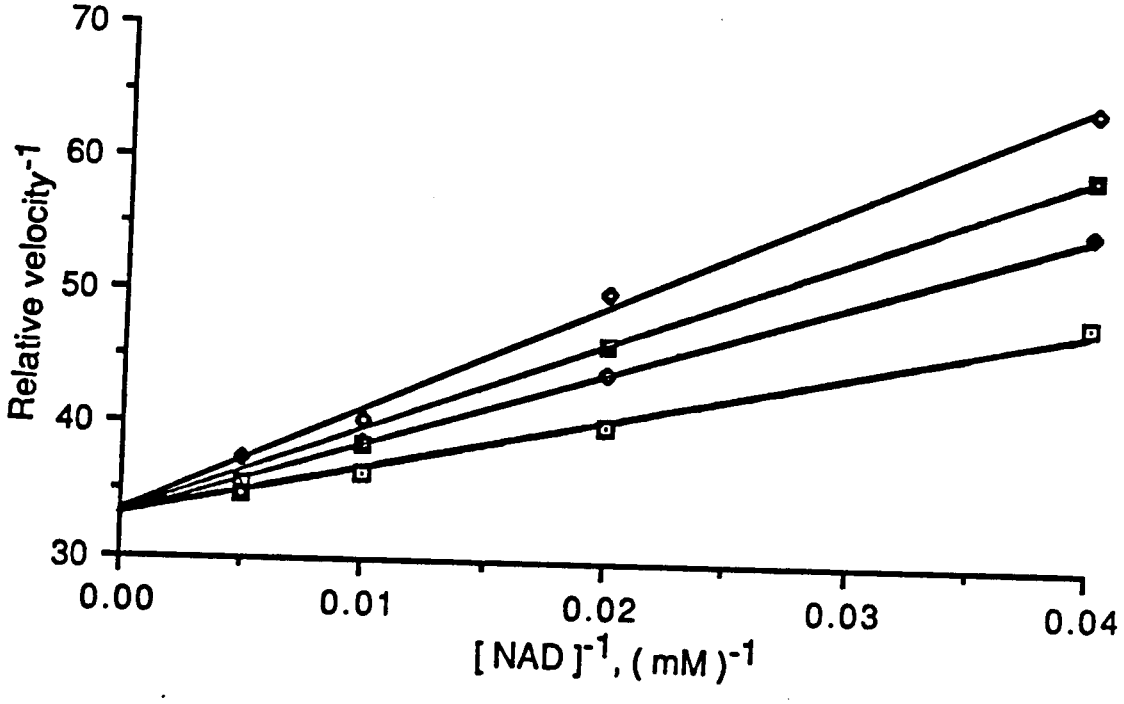


Figure 12. Inhibition of PD activity by 1,10-phenanthroline at fixed concentration of PL (0.5 mM). Results were obtained at concentrations of 1,10-phenanthroline of 0.08 mM (◆), 0.04 mM (■), 0.02 mM (◆), and 0 (⊡) at pH 8.0.

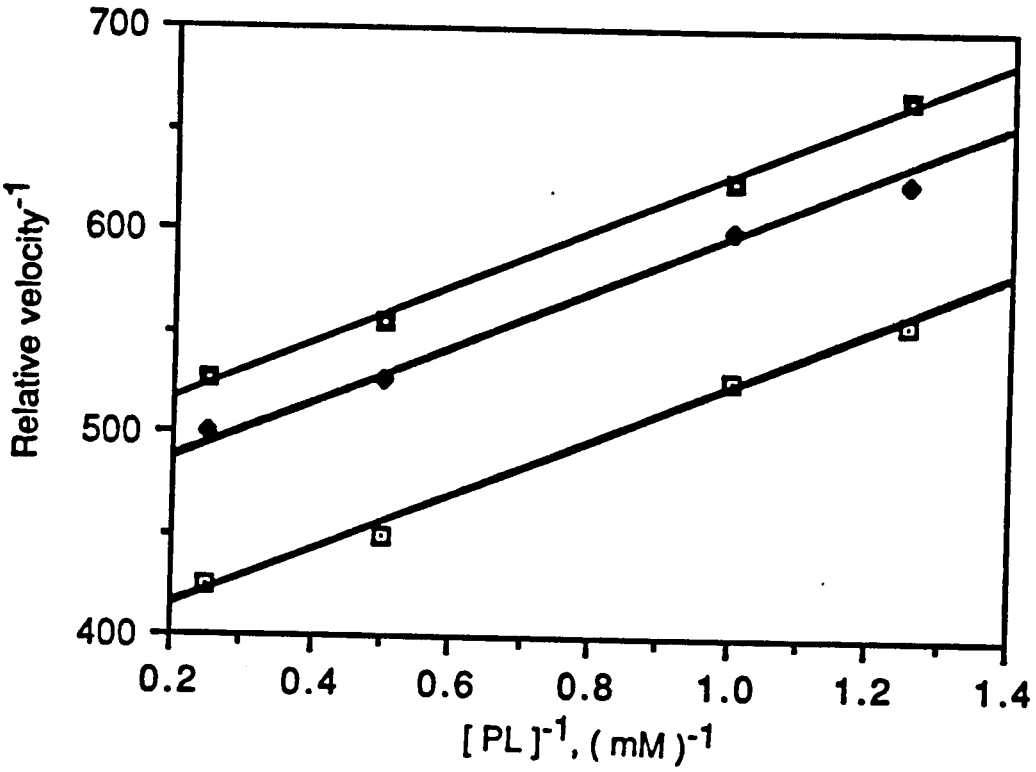


Figure 13. Inhibition of PD activity by NADH at fixed concentration of NAD (0.5 mM). Results were obtained at concentrations of 0.08 mM (■), 0.02 mM (◆), and 0 (⧈) at pH 8.0.

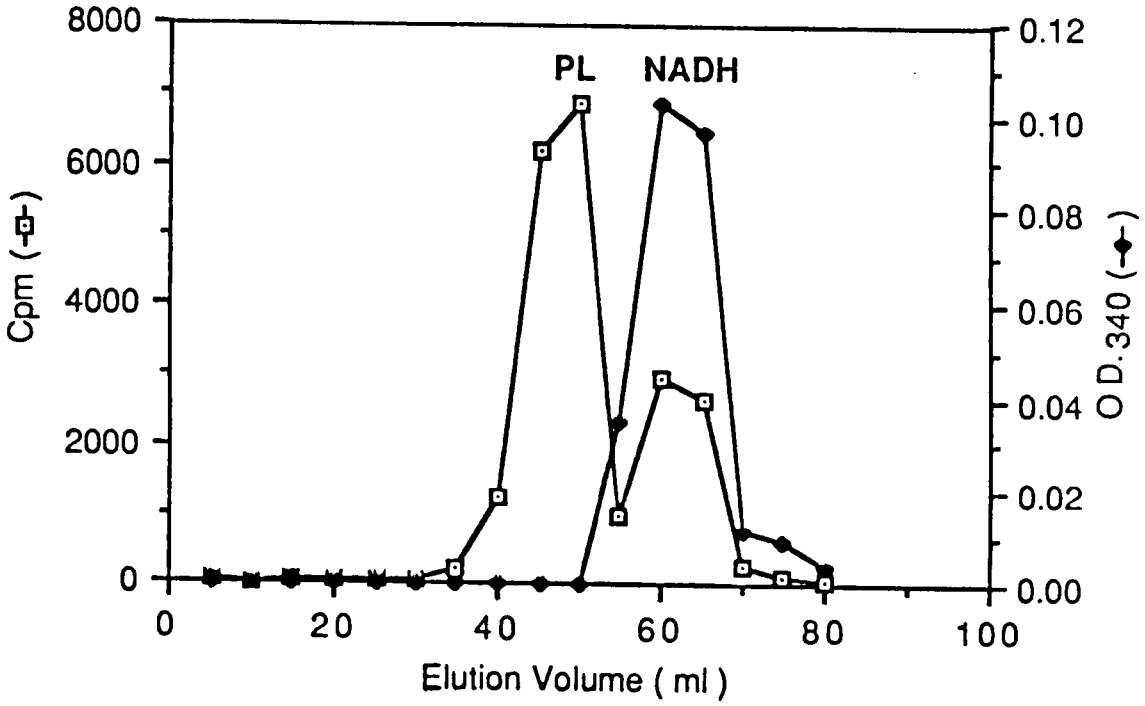


Figure 14. Elution profile of the radio-labeled PL and NADH by DEAE-Sephadex column (40 X 1.0 cm).

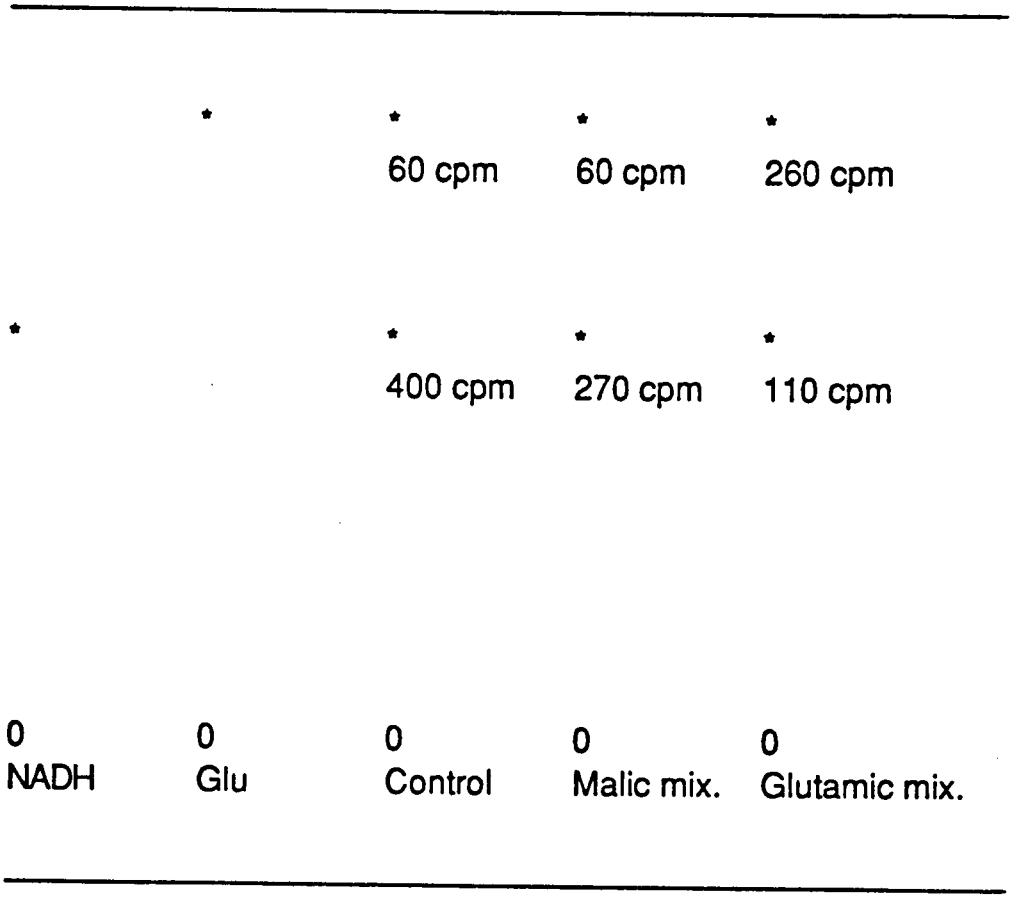


Figure 15. Stereochemistry of hydride transfer of PD determined by thin layer chromatography. The developing solvent system was 0.5M LiCL.

Malic mix.: $NAD^3H + \text{oxalacetate} + H^+ \xrightarrow{MD^a} \text{L-malate} + NAD.$

Glutamic mix.: $NAD^3H + \text{a-ketoglutarate} + NH_4^+ \xrightarrow{GD^b}$ glutamate + NAD.

MD^a: Malic dehydrogenase. GD^b: Glutamic dehydrogenase.

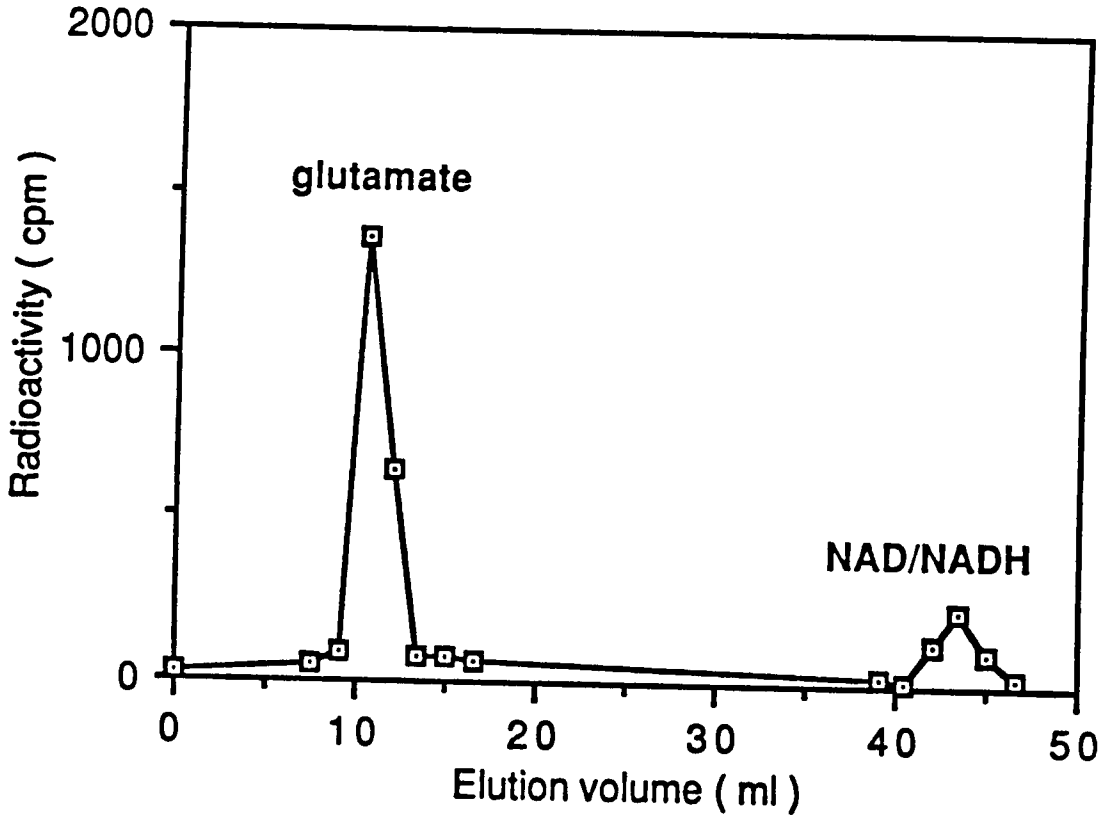


Figure 16. Elution profile of radioactivity by Dowex-1 X 8 column (27 X 1.0 cm). Sample: a-ketoglutarate, NH_4CL, NAD^3H, and glutamate dehydrogenase at pH 7.3.

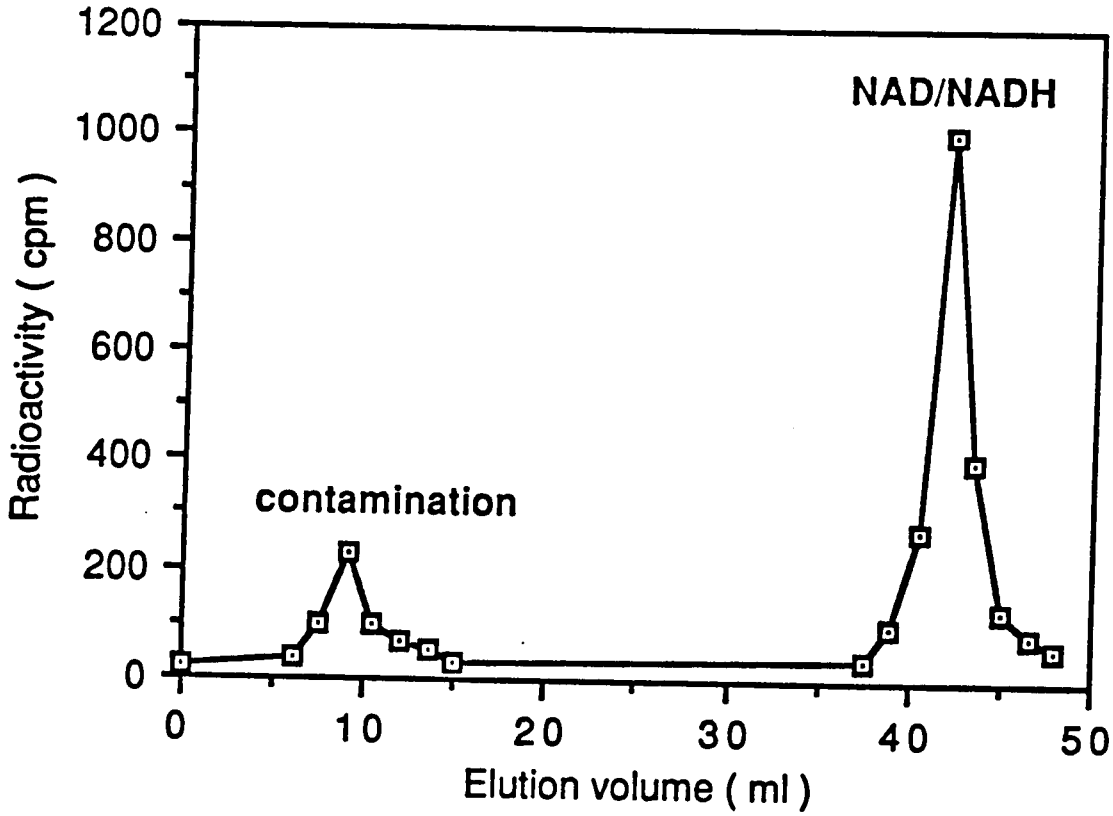


Figure 17. Elution profile of radioactivity by Dowex-1 X 8 column (27 X 1.0 cm). Sample: Acetaldehyde, NAD^{3}H, and alcohol dehydrogenase at pH 7.8.

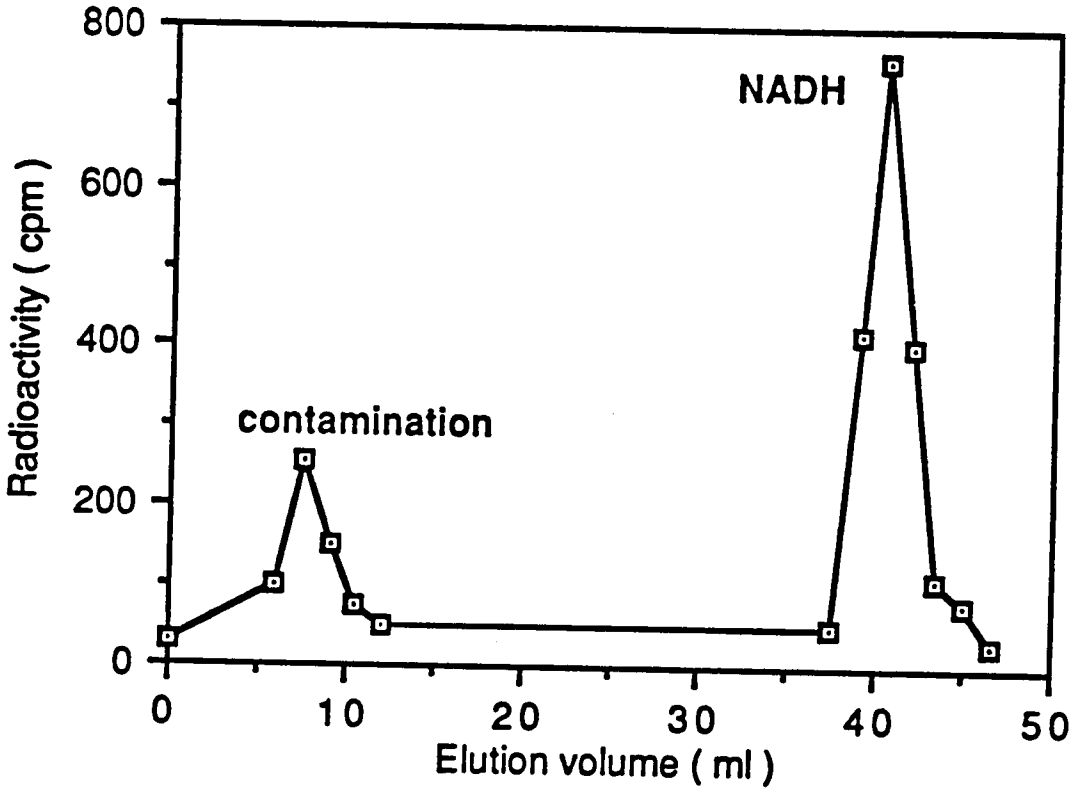


Figure 18. Elution profile of radioactivity by Dowex-1 X 8 column (27 X 1.0 cm). Sample: Control (NAD^{3}H only).

APPENDIX C

TABLES

Table I

Purification of pyridoxal dehydrogenase from porcine liver.

Treatment	volume (ml)	protein (mg/ml)	specific act. (unit/min. mg)	total act. (unit)	yield (%)
Homogenate	940	325	0.000495	151	100
40-60% of $(NH_4)_2SO_4$	120	137	0.00156	25.7	17
CM-Sephadex	81	12.1	0.01208	11.8	7.8
DEAE-A50	60	3.03	0.0216	3.93	2.6
Hydroxyl-apatite	16	0.53	0.1328	1.13	0.75
Sepharose-CL-4B	12	0.097	0.6781	0.79	0.52

Table II

Effect of pH on the spectroscopic properties of PA and PCL

Sample	pH	Absorption (nm)	Fluorescence (nm)	Life time (ns)
PA	7.30	314	433	13.6
PA	2.41	314	432	
PA	1.37	314	432	
PCL	7.30	349	436	14.0
PCL	2.60	349/316	426	
PCL	1.37	316	426	

Table III

Comparative activity of PL and related compounds (0.5 mM) as substrate of PD at pH 7.5.

Compound	Activity as substrate (%)
PL	100
PLP	0
PN	0
PM	0
Benzaldehyde[a]	0
P-nitrobenzaldehyde	0
Salicylaldehyde[a]	0
Acetaldehyde	6
Propionaldehyde	12

a. Saturated solutions of both benzaldehyde and salicylaldehyde were used for activity determination.

Table IV

Comparative activity of NAD and NAD analogous (0.5 mM) as cofactor of PD at pH 7.5.

Compound	Activity as cofactor (%)
NAD	100[a]
NADP	45.7[a]
APAD	66.5[a]
NAD	100[b]
NADP	30.4[b]
APAD	32.1[b]

a. pH was at 7.5. b. pH was at 9.0.

Table V

Kinetic parameters of PD for both NAD and PL at pH 7.5 and 8.0.

Data treatment	Km(NAD)[a]	Km(NAD)[b]	Km(PL)[c]	Km(PL)[d]
Direct Linear	75.5	62.5	2.50	1.54
Lineweaver-Burk	74.9	87.3	3.03	1.36
Hanes-Woolf	76.9	61.1	1.30	0.97
Eadie-Hofstee	75.7	73.6	2.99	1.37
Average	75.7	71.1	2.46	1.31

The unit of Km is uM.

a. PL as substrate at pH 7.5, Km was determined by spectrophotometric method.

b. Propionaldehyde as substrate at pH 7.5, Km was determined by spectrophotometric method.

c. PL as substrate at pH 7.5, Km was determined by spectrophotometric method.

d. PL as substrate at pH 8.0, Km was determined by fluorometric method.

Table VI

Comparison of turnover numbers of dehydrogenase and esterase reaction of PD at pH 8.0.

Turnover number	Amount (umoles/min.mg)	Substrate
Dehydrogenase	5.09	NAD + PL
Esterase	14.93	P-nitrophenyl acetate
	12.1	P-nitrophenyl acetate + NAD
	9.5	P-nitrophenyl acetate + NADH

VITA

Yongzhang Luo (羅永章) was born in Shandong Province, the People's Republic of China, on June 11, 1962. He graduated from Qixia No. 15 High School in July of 1980. He entered Lanzhou University in Lanzhou, Gansu Province, the People's Republic of China in September of 1981, and in July of 1985 received a Bachelor of Science degree in Chemistry.

In September of 1987, Mr. Luo enrolled at the University of Tennessee at Knoxville as a graduate student, and in August 1989 he received the degree of Master of Science, with a major in Biochemistry.